**TaDa! Analysing cell-type specific chromatin *in vivo* with Targeted DamID**

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**Abstract**

The emergence of neuronal diversity during development of the nervous system relies on dynamic changes in the epigenetic landscape of neural stem cells and their progeny. Targeted DamID (TaDa) is proving invaluable in identifying the genome-wide binding sites of chromatin-associated proteins *in vivo*, without fixation, cell isolation, or immunoprecipitation. The simplicity and efficiency of the technique has led to an ever-expanding TaDa toolbox. These tools enable profiling of gene expression and chromatin accessibility, as well as the identification of the genome-wide binding sites of chromatin complexes, transcription factors and RNAs. Here, we review these new developments, with particular emphasis on the use of TaDa in studying neuronal specification.

**Introduction**

The nervous system is composed of an enormous diversity of cell types that are linked together by an intricate network of connections. When studying genome-wide properties of chromatin in specific neural cell types, many techniques rely on the isolation of cells or nuclei from dissociated brain tissue [1–3]. These isolation steps are invasive and can lead to changes in the epigenome [4–6]. This is certainly the case for the nervous system, where cell-cell communication lies at the heart of its computational abilities. Furthermore, neural development relies on close contact between different cells to control the proliferation of neural stem cells, the orderly migration of young neurons and the directed outgrowth of axons and dendrites.

Targeted DamID (TaDa) can be used to probe the chromatin landscape in specific cells and tissues without the need for fixation, tissue dissociation and disruption of potentially important cell-cell contacts. When combined with next-generation sequencing (NGS), TaDa allows sensitive and specific identification of epigenetic modifications at a genome-wide scale.

**Targeted DamID for cell type specific chromatin analysis**

DNA adenine methylation identification, or DamID, was developed originally by van Steensel and Henikoff in 2000 [7,8] and has received increasing attention as a simple and robust method for identifying genome wide protein binding sites *in vivo*.DamID is based on the ability of the *E. coli* Dam methylase to methylate adenine residues in the sequence GATC [9,10]. By fusing Dam to a chromatin-associated protein, in a conformation which does not interfere with activity of the protein of interest [11], it is possible to methylate genomic DNA at the protein’s native binding sites [12,13]. While DNA adenine methylation has been observed in the genomes of various eukaryotic species (reviewed in [14]), endogenous levels are usually so low (less than 1 in 106 deoxy-adenines [15]) that they are insufficient to interfere with DamID. The absence of background DNA adenine methylation is critical for the high sensitivity and specificity of chromatin profiles acquired with DamID. In highly heterogeneous tissues, DamID methylation in even a small subset of the population can generate reproducible genome-wide chromatin signatures.

DamID has some limitations, including the need for ectopic expression of Dam protein fusions. DamID gives reduced sequence resolution, compared to ChIP-seq, as it depends upon the spacing of GATC-sites within the genome. Recently, MadID was developed to overcome this caveat and increase sequence resolution [16]. MadID makes use of the methylase M.EcoGII, which can methylate N6-adenosine in any sequence context. Expression of moderate to high levels of Dam methylase can also be toxic to cells [17] and for this reason, DamID experiments have often relied on low-level expression from basal, non-induced promoters [12]. Several attempts have been made to regulate levels of Dam-fusion protein expression, for example through insertion of recombination cassettes [18,19], the addition of a protein degradation signal [20], of a hormone-responsive element [21], or of a tamoxifen-inducible intein [22].

