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DNA Methylation in Mammalian and Non-Mammalian Organisms

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1. Introduction

Of particular interest in biology is how different chromatin states contribute to the complex regulation of gene transcription that is necessary to establish and maintain multi-cellular organisms. This is because (with few exceptions) each cell within an organism contains the same genomic sequence, meaning that the diversity of expression states is not due to variability in the underlying genetic sequence but could result from differences in chromatin landscape. This area of research comes under the umbrella of 'epigenetics', which is concerned with molecular processes involved in regulating gene expression that are transmittable and independent of changes in DNA sequence. However, perhaps owing to the ambiguity of this definition, the term often means different things to different people. A more precise definition of 'epigenetic' mechanisms is 'the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states' (Bird, 2007). Differentiated cells are said to have an 'epigenetic memory' imparted by epigenetic processes that can maintain a pattern of gene transcription through time and cell divisions. Understanding the mechanisms involved in setting up and maintaining these processes is an exciting area of research investigation.

The nucleosome is the basic repeating unit of chromatin and consists of 146bp of DNA wrapped 1.7 times around the histone octamer, which is composed of two molecules of each of four types of histone protein: H2A, H2B, H3 and H4 (An et al., 1998; Zlatanova et al., 2009). The core histones each have a distinct C-terminal, a structured globular domain and a flexible, unstructured N-terminal tail, which protrudes from the nucleosome (Luger et al., 1997; Schroth et al., 1990). The repressive effect of nucleosomes on transcription can be enhanced or reduced by combinations of histone post-translational modifications (PTM). Histone acetylation, methylation, ubiquitylation and other modifications play crucial roles in diverse biological processes, such as embryogenesis, development and maintenance of genome integrity. Recently, an integrated, mass spectrometry-based proteomics approach

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resulted in the identification of over 67 new PTM sites in histones including lysine crotonylation, expanding the total number of known histone PTMs by about 70% (Tan et al., 2011). Histone PTMs create docking sites for non-histone effector proteins that that can subsequently modify chromatin structure (Andrews & Luger, 2011). However in some cases, charge changes resulting from modifications can alter chromatin structure directly by disrupting the DNA-histone interaction. Linker histone modifications can also influence higher order chromatin structure and thus alter gene expression states.

Modification of DNA can alter its biological properties and involves enzymatic mechanisms that are sustained through cellular replication. Particular molecular signatures of DNA together with histone modifications are associated with active and repressed chromatin states (Barski et al., 2007). DNA methylation patterns are developmentally regulated and are thought to define tissue states in plants and animals (Feng et al., 2010b). In cancer as well as in embryos generated through somatic cell nuclear transfer, normal patterns of DNA methylation are altered implying that precise molecular pathways are involved in setting up and maintaining diverse patterns of modification (Hochedlinger & Jaenisch, 2006).

1.2 New modifications in the genome

The major form of epigenetic information in mammalian genomes is centred on DNA methylation, which now comes in the forms of 5-methylcytosine (5mc), 5-hydroxymethylcytosine (5hmC) and the more recently discovered 5-carboxylcytosine (5caC) and 5-formylcytosine (5fC) (He et al., 2011; Ito et al., 2011). The observed levels of both 5caC and 5fC are extremely low in ES cells (~3 5caC and ~18 5fC for every 10^6 C) (Ito et al., 2011) and these may represent transient intermediates in a demethylation pathway. In contrast to these low levels, far greater levels of the 5hmC modification were observed in ES cells - indicating that this mark may have additional functional roles. The presence of 5hmC, 5caC and 5fC in genomes is dependent on 5mC which is the substrate for conversion by the TET family (1-3) of Fe(II) and α-KG-dependent dioxygenases, which utilise molecular oxygen to convert 5mC to 5hmC, 5fC and 5caC (He et al., 2011; Ito et al., 2010; Ito et al., 2011; Ko et al., 2010; Koh et al., 2011; Tahiliani et al., 2009; Wossidlo et al., 2011).

1.3 The DNA methylation machinery

To generate 5mC, a methyl group is added covalently to the 5 position of cytosine by DNA cytosine methyltransferases (DNMTs), mostly within the context of CpG dinucleotides in somatic cells; however, non-CpG methylation also occurs at a high frequency in mouse and human embryonic stem (ES) cells (Lister et al., 2009;Ramsahoye et al., 2000). Non-CpG methylation may be a feature of the pluripotent state, as it is present in induced Pluripotent Stem (iPS) cells generated by transduction of a non-pluripotent somatic cell with stem cell-associated genes, which results in reprogramming of the recipient cell's epigenetic profile (Takahashi et al., 2007). The importance of 5hmC and its cousins in epigenetics is that the hydroxymethyl group is suggested to alter the biological properties of methylated DNA (Ndlovu et al., 2011). The rediscovery of 5hmC also presents an unanticipated experimental problem, as conventional techniques were originally unable to distinguish between 5mC and 5hmC in DNA (Nestor et al., 2010). Recent technical developments can now distinguish prominent 5hmC sites in the genome (C.X. Song et al., 2011). However, it is clear that 5hmC is less abundant than 5mC, and the latter is still the most prominent modification in

vertebrate DNA in many tissues. 5mC values are stable at a typical value of around 4.5% of all cytosine in tissues, whereas 5hmC values vary significantly (Munzel et al., 2010). This suggests that 5hmC has a specific function that is not absolutely correlated with 5mC levels. Initial analysis suggests that 5hmC is predominantly associated with the gene bodies of highly expressed genes (C.X. Song et al., 2011).

The presence of DNA methylation at regulatory sequences in somatic cells is generally associated with transcriptional repression, and potentially has a long term impact on the stability of gene expression states and on genome integrity (Sharma et al., 2010). Alterations in genomic methylation patterns underpin imprinting syndromes such as Beckwith-Wiedemann, Prader-Willi and Angelman, and have been implicated in a number of other disease conditions including cancer (Goll & Bestor, 2005). The enzymes responsible for targeting and maintaining global DNA methylation in mammals are constructed from a complex set of functional modules, broadly divided into the N-terminal 'regulatory' domain and the C-terminal 'catalytic' domain. The regulatory domain acts as an interaction platform for protein interactions, DNA binding and mediates its differential nuclear targeting during the cell cycle (Goll & Bestor, 2005). Not surprisingly, the localisation of the maintenance methyltransferase, Dnmt1, in mammals is co-ordinated with DNA replication so that newly synthesised hemi-methylated DNA is rapidly and fully methylated. Three methyltransferase enzymes, Dnmt1 along with the *de novo* methyltransferases Dnmt3a and Dnmt3b, coordinate the establishment and maintenance of DNA methylation patterns in mammals. The C-terminal domain of each enzyme comprises ten motifs responsible for the enzyme's catalytic activity; six of these motifs are conserved in nearly all cytosine methyltransferases from bacteria to mammals. Dnmt3a and Dnmt3b target cytosine methylation to previously unmethylated CpG dinucleotides, which the Dnmt1 preserves during cell division. Dnmt3a and 3b are thought to act with an equal preference for hemimethylated and unmethylated DNA *in vitro*, but *in vivo* they have differential targets which may be mediated by partner proteins such as transcription factors (Hervouet et al., 2009). They are necessary for *de novo* methylation of the genome during development and potentially newly integrated retroviral sequences (Okano et al., 1998b, 1999).

The N-terminal domain of Dnmt1 interacts with many chromatin-associated proteins including the *de novo* methyltransferases, methyl-CpG binding proteins (MeCPs) and histone modifying enzymes (Qin et al., 2011). It also contains a replication targeting region and a cysteine-rich Zn2+-binding domain that can potentially bind non-methylated CG rich DNA. Binding of the CXXC domain to unmethylated CpG DNA is thought to result in a repositioning of the CXXC-BAH1 linker between the DNA and the active site of DNMT1, thereby preventing *de novo* methylation (J. Song et al., 2011). In addition, a loop projecting from the BAH2 domain interacts with the target recognition domain (TRD), stabilising it in a retracted position so it cannot access the DNA major groove. Hemimethylated CpG dinucleotides that do not bind the CXXC domain can gain access to the active site of Dnmt1 by bypassing this molecular mechanism. Biochemical and molecular analyses of Dnmt1 suggest that it participates in multiple complex networks involved in gene regulation, epigenetic signalling and genome stability via the mismatch repair pathway. Dnmt1 is also post-translationally modified by the protein lysine methyltransferase SET7 which regulates its stability (Esteve et al., 2009). This modification on Lysine 142 is mutually exclusive with phosphorylation on Ser143; phosphorylated Dnmt1 is more stable than its methylated version (Esteve et al., 2011).

Dnmt3b is specialised in methylation of specific regions of the genome, such as pericentromeric repeats and CpG islands on the inactive X-chromosome, whereas Dnmt3a is required for maternal imprints of differentially methylated regions (DMRs), in addition to their general *de novo* roles (Kim et al., 2009). The PWWP domain of Dnmt3a specifically recognises the histone 3 lysine 36 trimethylation mark and this may be important for its subnuclear localisation (Dhayalan et al., 2010). Deletion of Dnmt3a in primordial germ cells disrupts paternal and maternal imprinting, whereas Dnmt3b is dispensable for mouse gametogenesis and imprinting (Kaneda et al., 2010;Kato et al., 2007). Protein interaction domains in the regulatory N-termini of Dnmt3a and Dnmt3b also mediate binding to transcriptional co-repressors (Qiu et al., 2002). Unlike Dnmt1 and Dnmt3a/b, the DNA methyltransferase Dnmt2 has only weak activity *in vitro* towards DNA, and its inactivation does not result in alterations to global CpG methylation levels (Okano et al., 1998b). A cofactor, Dnmt3L (DNMT3-Like), is expressed only in germ and ES cells. It is not a methyltransferase but enhances the *de novo* methyl transferase activity of Dnmt3a and 3b in mouse ES cells (Ooi et al., 2010).

