Tet proteins: master regulators of vertebrate body plan formation?

“A number of studies employing diverse model organisms have demonstrated the essential roles for Tet proteins during gastrulation and body plan formation.”

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DNA methylation dynamics & function

Early development is characterized by extensive epigenetic remodeling events that shape the regulatory landscape of the embryo. These changes drive and support cell-identity establishment and maintenance through the precise regulation of developmental pathways [1]. Perhaps one of the most conspicuous examples of such epigenetic remodeling is the global erasure and re-establishment of cytosine DNA methylation (mC; 5-methylcytosine) patterns in preimplantation embryos and primordial germ cells [2]. mC is a covalent modification of the DNA molecule typically associated with transcriptional repression. In vertebrates, this modification exists predominantly at the CpG dinucleotide and is involved in stable, long-term repression related to cellular processes, such as X chromosome inactivation, genomic imprinting and repetitive DNA silencing. The observation that *in vitro* methylated DNA is silenced when transfected into mammalian cells [3] led to the hypothesis that vertebrates might utilize such a system to dynamically regulate gene expression. However, DNA methylome maps generated by massively parallel sequencing technologies have not provided causal information to support such a scenario and the roles of developmental mC reorganization have remained largely elusive [4]. Notwithstanding these observations, a number of recent reports started to shed light on the diverse contributions of mC to developmental gene regulation [5–7].

Recent advances in the field have uncovered the family of ten-eleven translocation (Tet) methylcytosine dioxygenases (Tet1, Tet2, Tet3) as key contributors to developmental mC erasure in vertebrates. Tet proteins can sequentially oxidize mC to 5-hydroxymethylcytosine (hmC), 5-formylcytosine and 5-carboxylcytosine [8]. Thymine DNA glycosylases can excise 5-formylcytosine and 5-carboxylcytosine thereby creating abasic sites which are subsequently processed by the base-excision repair components, resulting in the reintroduction of unmethylated cytosines. The Tet protein family is known to play multiple roles in embryonic development, malignant transformation and gene regulation in differentiated cells.

Developmental roles of Tet proteins

The developmental requirements for Tet catalytic activity have proven particularly difficult to assess due to: Tet functional redundancy; noncatalytic roles of Tet proteins; experimental discrepancies in addressing loss of Tet activity; and possible distinct early and late Tet developmental functions. Furthermore, vertebrate species use diverse developmental strategies (i.e., internal vs external fertilization) that often result in the differential usage of gene regulatory pathways. For example, the extensive mC remodeling events observed in mammals, as well as phenomena, such as imprinting and X chromosome inactivation, are absent in anamniotes. These and other discrepancies often

Ozren Bogdanović

Harry Perkins Institute of Medical Research, University of Western Australia, Perth, WA 6009, Australia and

ARC Centre of Excellence in Plant Energy Biology, University of Western Australia, Perth, WA 6009, Australia
ozren.bogdanovich@uwa.edu.au

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make it difficult to extrapolate the results obtained in one species to a broader phylogenetic context.

Loss-of-function studies conducted in mice unraveled a function for Tet3 in the active demethylation of the paternal, and to a lesser extent maternal pronucleus in preimplantation embryos [9,10]. Importantly, deletion of maternal Tet3 did not affect the preimplantation development and embryos lacking maternal Tet3 could implant normally. The mutant embryos did, however, display a reduced rate of full-term development with a high frequency of degeneration and multiorgan abnormalities starting at midgestation (E8.5–E18.5) [9]. A subsequent study demonstrated through elegant genetic approaches that the phenotypes caused by maternal loss of Tet3 are due to Tet3 haploinsufficiency rather than defective paternal mC oxidation, which appears dispensable for mouse development [11]. Similarly, the combined loss of Tet1 and Tet2 in mice resulted in midgestation (E10.5–E16.5) abnormalities with some perinatal lethality, however, viable and fertile double knockout mice were also obtained [12]. Those mice, although viable and overtly normal, displayed globally reduced hmC and increased mC levels. Importantly, a notable upregulation of the Tet3 protein was observed in those knockout animals, suggestive of potential compensatory mechanisms. In total, these data unravel overlapping roles for Tet proteins during embryonic development and reveal difficulties in properly addressing Tet function in the presence of at least one functional Tet family member.

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Tet proteins control gastrulation & body plan formation

In nonmammalian vertebrates, such as zebrafish, the methylome reprogramming prior to totipotency establishment occurs in the absence of Tet activity and does not involve global mC erasure [13,14]. Tet expression in zebrafish starts during late gastrula stages coinciding with a global increase in hmC abundance [15]. In line with this observation, the triple Tet zebrafish mutant generated by transcription activator-like effector nuclease-mediated genome editing was compatible with early development but did not progress beyond the larval period due to combined mutations in Tet2 and Tet3 proteins [16]. The *tet2/tet3* mutant embryos advanced normally until 2 days post fertilization, when they started displaying phenotypes, such as altered brain development and morphology, reduced pigmentation, reduced eye size and trunk curvature.