Dam methylase toxicity precluded the ability to target expression with cell-type specific promoters *in vivo*. To overcome this hurdle and achieve cell-type specific, inducible expression of Dam-fusion proteins, we developed Targeted DamID (TaDa) [17] (**Figure 1**). TaDa enables the identification of genome-wide chromatin binding sites *in vivo* without cell isolation, fixation or immunoprecipitation. TaDa relies on expression of a bicistronic message lacking an Internal Ribosomal Entry Site (IRES). The first open reading frame (ORF1) encodes a fluorescent protein (i.e., mCherry [17,23]), or a Neomycin resistance gene [24]. This is followed by two stop-codons and a frameshift mutation (5’-UAA-UAA-C-3’) upstream of ORF2, the coding sequence of the Dam fusion protein. ORF1 is translated at high levels while ORF2 is expressed at minimal levels due to infrequent ribosome reinitiation [25] (**Figure 1a**). As a result, TaDa yields cell- or tissue-specific DNA methylation without toxicity (**Figure 1b**). TaDa is highly sensitive, robust and reproducible and does not require cell sorting. It has been used successfully to profile small populations of cells in complex tissues, for example, 100 of the 150,000 neurons in intact *Drosophila* heads [16]. TaDa was first developed for use in Drosophila [17], and has since been widely adopted [26–36]. TaDa has also been modified for inducible expression in mammalian systems (MaTaDa, Mammalian Targeted DamID; [23,24]). As DamID has been successful in yeast [37], plants [38], C. elegans [39] and zebrafish [40], it should be possible to adapt TaDa for use in these systems as well.

A powerful application of TaDa is the ability to profile active transcription by monitoring genome-wide RNA Polymerase II occupancy. Targeted expression of Dam-Pol II has been achieved with the GAL4 system in *Drosophila* and yields results similar to those generated by ChIP-seq [13].TaDa has also been used to define changes in the chromatin landscape in the progression from neural stem cells to neurons [28,41] , to map transcription factor binding sites in many different tissues [23,24,26,33–36] and to investigate the role of long-noncoding RNAs in gene regulation [42]. As such, it is making significant contributions in our understanding of the dynamic changes in chromatin states and the regulation of gene expression during nervous system development and in the generation of neuronal diversity. Here we discuss recent innovations that contribute to an ever-growing plug-and-play DamID toolbox for cell-type specific analysis of chromatin (**Figure 2**).

**A TaDa toolbox**

***TaDa for transcriptional profiling* |** TaDa was first used to profile RNA polymerase II occupancy in the developing *Drosophila* optic lobe [17] (**Figure 2b**) to compare the transcriptional profiles of symmetrically dividing neuroepithelial cells and asymmetrically dividing neuroblasts, two clonally related neural stem cell types. This revealed the signalling pathways that are differentially regulated between these cell types, such as Notch, JAK/STAT, and EGFR. More recently, several hundred quiescent NSCs in the developing *Drosophila* CNS were transcriptionally profiled with TaDa leading to the discovery of factors regulating stem cell quiescence [27]. Amongst these, the pseudokinase Tribbles was shown to be necessary for entry into quiescence, as well as crucially important for its maintenance. Most notably, Tribbles specifically marked quiescent NSCs arrested in the G2 phase of the cell cycle, rather than G0. Thus, TaDa not only paved the way for the identification of factors involved in quiescence, but ultimately revealed a novel type of quiescent stem cell.

***TaDa* *for transcription factors and chromatin complexes* |** DamID has proven particularly successful in analysing the genome-wide binding patterns of transcription factors (**Figure 2b**) and chromatin complexes (**Figure 2c**). While ChIP-seq resolves local binding events with finer resolution, DamID is superior in its simplicity and efficiency [23,43]. This enables DamID experiments to be scaled up to profile many different proteins of interest, as illustrated by the parallel screening of 53 chromatin proteins in tissue culture cells in a single study [44]. Remarkably, despite the great number of possible interactions between these factors, the Drosophila genome is dominated by 5 major protein combinations. These combinations define the chromatin landscape and influence transcriptional activity. Filion and colleagues assigned colours to these chromatin types [44], defining heterochromatic, repressive GREEN chromatin (Su(var)3-9, HP1, Lhr, HP6), repressive, Polycomb-dominated BLUE chromatin (PC, E(z), Pcl, Sce), and the two active, euchromatic states, RED (brm, Su(var)2-10, MED21) and YELLOW (MRG15). This analysis distinguished two distinct euchromatic states, but also uncovered a previously unknown third repressive state spreading across half of the genome, BLACK chromatin (histone H1, D1, aurB, SuUR). The power of TaDa was demonstrated by translating these findings to an *in vivo* system by profiling specifically proteins that act as proxies for each of these 5 chromatin states (Brm for RED, RNA PolII for YELLOW, HP1a for GREEN, Pc for BLUE and histone H1 for BLACK) [28]. Thus, genome-wide changes in chromatin states could be traced from neural stem cells via ganglion mother cells to neurons during neurogenesis in the *Drosophila* brain. In this way, conceptually novel chromatin transitions independent of Polycomb were uncovered during neuronal differentiation.