1.4 Role of DNA methylation in mammals

In general, repression by DNA methylation is considered to occur downstream of other epigenetic or trans-acting factors that signal the initial inactivation event. For example, initial repression of Pou5f1 during differentiation of mouse ES cells is mediated by sequence-specific transcription repressors such as GCNF leading to conversion of the 'active' histone modification state to an inactive one that is subsequently followed by *de novo* DNA methylation at its promoter (Cedar & Bergman, 2009). The number of potential genes that can be directly regulated by DNA methylation in a tissue and developmental specific manner may be quite small corresponding to 100–200 of annotated CpG island (CGI) genes in somatic cells (Meissner et al., 2008). However, new data suggests there are approximately 23,000 and 25,500 CGIs in the mouse and human genomes respectively, about half of which are associated with annotated transcription start sites for mainly constitutively expressed genes (Illingworth et al., 2010). The non-annotated or 'orphan' CGI's show higher levels of tissue specific methylation (14-20%) and may be directly regulated by DNA methylation in different tissues and developmental stages. The importance of the preservation of these patterns is highlighted by the observation that *de novo* methylation of promoter CGIs associated with tumour suppressor genes occurs in many neoplastic cells (Sharma et al., 2010). At the same time as *de novo* methylation of CGIs, global methylation levels associated with satellite repeats and retroposons are often reduced in cancers (Sharma et al., 2010). Recent results suggest that retrotransposons mobilise to protein-coding genes that are differentially expressed and active in the brain; suggesting that retrotransposition may result in somatic genome mosaicism and alteration in the genetic circuitry that underpins normal and abnormal neurobiological processes (Baillie et al., 2011).

1.5 Histone modifications and gene regulation

The combination of histone PTMs and their resulting effects on gene expression is often referred to as the "Histone Code" (Turner, 2007). Trimethylation of lysine 4 on histone H3 (H3K4me3) is enriched at transcriptionally active gene promoters, whereas trimethylation of H3K9 (H3K9me3) and H3K27 (H3K27me3) is present at inactive gene promoters. H3K9me3

can function in concert with DNA methylation whereas H3K27me3 may be exclusive of DNA methylation. Genome-wide studies of these histone marks in the genome have increased our understanding of how these diverse modifications act in a cooperative manner to regulate gene expression (Sharma et al., 2010). The polycomb complex (PRC), which mediates trimethylation of lysine 27 on histone H3 (H3K27me3) appears to be targeted specifically to genes involved in development and differentiation (Mikkelsen et al., 2007). Heterochromatin protein (HP1) binds H3K9me2/3 containing chromatin through its chromodomain (Dialynas et al., 2008). Interaction partners for HP1 include DNMT1, the histone H3K9 methyltransferases Suvar39H1 and G9a (Esteve et al., 2006; Smallwood et al., 2007), which may coordinate DNA and H3K9 methylation at genomic loci.

Binding of the PRC2 complex to specific genes, such as the Hox cluster, results in trimethylation of histone H3K27 by the histone methyltransferase EZH2 (Morey & Helin, 2010). However, polycomb target genes in ES cells can have a bivalent chromatin signature, being also marked by the activating modification H3K4me3 (Barski et al., 2007; Mikkelsen et al., 2007). These marks may be resolved as development proceeds leading to developmental and tissue specific patterns of gene expression (Barski et al., 2007; Boyer et al., 2006). Mature heterochromatin HP1alpha and H4K20me3 signatures do not arise until late in development (Wongtawan et al., 2011). Like DNA methylation, gene silencing via histone modification can be maintained *in vivo* through multiple cell divisions. It has been reported that CGIs that are aberrantly methylated in cancer cells coincide with sites targeted by polycomb in human ES cells (Schlesinger et al., 2007). Approximately 50% of tumor-specific methylated CGIs are H3K27 trimethylated in ES cells (Illingworth et al., 2010). These findings suggest that the mechanisms governing tumour-specific and normal directed CGI methylation are distinct.

It becomes clear that as our knowledge of the DNA methylation regulatory system in mammals and their component parts deepens, that this system functions out with the enzymatic modification of cytosine to its modified forms. In other words many of the enzymes involved have non-catalytic functions. Potentially this occurs in ways that we cannot predict, which is why study of DNA modification systems in other animal model systems may add mechanistic and biological insight into the role of DNA modification pathways in development and disease. In the following we undertake a short review of the role of DNA modification (primarily 5mC) in 4 organisms: frog, zebrafish, chicken, and honeybee. Now is a particularly relevant time to review 5mC in these organisms, with each of them benefitting from at least one recently completed genome-wide methylome.

2. Zebrafish – *Danio rerio*

Initial evidence for the existence of 5mC in zebrafish came from transgenesis studies using zebrafish. The expression of a chloramphenicol acetyltransferase (CAT) transgene was found to be variegating; an expression pattern that would be consistent with transgene methylation (Stuart et al., 1990). It was subsequently shown that treatment with 5-azacytidine (an anologue of cytidine, with methyltransferase inhibitory action (Friedman, 1979)) significantly increased the expression of transgenes, strongly implying that transgene repression could be mediated by DNA methylation. The above experiment suggested that a working *de novo* methylation process was present in zebrafish, and this was confirmed when the CpG island of the *ntl* (notail) gene of zebrafish larvae was found to undergo *de novo* methylation (Yamakoshi & Shimoda, 2003). As discussed earlier, the DNMT enzymes can be

categorised as either *de novo* or maintenance in function. Surprisingly, the zebrafish genome contains at least 8 potential DNMTs (see Table 1 and Figure 1), including the tRNA methylase Dnmt2, but does not have an obvious DNMT3L homologue.

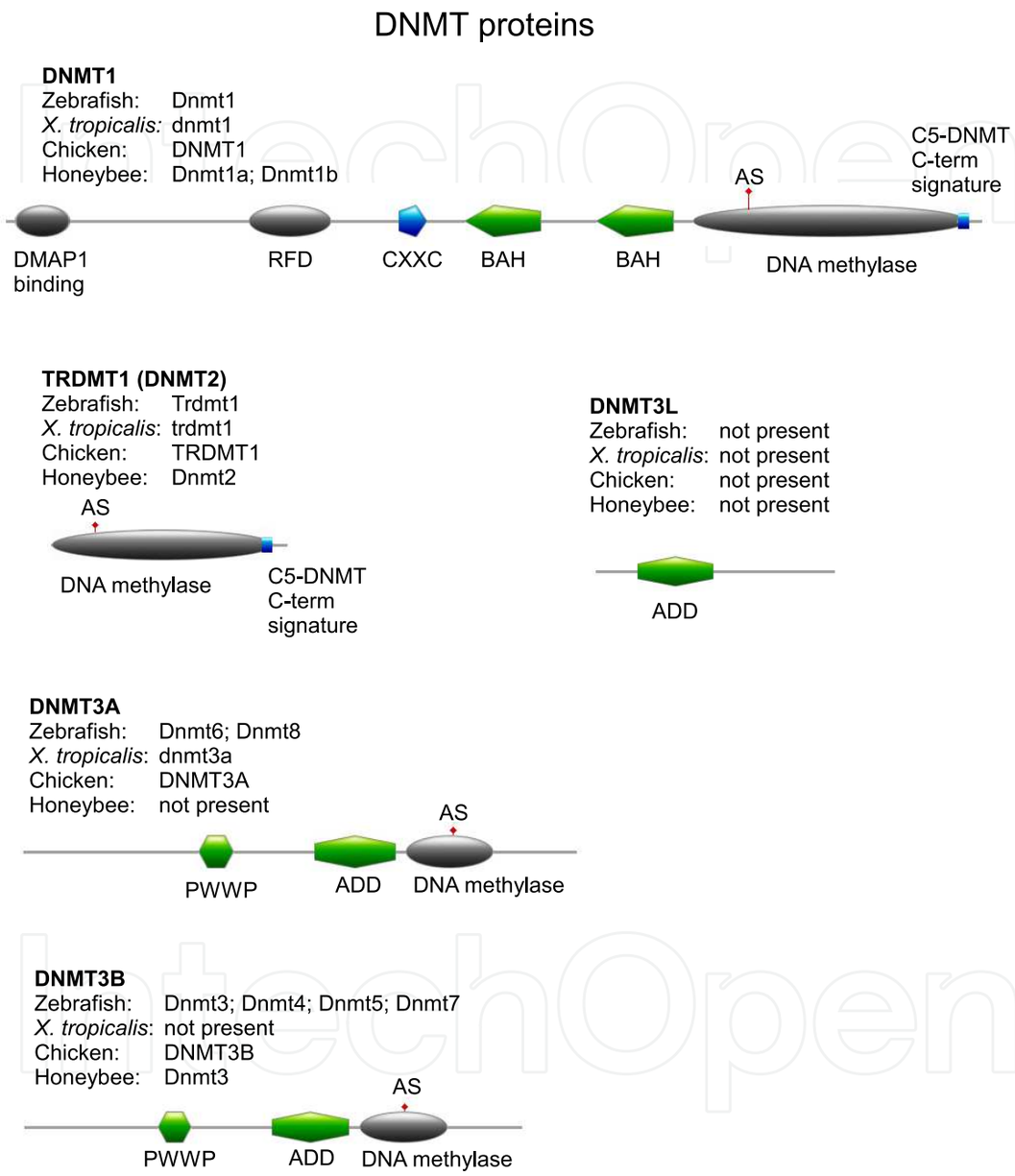

Fig. 1. **DNMT proteins.** Domains were identified by searching PROSITE (http://prosite.expasy.org/) and the Sanger pfam database (http://pfam.sanger.ac.uk/) using the sequences belonging to the accession numbers shown. Diagrams were constructed using the output from PROSITE searches, and pfam output added to the diagrams using the MyDomains tool in PROSITE. ADD: ATRX-DNMT3-DNMT3L domain; AS: active site; BAH: Bromo-adjacent homology; CXXC: CXXC zinc finger domain; PWWP: Pro-Trp-Trp-Pro domain; RFD: Cytosine specific DNA methyltransferase replication foci domain