A recent study that employed base-resolution methylome sequencing of blastula, gastrula, pharyngula and tailbud/fetal stages in zebrafish, *Xenopus* and mice unraveled highly conserved mC dynamics in the three species [6]. All three organisms displayed a developmental increase in the number of hypomethylated regions marked by hmC, coincident with the phylotypic stage (pharyngula), the most conserved phase of vertebrate development associated with body plan formation [17]. Importantly, these hypomethylated regions frequently overlapped with developmentally activated enhancer elements located near crucial genes implicated in TGF- β , Notch/Delta and Wnt signaling, suggesting a role for the modulation of enhancer mC state in the activation of this essential developmental toolkit. Morpholino injections targeting all three Tet transcripts (*tet1*, *tet2* and *tet3*), resulted in increased mC levels and reduced chromatin accessibility specifically at these regions, suggestive of high Tet specificity during these developmental stages, and a role for DNA demethylation in the activation of these enhancers. The triple Tet morphants displayed embryonic lethality, with 77% of embryos not surviving gastrulation, whereas the surviving embryos exhibited short and blended axes, malformed head structures, smaller eyes and reduced pigmentation. These phenotypes, although more severe, have some similarities with those observed in the *tet1/2/3* mutants [16]. The differences observed between the two loss-of-function strategies could be explained by the different sensitivity of these techniques to the maternal contribution of *tet1* mRNA [15], or potentially different levels of reduction in Tet function.

A more recent study employed germline deletion of all three Tet proteins in the mouse model system and similarly demonstrated strong requirements for Tet proteins during gastrulation [7]. Importantly, the triple Tet knockouts developed normally until E7.5 when they started displaying defects associated with impaired mesoderm migration. At E8.5 the embryos lacked structures characteristic of the early somite stage such as heart, somites, gut tube and headfolds. RNA-seq profiling of wild-type and mutant embryos identified *Lefty1* and *Lefty2* genes as significantly downregulated in the mutant. *Lefty* genes are antagonists of Nodal signaling and members of the TGF- β superfamily of regulatory proteins essential for mesoderm formation. Genes belonging to the TGF- β pathway have previously been shown to be enriched in regulatory elements associated with active, Tet-dependent demethylation [6]. Finally, the study by Dai *et al.* demonstrated that the requirements for Tet function are due to their catalytic activity, as proteins harboring point mutations in catalytic domains were not able to rescue the severe developmental phenotypes.

Taken together, the data obtained from the interrogation of triple Tet loss-of-function phenotypes in zebrafish and mice strongly suggest a previously underappreciated role for Tet proteins during vertebrate gastrulation and body plan formation associated with the activation of key developmental pathways. In both studies [6,7] a notable increase of mC in the triple Tet mutants/morphants was found on regulatory elements linked to developmental genes, indicative of an upstream role mC might play in the regulation of early embryogenesis.

Outlook

Recent advances in massively parallel DNA sequencing technologies have facilitated valuable insights into the developmental functions of the Tet protein family. A number of studies employing diverse model organisms have demonstrated the essential roles for Tet proteins during gastrulation and body plan formation. Nevertheless, many open questions remain. For example, despite a large number of studies, very little is known about the transcriptional regulation of Tet proteins during development and disease. A recent report demonstrated the sensitivity of Tet proteins to retinoic acid (RA) signaling through a conserved RA-responsive enhancer located within the *Tet2* locus [18]. These data provide further evidence as to the functions of Tet proteins in the early embryo, as RA is known to play essential roles during elongation of the embryonic body axis and organ formation. Subsequent studies will likely reveal the molecular components implicated in the regulation of other Tet family members. Another vastly underexplored aspect of Tet function is the mechanism of their recruitment to genomic target sites. Very little

is currently known regarding protein–protein interactions between the Tet family and transcription factors, or other molecular mediators, that can provide the specificity for targeted recruitment. In vertebrate embryos, sites of active, Tet-dependent demethylation are CpG rich [6], however, it is currently unclear whether such sequence composition is enough for the recruitment of Tet proteins, or whether additional factors are needed for this process. For example, a recent study demonstrated that the transcription factor Tex10 interacts with Tet1 to regulate super-enhancer activity in embryonic stem cells [19]. Finally, Tet proteins have also been shown to carry out functions independent of their catalytic activity [20]. These and other questions regarding Tet proteins have the potential to be resolved in the near future using a combination of precise (epi) genome editing tools, sensitive proteomics assays and sequencing technologies.

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