***RNA-DamID* |** Long non-coding RNA (lncRNA)–chromatin interactions are thought to play important roles in development and disease. Whereas lncRNAs encoded by the dosage compensation genes *Xist* in humans and *roX* in Drosophila have long been known to play key roles in epigenetic modifications, the function of many lncRNAs remains a mystery. Approaches for easily profiling lncRNA-chromatin associations *in vivo* had been lacking, in particular for cell-type specific analysis. RNA-DamID was developed as a simple and sensitive technique to detect lncRNA–genome interactions in a cell-type-specific manner *in vivo*.

RNA-DamID takes advantage of the bipartite, bacteriophage MS2-MCP system: the MS2 coat protein (MCP) tandem dimer is fused to Dam methylase, while the lncRNA of interest is tagged with three MS2 RNA stem–loops [42]. Upon co-expression, MCP recruits Dam to the chromatin-associated lncRNA by binding to its MS2-tag (**Figure 2e**).

RNA-DamID has been used to perform genome-wide profiling of the *roX1* and *roX2* lncRNAs in *Drosophila* leading to the intriguing observation that dosage compensation may vary between cell-types [42]. Long-standing questions regarding the impact of lncRNAs in shaping the chromatin landscape and thereby influencing genome-wide transcriptional activity can now be addressed by leveraging the full potential of the TaDa toolbox: screening simultaneously the genomic sites occupied by lncRNAs, chromatin markers and RNA PolII. In this way the impact and function of lncRNAs can be profiled in unprecendented, genome-wide detail.

***Chromatin accessibility TaDa (CATaDa)* |** Before the introduction of DamID [7,8], Dam itself was used to study chromatin accessibility and nucleosome positioning in yeast and Drosophila [45,46]. These studies led to the observation that Dam can label all GATC-sites within the genome, but does so primarily in regions of low histone density and compaction. More recently, Dam-methylation has been used as a genome-wide proxy for chromatin accessibility in C. Elegans [39]. One of the latest additions to the TaDa toolbox, Chromatin Accessibility Targeted DamID (CATaDa), also exploits this intrinsic preference of the Dam enzyme. By expressing Dam with TaDa in a cell-type specific manner, sites of open chromatin are labelled on a genome-wide scale [41] (**Figure 2f**). This generates results which are highly comparable to FAIRE-seq and ATAC-seq [41]. As a result, TaDa experiments deliver two for one: in every DamID experiment the Dam-alone control yields valuable information on chromatin accessibility in parallel with the binding profile of the investigated protein.

**At the intersection between DamID and TaDa**

***DamC*** **|** The adaptability of the core concept of DamID made it possible to propose a replacement of 3C-derived methods for probing chromatin interactions [47,48]. In order to assess quantitatively chromosome structures *in vivo*, damC relies on Dam methylation signatures derived by recruiting Dam to experimentally defined binding sites. DamC works without crosslinking, DNA-digestion and religation. Thus, damC can for the first time validate chromosome folding, topologically associated domains (TADs), and CTCF-cohesin-associated loops in their native, nuclear environment.

DamC makes it possible to modify local chromatin looping by introducing novel binding sites for Dam constructs together with CTCF binding sites. Chromosomal interactions in the presence or absence of these newly generated loops can be followed during differentiation. These types of experiments promise to shed light on the significance of chromosomal interactions within TADs and their regulation in tissues throughout development.

***Single cell DamID* |** The nuclear envelope protein, LaminB1, is stably associated with chromatin and results in strong methylation when fused to Dam. In addition, LaminB1-Dam profiles are strikingly anti-correlated with the Dam-only binding pattern, which increases specificity in detecting LaminB1-bound regions. As a result it has been possible to detect LaminB1-Dam methylation in single-cells [49]. This is not yet the case for most other chromatin proteins and transcription factors, which tend to be bound more transiently. Moreover, the binary nature of either methylated or non-methylated GATC motifs limits quantitative analysis.