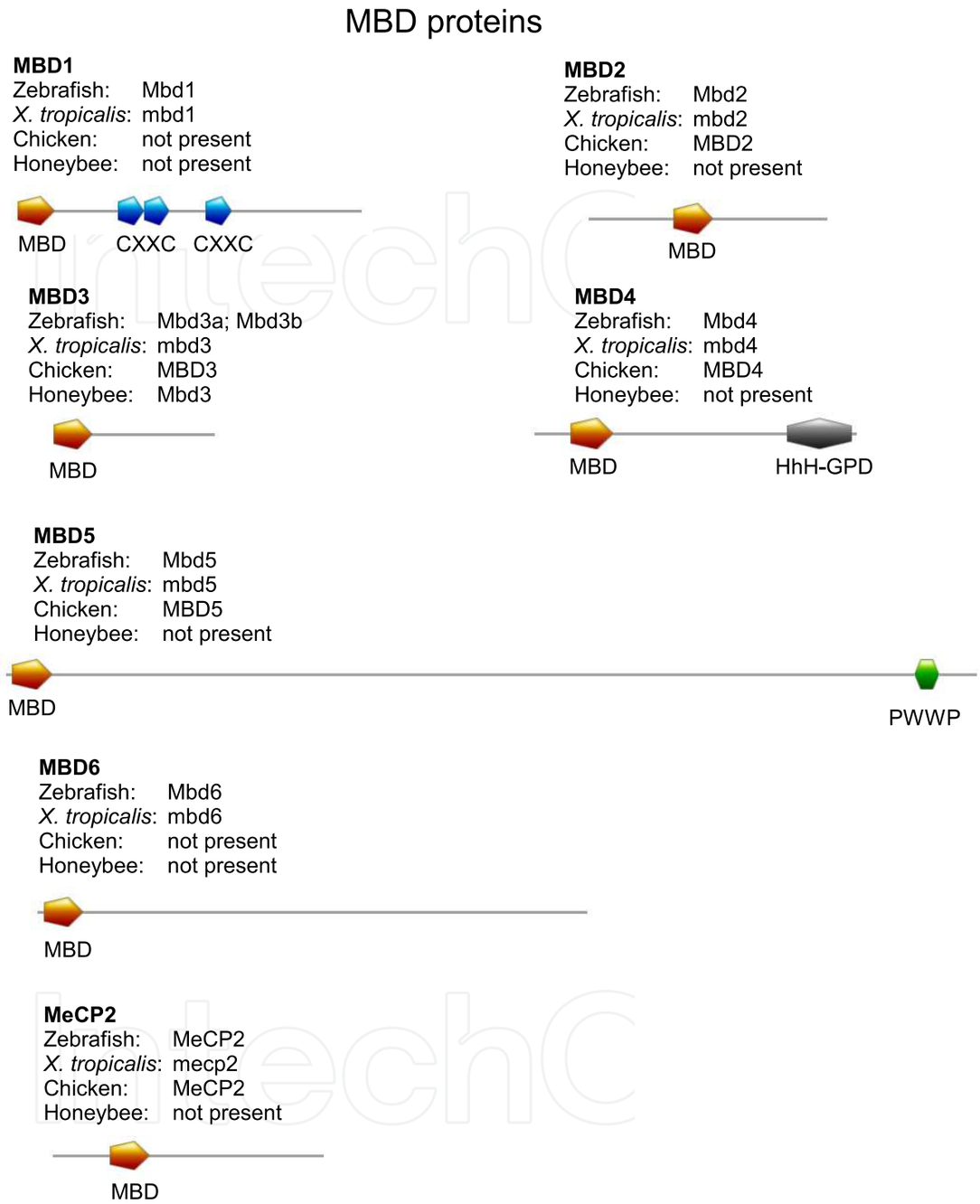

Fig. 2. **MBD proteins in mouse.** Domains were identified by searching PROSITE (http://prosite.expasy.org/) and the Sanger pfam database (http://pfam.sanger.ac.uk/) using the sequences belonging to the accession numbers shown. Diagrams were constructed using the output from PROSITE searches, and pfam output added to the diagrams using the MyDomains tool in PROSITE. CXXC: CXXC zinc finger domain; HhH-GPD: Helix-hairpin-helix-gly-pro-asp superfamily base excision repair domain. MBD: methyl binding domain; PWWP: Pro-Trp-Trp-Pro domain

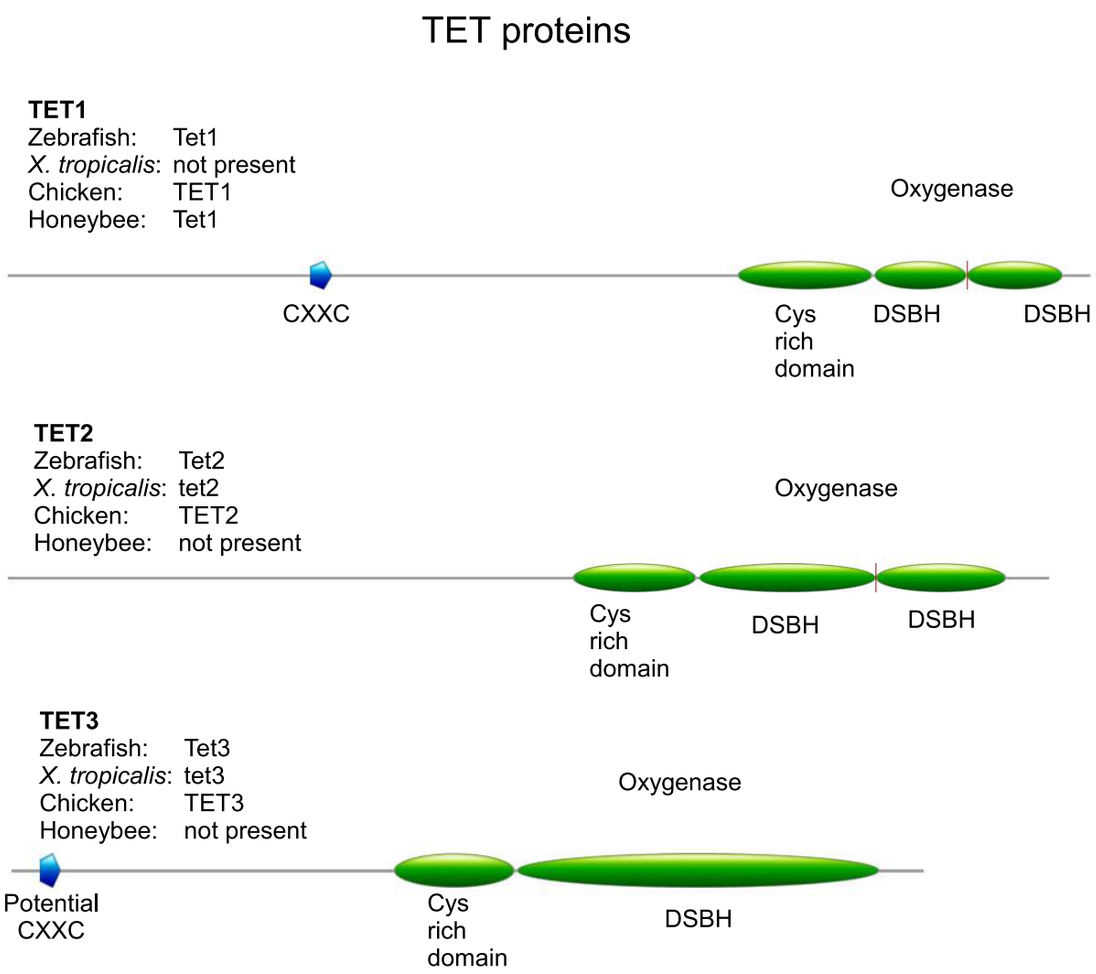

Fig. 3. **TET proteins in mouse.** Domains were identified by searching PROSITE (http://prosite.expasy.org/) and the Sanger pfam database (http://pfam.sanger.ac.uk/) using the sequences belonging to the accession numbers shown. Diagrams were constructed using the output from PROSITE searches, and pfam output added to the diagrams using the MyDomains tool in PROSITE. CXXC: CXXC zinc finger domain; DSBH: double stranded beta helix fold of oxygenase domain of TET proteins.

A homology search revealed that 6 of the zebrafish DNMTs exhibit a high degree of similarity to DNMT3A/B, termed Dnmt3-8 (Shimoda et al., 2005). Interestingly, knockdown experiments suggested that Dnmt7 was responsible for *de novo* methylation of *ntl* but not other forms of *de novo* methylation, such as at transgenes (Shimoda et al., 2005). The homologues most similar to Dnmt3A are Dnmt6 and Dnmt8, and it is of note that Dnmt3A is expressed as 2 major isoforms in mammals. The expression profile of zebrafish Dnmt6 and Dnmt8 has been shown to be similar to DNMT3A; while Dnmt3-4,7 is similar to DNMT3B (Smith et al., 2011). This suggests that the 6 potential *de novo* methyltransferases are functionally equivalent to DNMT3A/B in mammals.

In mammals, *de novo* methylation at imprinted regions is dependent upon DNMT3L (Bourc'his et al., 2001). Zebrafish do not possess any DNMT3L homologue, but are also not

known to use imprinting extensively. Interestingly, it has been reported that a transgene in the zebrafish shows parent-of-origin dependent DNA methylation (Martin & McGowan, 1995). In line with this, parent-of-origin effects have been reported in interspecific crosses of

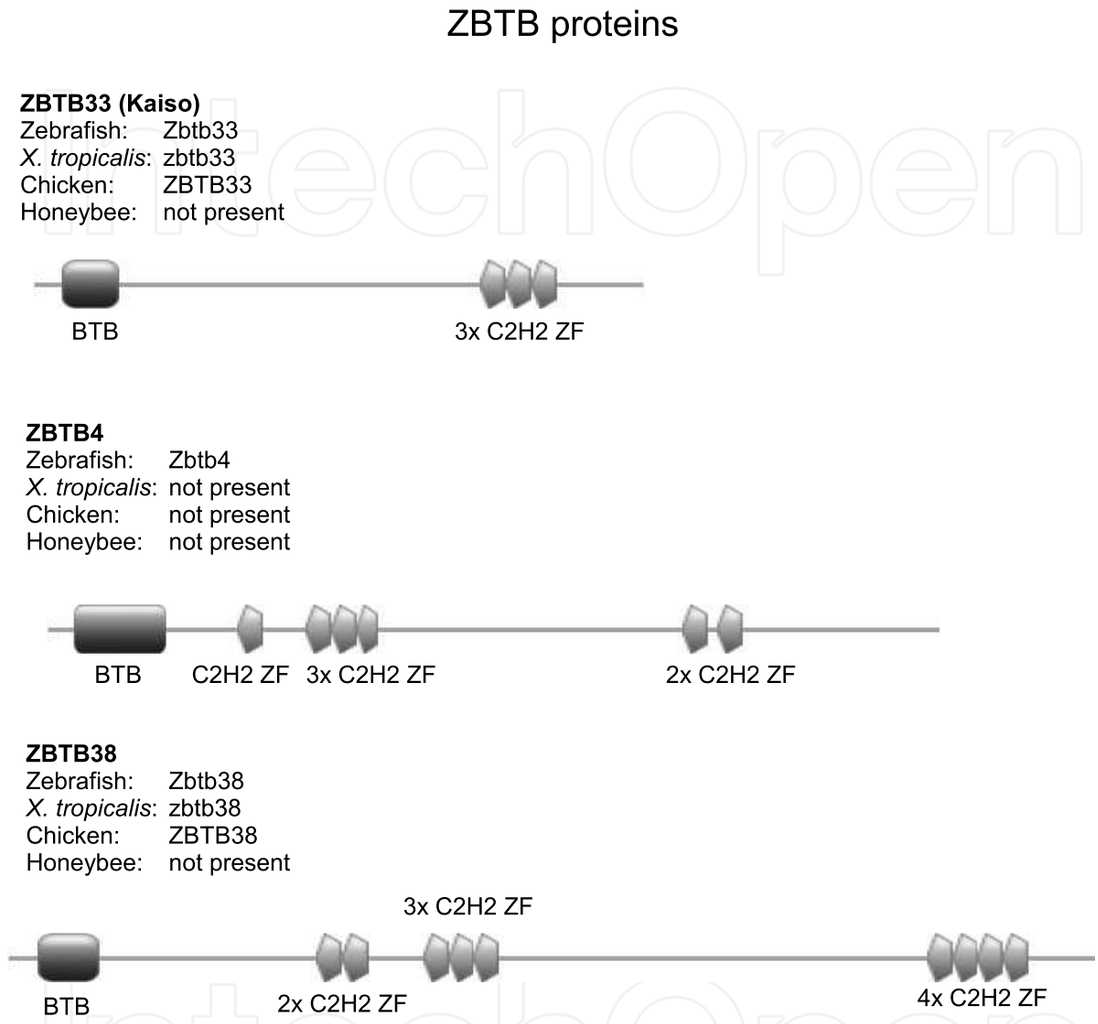

Fig. 4. **methyl binding ZBTB proteins in mouse.** Domains were identified by searching PROSITE (http://prosite.expasy.org/) and the Sanger pfam database (http://pfam.sanger.ac.uk/) using the sequences belonging to the accession numbers shown. Diagrams were constructed using the output from PROSITE searches.

birds, frogs, and fishes. However, in zebrafish and frogs it is possible to create viable uniparental diploids (Cheng & Moore, 1997). Recent work (Gertz et al., 2011) suggests that in humans 8% of heterozygous SNPs are associated with differential methylation in *cis*. In these cases, the vast majority of differential methylation between homologous chromosomes (>92%) occurs on a particular haplotype, as opposed to being associated with the gender of the parent of origin. This indicates that genotype affects DNA methylation far more than gametic imprinting does. Overall, this suggests that the influence of genotype on patterns of DNA methylation is widespread in the genome, and greatly exceeds the influence of

imprinting on genome-wide methylation patterns. DNMT3L has also been shown to be important for the methylation of transposable elements (Bourc'his & Bestor, 2004), which are reported to be methylated in zebrafish (Feng et al., 2010a). It would be of interest to find whether any of the previously mentioned zebrafish Dnmts can methylate transposable elements, and whether their perturbation results in lack of methylation and reactivation of such elements. The existence of alternative methylation mechanisms in zebrafish is made more appealing by the existence of apparent calponin homology domains in Dnmt3 and Dnmt7 (Figure 5), although what these mechanisms could be is currently a mystery and the role (if any) of these domains in Dnmt function is currently unknown.

Organism	DNMTs	MBDs	TETs	ZBTBs
Mouse	DNMT1 [ENSMUSP00000004202] TRDMT1 (DNMT2) [ENSMUSP00000114572] DNMT3A [ENSMUSP00000020991] DNMT3B [ENSMUSP00000051830] DNMT3L [ENSMUSP00000121562]	MBD1 [ENSMUSP00000025446] MBD2 [ENSMUSP00000073701] MBD3 [ENSMUSP00000089948] MBD4 [ENSMUSP00000032469] MBD5 [ENSMUSP00000036847] MBD6 [ENSMUSP00000026476] MeCP2 [ENSMUSP00000033770]	TET1 [ENSMUSP00000133279] TET2 [ENSMUSP00000043977] TET3 [ENSMUSP00000087049]	ZBTB4 [ENSMUSP00000104279] ZBTB33 [ENSMUSP00000110795] ZBTB38 [ENSMUSP00000121753]
Zebrafish	Dnmt1 [ENSDARP00000013243] Trdmt1 (Dnmt2) [ENSDARP00000048632] Dnmt3 [ENSDARP00000110904] Dnmt4 [ENSDARP00000053417] Dnmt5 [ENSDARP00000118597] Dnmt6 [ENSDARP00000104553] Dnmt7 [ENSDARP00000108732] Dnmt8 [ENSDARP00000029456]	Mbd1 [ENSDARP00000031774] Mbd2 [ENSDARP00000104037] Mbd3a [ENSDARP00000082941] Mbd3b [ENSDARP00000048774] Mbd4 Mbd5 [ENSDARP00000077389] Mbd6 [ENSDARP00000105745] MeCP2 [ENSDARP00000109399]	Tet1 [ENSDARP00000104252] Tet2 [ENSDARP00000101295] Tet3 [ENSDARP00000115297]	Zbtb4 [ENSDARP00000068451] Zbtb33 [ENSDARP00000096464] Zbtb38 [ENSDARP00000102342]
Chicken	DNMT1 [Q92072] TRDMT1 (DNMT2) [ENSGALP00000014121] DNMT3A [ENSGALP00000006352] DNMT3B [Q4W5Z3]	MBD2 [NP_001012403.1] MBD3 [ENSGALP00000001652] MBD4 [F1P366] MBD5 [F1NE18] MeCP2 [O42403]	TET1 [XP_421571.2] TET2 [ENSGALP00000017216] TET3 [ENSGALP00000021873]	Zbtb33 [ENSGALP00000038290] Zbtb38 [ENSGALP00000004524]
Xenopus tropicalis	dnmt1 [ENSXETP00000047703] trdmt1 (dnmt2) [ENSXETP00000057609] dnmt3a [ENSXETP00000002659]	mbd1 [Q66JI6] mbd2 [ENSXETP00000029954] mbd3 [ENSXETP00000029788] mbd4 [Q28HB5] mbd5 [ENSXETP00000062167] mbd6 [ENSXETP00000053901] mecp2 [ENSXETP00000003337]	tet2 [ENSXETP00000030770] tet3 [ENSXETP00000054061]	Zbtb33 [ENSXETP00000009735] Zbtb38 [ENSXETP00000056311]
Honeybee	Dnmt1a [GB19865-PA] Dnmt1b [GB15130-PA] Dnmt2 [GB10767-PB] Dnmt3 [D7RIF7]	Mbd3 [XP_392422.2]	Tet1 [GB13880-PA]	(none)

Table 1. DNMTs, MBDs, TETs and ZBTBs found in mouse, zebrafish, chicken, *X. tropicalis* and honeybee.