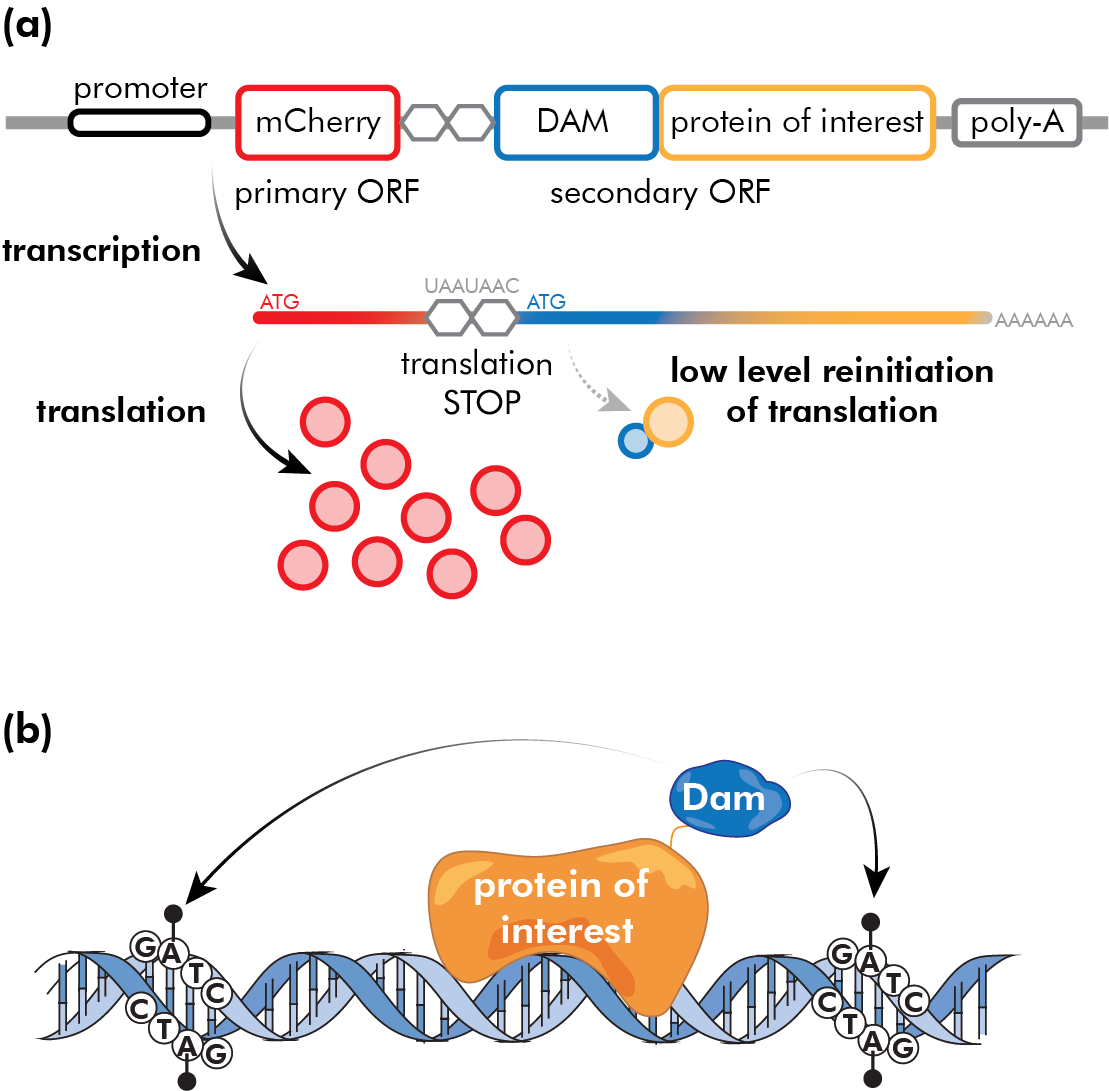
***Split DamID (SpDamID)*** | The Dam methylase can be separated into two complementary halves that by themselves are inactive; upon reconstitution the protein regains its catalytic activity. This behaviour is exploited in the Split DamID approach, in which the separate Dam-halves are fused to two different proteins that are suspected to physically interact when bound to DNA or chromatin [21]. To enable Dam reconstitution and DNA methylation, the two DNA binding proteins need to bind simultaneously and in close proximity. SpDamID has served to decipher the combinatorial binding of Notch, RBPJ, MAML and p300 during Notch-dependent alteration of chromatin accessibility and transcriptional activation *in vitro*. Its capacity to decipher the composition of transcriptional complexes *in vivo* remains to be explored.