Maintenance of DNA methylation is generally more widely studied than *de novo* methylation. In mice, hemimethylated DNA is recognised and bound by UHRF1, which is required for the subsequent recruitment of DNMT1 (Sharif et al., 2007). Both of these proteins are conserved in zebrafish, and mutation of either results in a global reduction of 5mC levels (Goll et al., 2009;Tittle et al., 2011). Furthermore, Uhrf1 mutation phenocopies certain aspects of Dnmt1 mutation. However, it is not clear if there are similar methylation dependant patterns of misexpression in these mutants.

Sequencing of the zebrafish genome allowed a genome wide profile of 5mC to be obtained. Bisulfite sequencing (BS-seq) was used to profile the distribution and abundance of 5mC within 5dpf zebrafish embryos (Feng et al., 2010a). Methylation was found in all 3 sequence contexts – CpG (80.3%), CHG (1.22%), CHH (0.91%); where H stands for C, T or A. These levels were higher than is seen in mouse embryos for each category (CpG, 74.2%; CHG, 0.30%; and CHH, 0.29%); but roughly equivalent to E14 mouse ES cells (Feng et al., 2010a).

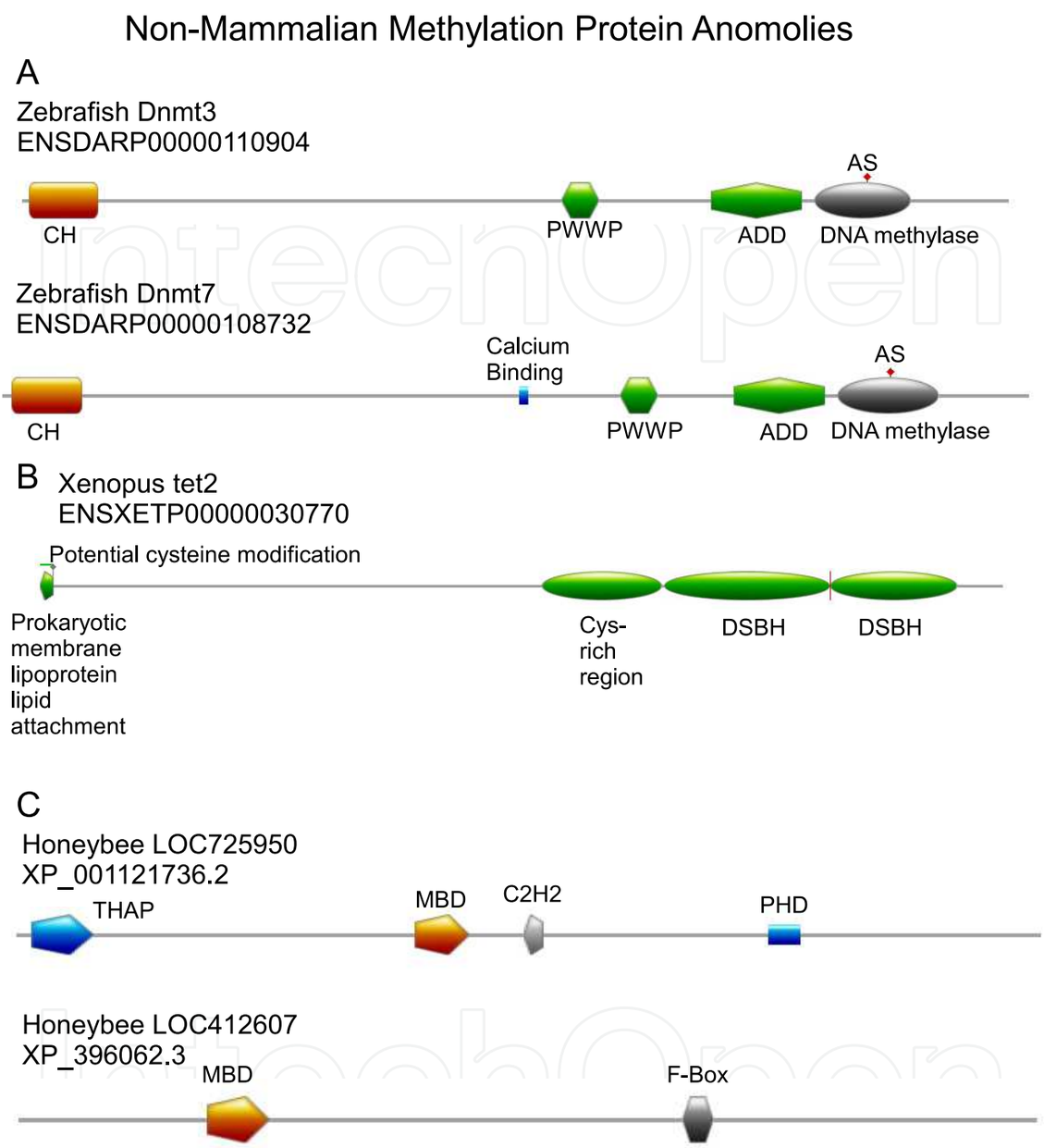

Fig. 5. **Non-mammalian methylation protein variants.** Domains were identified by searching PROSITE (http://prosite.expasy.org/) and the Sanger pfam database (http://pfam.sanger.ac.uk/) using the sequences belonging to the accession numbers shown. Diagrams were constructed using the output from PROSITE searches, and pfam output added to the diagrams using the MyDomains tool in PROSITE. A: Zebrafish Dnmt3 and Dnmt7 each contain a calponin homology (CH) domain of unknown function, not seen in other species. Dnmt7 also contains a calcium binding domain. B: *Xenopus tropicalis* tet2 contains a potential lipid attachment site normally seen in prokaryotes. C: In *Apis mellifera*, MBDs are present in architectures found in other flying insects, but not other animals (MBD-FBOX and THAP-MBD / THAP-MBD-PHD)

Especially interesting is the increase in non-CpG methylation seen in zebrafish embryos compared to mouse embryos, equivalent to a 3-4 fold increase; but similar to mouse ES cells. BS-seq showed that 5mC is modestly enriched at repetitive elements and within gene bodies, but depleted at CpG islands covering TSSs. Overall, the organisation of the 5mC methylome is similar in all vertebrates, which have methylation throughout the genome except at CpG islands (Feng et al., 2010a). Gene body methylation is conserved with clear preference for exons in most organisms.

Mammalian development is known to be highly dependent upon methylation and there are many studies which also highlight the importance of methylation in zebrafish. First, the apparent presence of maternal Dnmt1 in zebrafish oocytes points towards an essential role of 5mC in zebrafish development (Goll et al., 2009). Treatment of embryos with 5-azacytidine resulted in abnormal tail development, and incorrect patterning of somites (Martin et al., 1999). Morpholino knockout (zdnmt1MO) of Dnmt1 in zebrafish embryos has been shown to interfere with terminal differentiation of the intestine, exocrine pancreas and retina; but not the liver or endocrine pancreas (Rai et al., 2006). This is interesting as many embryos survive past gastrulation, unlike in mice. Surviving zdnmt1MO embryos exhibit developmental defects including curled tails, pericardial oedema, and jaw defects; with eyes appearing normal. This may suggest altered dependence on DNA methylation or Dnmts in zebrafish, or alternative mechanisms of DNA methylation maintenance in non-mammalian animals. It chimes with the situation in *Xenopus*, where certain key roles of DNMT1 do not depend on it methylating DNA (Dunican et al., 2008; Stancheva et al., 2001). Genetic analysis reinforces this difference, as zebrafish mutants in uhrf1 and dnmt1 also have defects in lens development and maintenance and they are not embryonic lethal, despite being hypomethylated (Anderson et al., 2009; Tittle et al., 2011). This suggests that attributing developmental defects in these mutants to hypomethylation may be premature, as the biology may be more complex. Here it is worth noting that Lsh mutants in mice are as globally hypomethylated as Dnmt1 mutants, yet certain Lsh -/- mutant mice exhibit growth retardation and a premature aging phenotype (Sun et al., 2004). This may be partly due to the different classes of gene misexpression compared to Dnmt1 mutants in mice (Myant et al., 2011). This implies that the role of DNA methylation and its associated components is still unclear, although DNA methylation patterns at promoters are highly correlated with transcription state.