***Methyl adenine identification (MadID*)|** MadID relies on the more promiscous methyltransferase, M.EcoGII [16]. This enzyme catalyses N6-adenine methylation irrespective of the surrounding sequence and is thus not limited to methylation of GATC sites [50,51]. MadID overcomes the bias in genome coverage dictated by the genomic distribution of GATC sites.. As proof of principle, MadID revealed binding to telomere sequences devoid of GATC-motifs by proteins of the inner nuclear envelope, such as LaminB1. If the specificity and sensitivity of the antibody for m6A-sites is sufficient, the proposed MadID-workflow could be adapted as a targeted approach.

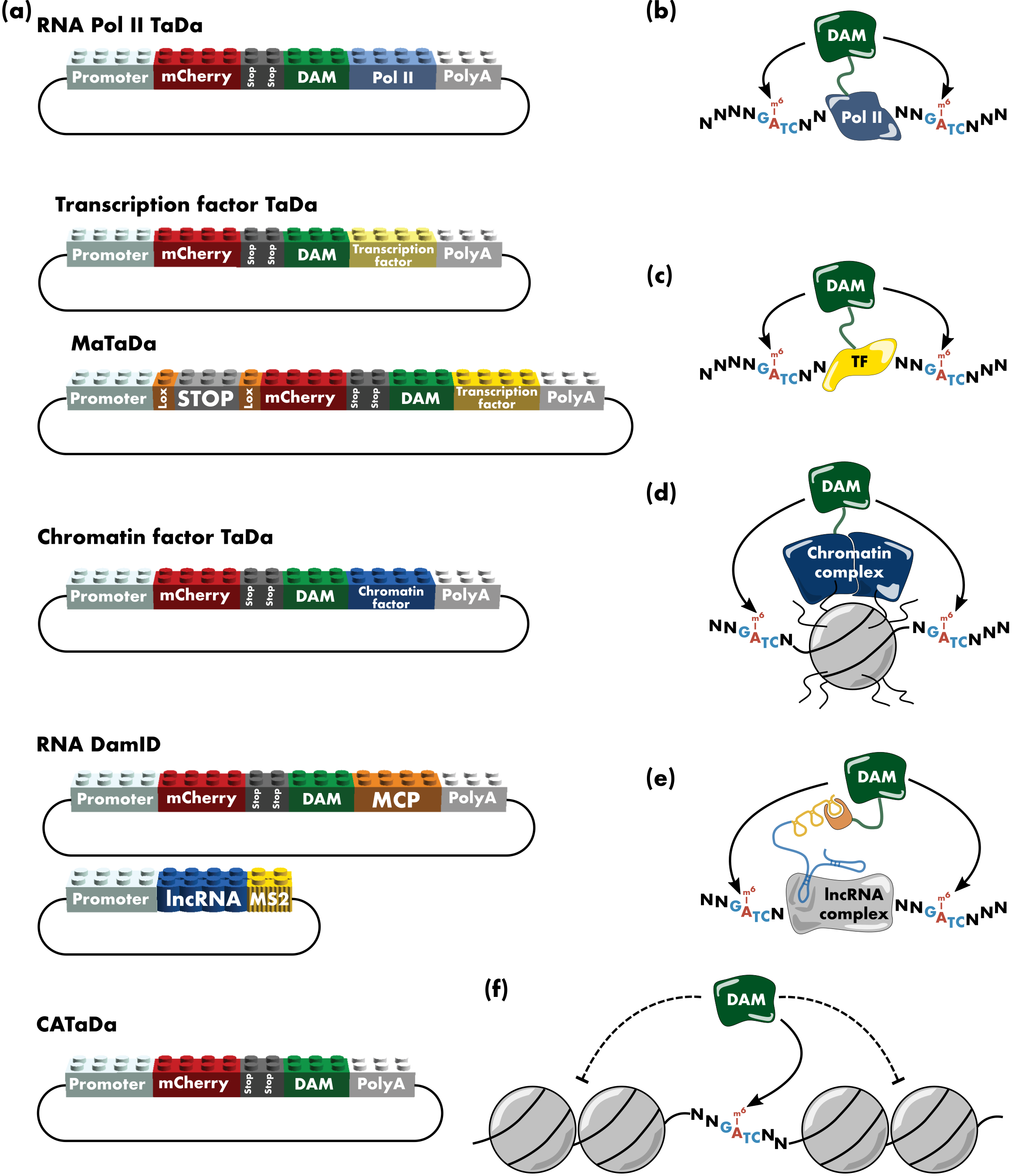
**Conclusion and future perspectives**

Since its introduction almost two decades ago [7,8], DamID has inspired technical innovations to enable genome wide profiling of a wide variety of chromatin binding proteins. A notable landmark in this series of innovations was the development of Targeted DamID for cell type analysis in intact organisms [17]. While the powerful genetics in *Drosophila* has clearly facilitated the use of TaDa, the technology has already been adapted for mammalian systems [23,24]. DamID has inspired new applications such as DamC to quantitatively assess chromosomal interactions [48]. In parallel, computational tools and pipelines are continuously deployed to model and interpret the raw data and intersect with datasets obtained using other techniques [40,48,52,53, our unpublished results]. Together, these are part of an ever-growing plug-and-play toolbox of DamID-related constructs, which may eventually ‘cater for all’. In the wake of single cell-based sequencing approaches, TaDa will prove to be an invaluable tool to validate gene-regulatory networks and probe the chromatin landscape in cell subtypes identified by scRNA-seq [54–56].

**Figures**



**Figure 1. Targeted DamID.** (A) Targeted DamID is a genetically encoded tool which allows cell-type specific expression of a protein of interest fused to E.coli Dam methylase. A promoter drives the transcription of a bicistronic mRNA transcript where two coding sequences are separated by two stop codons and a frameshift (UAA-UAA-C). The upstream open reading frame (primary ORF, mCherry in this case) is expressed at high levels when the promoter is active. Translation terminates at the stop codon (UAA), but very infrequently ribosomes reinitiate translation of the secondary ORF (Dam fused to the protein of interest). This results in low-level expression of the Dam fusion protein. (B) This low-level expression is sufficient to generate detectable methylation of GATC motifs near genomic binding sites of the protein of interest.

**Figure 2. A plug-and-play toolbox for Targeted DamID.** (A) Many different properties of chromatin can now be identified using TaDa; these innovations contribute to an ever-growing toolbox. (B) Fusion of Dam to RNA Polymerase II allows profiling of gene expression. (C,D) Fusion of Dam to a transcription factor of interest (C), or a subunit of a complex involved in reading or writing chromatin modifications (D), reveals the sites at which these proteins are bound to chromatin. (E) In RNA DamID, Dam is fused to the MCP (MS2 coat protein), which has a very high affinity for MS2 RNA -loops. When these MS2-loops are fused to a specific long noncoding RNA (lncRNA), co-expression of the Dam-MCP fusion protein will report where the lncRNA is associated with the chromatin. (F) CATaDa relies on the intrinsic affinity of Dam for all genomic GATC sites that are not shielded by other proteins, such as nucleosomes (closed chromatin). It therefore reports regions of open chromatin across the genome.

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**Highlights**

* Generation of neuronal diversity is tightly linked to epigenetic modifications
* Targeted DamID (TaDa) allows cell-type specific analysis of chromatin modifications
* An ever-growing toolbox of TaDa-related applications

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\* This paper not only characterises histone marks in specific cell types during neurogenesis, but also studies the functional role of a specific mark on one genomic locus through CRISPR-Cas9-based epigenome editing.