Demethylation of DNA may occur either actively or passively, with passive demethylation occurring via the absence of methylation maintenance upon cell division and active methylation via direct enzymatic action resulting in the reversion of 5mC to C (Wu & Zhang, 2010). Active demethylation in the mouse is most obvious during early development, where detection of 5mC is rapidly lost in the male pronucleus (Santos et al., 2002), and also appears to occur during PGC development (Monk et al., 1987). Whether an equivalent process occurs within zebrafish is still unclear. Early experiments looking for 5mC changes during early zebrafish development did not detect any (Macleod et al., 1999), though this investigation was not comprehensive; while a more recent report did (Mhanni & McGowan, 2004). Promising results were obtained using immunological methods in zebrafish, and it was seen that methylation is lost around 1.5-2 hpf (cleavage and early blastula stages), and increases by 4 hpf (MacKay et al., 2007). This result is particularly encouraging, as the study made use of a similar technique to that used to obtain positive results in mouse (Santos et al., 2002). The authors suggest possible explanations for negative

results reported by other groups; including the relevant time point being missed, and potential contamination of samples with hypermethylated mitochondrial DNA (MacKay et al., 2007). In any case, the lack of large scale demethylation events would not prevent the existence of active demethylation, and there is evidence that such non-global processes occur in zebrafish. It has been shown that a methylated plasmid is demethylated when inserted into a one-cell zebrafish embryo, independently of DNA replication (Collas, 1998). It was also reported that the demethylase activity was dependent upon RNA, due to its inhibition by RNaseH. Upregulation of both MBD4 and AID results in global DNA demethylation, and both are found to be recruited to methylated DNA by Gadd45a (Rai et al., 2008). AID is a 5mC-deaminase which converts 5mC to T; and MBD4 has G:T mismatch thymine glycosylase. These enzymes are thus expected to promote demethylation via the conversion of 5mC to T, and the subsequent substitution of T to C. Interestingly, Gadd45a has been shown to bind neither single or double stranded DNA whether methylated or unmethylated (Sytnikova et al., 2011). However, RNA binding activity was observed, indicating that recruitment of the demethylation couplet may proceed via an RNA intermediate, explaining the RNA dependency reported.

Mammalian demethylation studies have recently benefited from a flurry of papers detailing 5hmC and its potential role in demethylation. This base is produced from the further modification of 5mC by TET enzymes (TET1-3), and appears to allow demethylation to occur through a series of intermediate conversions (He et al., 2011; Ito et al., 2011; Kriaucionis & Heintz, 2009; Tahiliani et al., 2009). It has been suggested that 5hmC is converted to 5-formylcytosine (5fC), then 5-carboxylcytosine (5caC); which is subsequently substituted for cytosine by the action of TDG (Ito et al., 2011; He et al., 2011). This pathway is yet to be studied in zebrafish, though at least one unpublished report exists claiming the presence of 5hmC in zebrafish (Yen & Jia, 2010). All 3 mammalian TET proteins have homologous sequences in zebrafish (see Table 1). Mammalian 5hmC could be suggested to have a dual functional role, as it does not appear to be immediately converted, and is present at considerably greater levels than 5fC and 5caC. Such roles could include repression of transcription SIN3A recruitment (Williams et al., 2011) and hypomethylation of target regions allowing PRC2 recruitment (Wu et al., 2011). It will be interesting to explore the distribution of 5hmC and TET binding/expression profiles in zebrafish. Immunostaining of mitotic chromosome spreads of mouse pre-implantation embryos demonstrated that paternal 5hmC is gradually lost during pre-implantation development (Inoue & Zhang, 2011). Here it is suggested that although the conversion of 5mC to 5hmC in zygotes is an enzyme-catalysed process, loss of 5hmC during pre-implantation may be a DNA replication dependent but passive process.

3. Chicken – *Gallus gallus*

DNA methylation has long been studied in the chicken, with the globin genes being the primary model of study; and is complimented by structural studies (Heitmann et al., 2003; Scarsdale et al., 2011).

The first evidence for functionally relevant DNA methylation in chicken came when methylation sensitive restriction enzymes were used to study the methylation profile around the chicken beta globin gene (McGhee & Ginder, 1979). Lack of methylation was observed in tissues expressing the gene; while tissues not expressing it showed high

methylation in the region. Soon after this initial study, it was found that around the genes encoding ovalbumin, conalbumin, and ovomucoid; DNA methylation was reduced in the oviduct when they are expressed (Mandel & Chambon, 1979). It was also noted that the highest levels of methylation were found in sperm. The observation that methylation is particularly high in sperm was replicated in a study reporting a negative correlation between methylation and expression of the adult and embryonic alpha globin genes during chicken development (Haigh et al., 1982). Despite these results linking methylation to expression for globin genes, *in vivo* demethylation by 5-azacytidine treatment in adult chickens did not result in reactivation of embryonic alpha globin (Ginder et al., 1983); it was also subsequently shown that a difference in methylation profile does not necessarily correspond to a difference in expression level (Cooper et al., 1983). The link between methylation and expression was nevertheless enhanced by experiments showing by HPLC and restriction analysis that chicken methylation profiles appear to change in an age- and tissue-dependent manner (Harasawa & Mitsuoka, 1984). This idea is strengthened by a more recent study showing significant differences in global methylation levels between different chicken tissues (Xu et al., 2007), but this may only be true for a subset of genes, as no correlation was observed between methylation of the lysozyme promoter and its transcriptional activity (Wolfl et al., 1991).

Structural evidence for a link between methylation and expression of target genes in chicken came when it was shown that the presence of methylation at just 3 CpGs in the beta globin promoter was sufficient to exclude binding of histone proteins (Davey et al., 1997). The finding that a small number of CpGs could be the major determinant in gene expression changes was also seen when chickens were transfected with GFP under the control of the RSV promoter (Park et al., 2010). It was found that GFP expression varied between tissues, but didn't appear to have inserted into a tissue specific gene cluster. To test if methylation was involved in this, the methylation status of the promoter was tested, and was seen to be slightly lower in tissues with higher GFP expression, but mainly at the set of CpGs at the very start of the promoter, where the majority of CpGs were unmethylated.

The methylation of the GFP reporter construct demonstrates *de novo* methylation in chicken. The chicken genome contains homologues of each mammalian DNMT (Table 1) with the exception of DNMT3L (Yokomine et al., 2006) each having high conservation with mammalian proteins; with DNMT1 having 94% identical amino acid sequence (Tajima et al., 1995).

The chicken genome consists of 39 chromosomes, 33 of which are classed as microchromosomes (McQueen et al., 1998). These microchromosomes are gene rich, early replicating and enriched for CpG islands (McQueen et al., 1996, 1998); but remain incompletely sequenced due technical difficulties (Dodgson et al., 2011). The sequencing of the chicken genome (International Chicken Genome Sequencing Consortium, 2004) allowed a genome-wide methylation profile to be obtained. Liver and muscle tissue was analysed from two breeds of chicken, and found to be enriched within gene bodies and at repetitive sequences but depleted at TSS and TTSs. The majority of CpG islands were in an unmethylated state, and promoter methylation correlated with gene expression. No differentially methylated regions were found, consistent with the previous lack of evidence for imprinting in birds and the lack of a DNMT3L homologue in the chicken genome sequence. UHRF1 appears in the chicken genome, as do several MBDs (Table 1 and Figure 2).

A potential active demethylation system in chicken was first seen when chicken embryonic nuclear extracts were shown to be capable of demethylation, and that the extent of demethylation was different across developmental time points (Jost, 1993). This action was subsequently suggested to involve the combined action of glycosylases, (Jost et al., 1995) and repair pathways (Jost et al., 1995; Zhu et al., 2000) in an RNA dependant manner (Fremont et al., 1997; Jost et al., 1997). There is potential for 5hmC to be involved in this pathway, as the chicken genome contains potential homologues for all three TET proteins, which share all domains with their mouse equivalents (Table 1 and Figure 3).

A particularly interesting region found in the chicken is the chicken male hypermethylation region (cMHM). This region is hypermethylated in males but hypomethylated in females where the region is transcribed into ncRNAs which accumulate at DMRT1 – a gene required for chicken testis differentiation; and in a region enriched for dosage compensated genes. A recent study investigated this region in chickens subject to sex-reversal (Yang et al., 2011). Female chickens were sex reversed by the injection of fadrozole into eggs, and grouped into different states of sex reversal: slightly sex reversed, or highly sex reversed and compared to standard males and females. The cMHM in gonad cells was seen to be highly methylated in males and hypomethylated in standard females, as expected. Methylation was slightly increased in the slightly sex-reversed group of females, and there was no significant difference in cMHM methylation between highly sex-reversed chickens and males. However, in liver the cMHM was hypermethylated in males, and low in females but remained low in each of the sex-reversed groups. DMRT1 expression was seen to increase towards male levels upon sex-reversal, but the expression of other sex specific genes changed less dramatically. A link between chicken DNA methylation and development was further seen by the interesting regulation of DNMTs by miRNAs during PGC development (Rengaraj et al., 2011), a finding similar to that seen in mouse PGCs (Takada et al., 2009).

Due to the extensive use of chick in agriculture, study of disease in the organism is relevant not only as models for human diseases; but also due to potential economic benefits. A recent study sought to find links between DNA methylation and neoplastic diseases in chickens using two lines, one susceptible to tumours and one resistant (Yu et al, 2008a, 2008b). Possible links between methylation of viral DNA (Yu et al., 2008a) and DNMT genes (Yu et al., 2008b) were reported. The general importance of methylation to chicken health was seen when *in vivo* treatment with 5-azacytidine negatively affected lymphatic organs, and increased the prevalence of autoimmune diseases in chicks (Schauenstein et al., 1991). Findings particularly relevant to humans came from experiments which used betaine as a dietary supplement (Xing et al., 2011) - a methyl donor known to reduce fat deposition. A modest reduction and change in pattern of promoter methylation was noted for the LPL gene, involved in lipoprotein catabolism; which coincided with a change in LPL levels.

4. Frog – *Xenopus tropicalis / Xenopus laevis*

Xenopus laevis has a solid history in methylation research, and was used in the experiments which pioneered the use of methylation-sensitive restriction enzymes to analyse gene expression (Bird & Southern, 1978). This method allowed extensive discoveries to be made and permitted functional DNA methylation studies to begin.

Although both *X. laevis* and *X. tropicalis* have a DNMT3 homologue, *de novo* methylation in *Xenopus* is not well studied, and it is notable that they do not appear to have a DNMT3B homologue (Table 1). When *Xenopus* eggs were injected with *Xenopus* globin transgenes, no *de novo* methylation was observed (Bendig & Williams, 1983; Harland, 1982). *In vitro* methylation of the transgenes did, however, result in the maintenance of transgene methylation, and importantly, low transgene expression was observed regardless of methylation status (Bendig & Williams, 1983).

It has been shown that when *in vitro* methylated plasmids are injected into *Xenopus* embryos, the methylation is maintained (Harland, 1982) and that adenoviral genes injected into *Xenopus* were repressed by methylation (Vardimon et al., 1982b). Further studies went on to show that methylation is only repressive at certain CpG sites (Langner et al., 1984; Vardimon et al., 1982a, 1983). An attempt to link gene induction by estrogen to methylation was unsuccessful, as no methylation change was detected (Folger et al., 1983).

Dnmt1 was confirmed to be expressed in *Xenopus* (Kimura et al., 1996) and is accumulated in oocytes (Kimura et al., 1999), suggesting an *in vivo* importance during development. Functional evidence for such a role came when dnmt1 depletion in embryos resulted in temporal misexpression of genes, with various developmental markers being expressed early (Stancheva & Meehan, 2000); and misexpression of dnmts was linked to activation of apoptosis by various studies (Kaito et al., 2001;Kimura et al., 2002; Stancheva et al., 2001). However, it has recently been shown that xDnmt1 can alter gene expression in a methylation independent manner (Dunican et al., 2008), making it possible that the apoptotic phenotypes were also methylation independent. Further, when the function of dnmt1 oocyte accumulation was probed using monoclonal antibodies for xDnmt1, the resulting inhibition of cell division was also found to be unrelated to methylation (Hashimoto et al., 2003). Nevertheless, several studies link methylation to expression in *Xenopus*, such as the finding that methylation directly inhibits transcription (Harvey & Newport, 2003; Lopes et al., 2008); 5mC promotes HDAC mediated transcriptional repression (Jones et al., 1998; Wade et al., 1999); and the finding that the methyl CpG binding activity of the transcription factor Kaiso is specifically required during development; although some of the Kaiso targets do not appear to be directly regulated by promoter methylation (Ruzov et al., 2009a, 2009b). Kaiso can also interact directly with components of the Wnt signalling pathways in development and cancer, which may expand its functional repertoire (Ruzov et al., 2009a). It was demonstrated that the zinc finger regions (ZF1-3) of xKaiso (*Xenopus*), dKaiso (*Drosophila*) and gKaiso (chicken) are sufficient for direct interaction with a terminal component of the canonical Wnt pathway, xTcf3, via its HMG domain *in vitro* (Ruzov et al., 2009a); suggesting that the interaction between Kaiso and Tcf3/4 is mutually exclusive of their DNA binding. Over-expression of xKaiso in developing *Xenopus* embryos actually mimics certain aspects of xTcf3 depletion, such as ectopic Siamois expression. A potential intersection of Kaiso with Wnt signalling pathways may occur in cancer, where over-expression of Kaiso could attenuate constitutive Wnt signalling, while at the same time promoting cancer progression through silencing of *de novo* methylated tumour suppressor genes (Lopes et al., 2008). Recently published work shows that disruption of the Tcf4:Kaiso interaction in human colon cancer cell lines releases Tcf4, enabling its mutual association with β-catenin and the formation of a transcriptional complex (Del Valle-Perez et al., 2011). This also permits Kaiso binding to the methylated CDKN2A promoter in cells, leading to its decreased expression.

xMBD3 is highly expressed in *X. laevis* embryos, in a spatially distinctive pattern which overlaps significantly with DNMT1 expression (Iwano et al., 2004). Knockdown of xMBD3 resulted in the eye developmental transcription factor Pax6, consistent with the eye being one of the regions with particularly high xMBD3 expression. This is in contrast with Mbd3 being indispensible for mouse development (Hendrich et al., 2001) The *X. tropicalis* genome appears to contain multiple MBD proteins (Table 1).

The xDnmt1 sequence has all the hallmarks of a maintenance methyltransferase which can propagate pre-existing patterns of methylation that are present in the early *Xenopus* embryos (Stancheva & Meehan, 2000). Transient anti-sense RNA depletion of xDnmt1 levels by 90% results in DNA hypomethylation and premature activation of gene expression before the mid-blastula transition (MBT), a developmental landmark that coincides with general zygotic gene activation (Dunican et al., 2008; Newport & Kirschner, 1982; Stancheva & Meehan, 2000). Loss of xDnmt1 results in embryonic lethality due to activation of a programmed cell death pathway (Jackson-Grusby et al., 2001; Stancheva & Meehan, 2000). DNA methylation levels recover after anti-sense RNA depletion of xDnmt1 suggesting that a functional de novo methylation pathway is present ion *Xenopus laevis* (Stancheva & Meehan, 2000). Morphant knockdown of xDNMT1 (xDMO) matched the anti-sense RNA phenotype by exhibiting p53 dependant apoptotic embryo lethality and premature activation of zygotic transcription before the MBT. Very surprisingly, this occurred without global changes in DNA methylation levels. The underlying explanation was that a moderate reduction in xDnmt1p levels was sufficient to prematurely activate gene expression in *X. laevis* embryos independently of changes in DNA methylation levels or histone modifications (Dunican et al., 2008). Crucially, repression of target genes was re-imposed in xDMO morphants by co-injection of mRNA encoding a catalytically inactive form of DNMT1. In addition, it was observed that histone modifications (H3K4me3, H3K9me2 and H4K20me3) accumulate after the MBT and are not prematurely accrued when xDnmt1 levels were reduced and transcription occurs *before* the MBT (Akkers et al., 2009; Dunican et al., 2008). Here there is separation between the transcriptional outcome and specification by the histone code. A model was proposed in which xDnmt1 has a major silencer role in early *Xenopus* development as a chromatin bound non-enzymatic protein that regulated the timing of zygotic gene activation (Dunican et al., 2008). xDnmt1p may serve as a titratable repressor component that has been previously invoked for *Xenopus* embryos (Almouzni & Wolffe, 1995; Newport & Kirschner, 1982; Prioleau et al., 1994)

The study of DNA methylation in amphibians is particularly interesting due to the possibility of regenerative mechanisms being elucidated. Methylation of the enhancer of *Shh* - the ZRS (Lettice et al., 2003), was found to be low in tadpoles, which have full limb regeneration ability, but high in adult frogs capable of only limited regeneration (Yakushiji et al., 2007). Re-expression of *Shh* has been shown to occur during limb regeneration in other amphibians (Imokawa & Yoshizato, 1997; Torok et al., 1999).

Although the majority of frog DNA methylation studies have been performed in *Xenopus laevis*, the recent sequencing of the *Xenopus tropicalis* genome (Hellsten et al., 2010) has allowed the 5mC profile of this organism to be investigated. Genome wide 5mC profiling in embryos revealed that 5mC is mainly present in the CpG context, 45% of which are methylated, but also as CpA methylation. It appeared particularly enriched within repetitive

regions (except for CpG depleted microsatellites), gene bodies and promoters; with TSSs being hypomethylated (Bogdanovic et al., 2011).

H3K4/27 trimethylation and RNA polymerase II (RNAPII) maps identify promoters and transcribed regions in *Xenopus tropicalis* (Akkers et al., 2009). Spatial differences in H3K27me3 deposition are predictive of localised gene expression. In agreement with a previous study, the appearance of K3K4me3 coincides with zygotic gene activation, whereas H3K27me3 is predominantly deposited upon subsequent spatial restriction or repression of transcriptional regulators (Akkers et al., 2009; Dunican et al., 2008).

Deep sequencing of purified methylated DNA obtained from early *X. tropicalis* embryos demonstrates that its genome is heavily methylated during blastula and gastrula stages (Bogdanovic et al., 2011). DNA methylation is absent in large H3K27me3 domains, indicating that these two repression pathways may be mutually antagonistic. Strikingly, genes that are highly expressed in *X. tropicalis* embryos but not in differentiated cells exhibit relatively high DNA methylation. Direct testing with reporter template demonstrates that methylated promoters are robustly transcribed in blastula- and gastrula-stage embryos, but not in oocytes or late embryos. This complements the situation in *X. laevis*, in which depletion of xDnmt1 leads to premature zygotic activation on a background of global methylation and a *tabula rasa* of histone modifications (Dunican et al., 2008). These findings have implications for epigenetic regulation of gene expression in early embryos and subsequent differentiation. It is noteworthy that mouse ES cells that lack *Dnmt1*, *Dnmt3a* and *Dnmt3b* survive in culture very well, but cannot differentiate properly (Tsumura et al., 2006).

It has been shown that global loss of 5mC does not occur during early *Xenopus* development (Stancheva et al., 2002; Veenstra & Wolffe, 2001). However, evidence exists for active demethylation; for example, demethylation of exogenous mouse *Oct4* is required for its transcription when micro injected into *X. laevis* oocytes (Simonsson & Gurdon, 2004), and gadd45a recruitment occurs at the demethylated region, with demethylation proceeding via action of the repair enzyme XPG (Barreto et al., 2007). It has subsequently been shown that *Xenopus* demethylation makes use of the NER system rather than BER (Schafer et al., 2010). Interestingly, gadd45a has been implicated in the switch from pluripotency to differentiation in the early *Xenopus* embryo (Kaufmann & Niehrs, 2011). *X. tropicalis* appears to possess homologues for at least TET2 and TET3 (Table 1 and Fig 3), but again the 5hmC system has not yet been investigated in *Xenopus*; though the results of such investigation would certainly be of interest. The apparent absence of a TET1 homologue (the only TET with a definite CXXC domain), is particularly interesting and could represent a demethylation mechanism absent in this species; although the function of the TET1 CXXC domain is still unclear (Frauer et al., 2011). Also notable is the presence of a potential lipid attachment site in Tet2, which is normally found in prokaryotes (Figure 1B).

5. Honeybee – *Apis mellifera*

The sequencing of the genome of the honeybee *Apis mellifera* (Honeybee Genome Sequencing Consortium, 2006) allowed potential DNA methylation genes to be identified. This was a significant turning point in the study of insect DNA methylation, with honeybee being the first insect shown to contain at least one homologue of DNMT1, 2 and 3 (Wang et

al., 2006). In contrast with mammals, the honeybee genome contains two homologues of the DNMT1 maintenance methylase, and one for the DNMT3 *de novo* methylases (Table 1); with each having high sequence conservation with the mammalian genes (Wang et al., 2006). The *Apis mellifera* genome also contains UHRF1, possibly indicating a conserved DNA maintenance methylation mechanism. Inevitably, once the genome sequence was available, a methylome was obtained (Zemach et al., 2010). This initial analysis was performed on DNA obtained from whole bodies of worker bees, and revealed that overall methylation levels in honeybee are low, with CpG methylation being 0.51%; CHG methylation 0.11%; and CHH methylation 0.16%. Hypomethylation of transposable elements was observed, with methylation mainly being observed within gene bodies, and no enrichment seen at promoters. Interestingly, when transcriptional activity was compared to CpG methylation it was seen that the methylation profile peaked at around the 50th percentile of transcriptional activity, with lowly expressed genes and highly expressed genes sharing a relatively low amount of CpG methylation. Due to this study being from whole bodies of honeybees, it is possible that the results are not biologically relevant, and are an artefact of the amalgamation of all cell types and tissues being analysed together. More informative honeybee methylomes were obtained when BS-seq was used to map the 5mC profile of *Apis mellifera* in brains from both worker and queen bees (Lyko et al., 2010). This allowed both the study of 5mC within a distinct tissue; and the comparison of 5mC profiles between two genomically identical castes with massive behavioural and physiological differences. This study replicated the finding that global levels of DNA methylation are low in honeybees, with approximately 70,000 cytosines methylated from a total of 60,000,000 in the genome (although it remains possible that methylation levels are high in other tissues), and showed that honeybee cytosine methylation is almost exclusive to CpG dinucleotides. Further, the vast majority of 5mC (over 85% in both workers and queens), was within gene bodies; most abundant in exons (over 75% in both castes) and found significantly at splice sites. The lack of methylation of repeats and transposons was also confirmed by this study, with the authors suggesting that it may represent that these elements do not confer genome instability in honeybees. Interestingly, the genes found to be methylated showed higher sequence conservation across a wide range of species than non-methylated genes. Finally, the authors found a significant group of genes to be differentially methylated between worker and queen bees, pointing towards DNA methylation being one mechanism of genetic control contributing to the differences between these castes. Interestingly, when Dnmt3 is knocked down in honeybees using siRNA, they are born with queen-like characteristics such as developed ovaries, mimicking the effects of a royal jelly diet (Kucharski et al., 2008). This study was complimented by the finding that DNMT3 activity reduced with each day that larvae were fed royal jelly (tested at days 3,4 and 5) (Shi et al., 2011). The existence of MBDs in the honeybee is unclear, with Mbd3 being the only classical-type MBD apparent in the genome (Table 1). However, MBD domains are seen in other contexts (Figure 1C). The hypothetical honeybee protein LOC725950 contains an MBD domain and shares most amino acid similarity in mouse with the PHF20L1 protein, which lacks any MBD. Another hypothetical protein in Apis, LOC412607, shares most amino acid similarity in mouse with the FBXL7 protein, with which it shares an F-BOX domain but no MBD. In fact, the MBD:F-BOX architecture appears to be unique to insects, based on the Sanger pfam

database. In both of these examples, the compared mouse proteins are probably not homologues, with similarity only being due to the shared domain. It is therefore likely that DNA methylation in honeybees is acted on via mechanisms unseen in non-insects. Further evidence for novel epigenetic systems in honeybee include the finding that hypermethylated genes tend to be significantly longer than hypomethylated genes (Zeng & Yi, 2010). Other potential MBD-containing proteins in honeybee include BAZ2B-like, and SETDB1, both of which appear to have legitimate homologues in mouse, which they share MBD and other domains with. Interestingly, honeybee appears to have a TET1 homologue complete with CXXC domain, and may therefore may have an active demethylation system making use of this protein, but not TET2/3 homologues (Table 1). Alternatively, TET1 may function non-catalytically to recruit/block other proteins.

6. Conclusions

Although interspecific 5mC distributions can vary, methylation of cytosine offers a selective potential as a mechanism for regulating gene expression across a wide range of species. The fundamental mechanism of DNA methylation mediated repression seems to be conserved, with active DNMT enzymes appearing in each of the species discussed, and each having homologues of UHRF1 and MBDs. Additionally, there is genomic evidence for active demethylation mechanisms via 5hmC in each of the species discussed, with each having at least one TET homologue present; although it is unclear whether large-scale active demethylation events such as those seen in mammalian development also occur in non-mammalian animals. It will be particularly interesting to find roles of active demethylation in non-mammalian animals; and to see whether TETs/5hmC are involved. Additionally, it will be interesting to investigate potential roles for domains apparently unique to non-mammalian methylation enzymes; such as the CH domain of zebrafish dnmt3/7; and the potential lipid attachment of *Xenopus* Tet2. However, attribution of the molecular pathology of mutants in components of DNA methylation machinery is not always so clear, especially as non-catalytic versions (e.g. in xDnmt1) can rescue phenotypes. For example, a model for the activation of xDnmt1-mediated apoptosis in *X. laevis* embryos has been proposed in which a chromatin associated complex of xDnmt11, xMbd4 and xMlh1 in embryos responds to DNA damage or replication stress by either repairing the lesion or to activate an apoptotic response through the activation/release of Mbd4/Mlh1 from chromatin-bound xDnmt1 (Ruzov et al., 2009c). The Mbd4/Mlh1 complex signals, perhaps via the DNA-damage kinases ATM and ATR, to activate the p53-dependent programmed cell death pathway.

A recent report has validated this model in human cells by demonstrating that DNMT1 is rapidly but transiently recruited to double stranded breaks (DSBs) (Ha et al., 2011), dependent on its ability to interact with both PCNA and CHK1, but independent of its catalytic activity. What is the potential importance of the Dnmt1 signalling mechanism? Cancers arise from the sequential acquisition of genetic alterations in specific genes leading to cellular transformation in step with epigenetic alterations (Fang et al., 2011; Figueroa et al., 2010). One possibility is that Dnmt1 is part of a signalling cascade that activates a barrier against tumour progression. Cellular sensitivity to changes in DNMT1 levels are lost when components of the signalling cascade are either absent or mutated, contributing to the generation of an altered cancer epigenome in tumours and cell lines. In a recent paper it was shown that depleting DNMT1 in proliferating human fibroblasts is sufficient to cause

mismatch repair defects and increased mutation rates at a CA17 microsatellite (Loughery et al., 2011). This is associated with decreases in mismatch repair protein levels, including MBD4 following activation of the DNA damage response (DDR). Blocking the DDR, and in particular PARP over-activation, also increases survival of the DNMT1 knockdowns.

Thanks to the study of DNA modification patterns, their generation and the consequences of inactivating components of the epigenetic pathways in different model organisms we are gaining a fuller understanding of their function in mammalian and especially human disease models.

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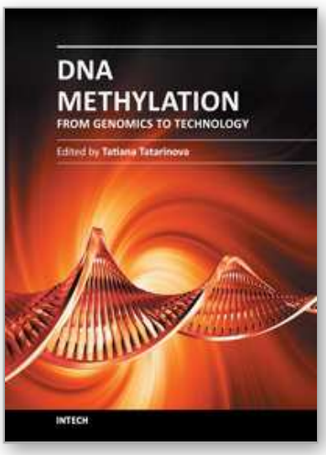

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Epigenetics is one of the most exciting and rapidly developing areas of modern genetics with applications in many disciplines from medicine to agriculture. The most common form of epigenetic modification is DNA methylation, which plays a key role in fundamental developmental processes such as embryogenesis and also in the response of organisms to a wide range of environmental stimuli. Indeed, epigenetics is increasing regarded as one of the major mechanisms used by animals and plants to modulate their genome and its expression to adapt to a wide range of environmental factors. This book brings together a group of experts at the cutting edge of research into DNA methylation and highlights recent advances in methodology and knowledge of underlying mechanisms of this most important of genetic processes. The reader will gain an understanding of the impact, significance and recent advances within the field of epigenetics with a focus on DNA methylation.

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