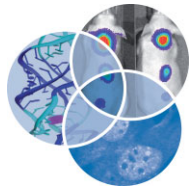
Advanced Review



The Bin3 RNA methyltransferase targets 7SK RNA to control transcription and translation

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Bicoid-interacting protein 3 (Bin3) is a conserved RNA methyltransferase found in eukaryotes ranging from fission yeast to humans. It was originally discovered as a Bicoid (Bcd)-interacting protein in *Drosophila,* where it is required for anterior–posterior and dorso-ventral axis determination in the early embryo. The mammalian ortholog of Bin3 (BCDIN3), also known as methyl phosphate capping enzyme (MePCE), plays a key role in repressing transcription. In transcription, MePCE binds the non-coding 7SK RNA, which forms a scaffold for an RNA–protein complex that inhibits positive-acting transcription elongation factor b, an RNA polymerase II elongation factor. MePCE uses *S*-adenosyl methionine to transfer a methyl group onto the γ-phosphate of the 5′ guanosine of 7SK RNA generating an unusual cap structure that protects 7SK RNA from degradation. Bin3/MePCE also has a role in translation regulation. Initial studies in *Drosophila* indicate that Bin3 targets 7SK RNA and stabilizes a distinct RNA–protein complex that assembles on the 3′-untranslated region of *caudal* mRNAs to prevent translation initiation. Much remains to be learned about Bin3/MeCPE function, including how it recognizes 7SK RNA, what other RNA substrates it might target, and how widespread a role it plays in gene regulation and embryonic development.

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INTRODUCTION

Bicoid-interacting protein 3 (Bin3) was first identified in the 1990's by a graduate student as part of her thesis project aimed at understanding how Bicoid (Bcd) directs anterior development in the fruit fly.[1] At the time, it was a novel protein, of large size (1368 aa) whose function was completely unknown. Bin3 is highly conserved and turns out to be very important; it is required for axis formation during *Drosophila* embryonic development[2] and plays critical roles in transcription and translation regulation. What helped set the field in motion was the discovery in 2007 of the human homolog [BCDIN3/methyl phosphate capping enzyme (MePCE); 689 aa], and the demonstration that it uses RNA rather than a protein as a substrate for methylation.[3] Its main target, the ubiquitous non-coding 7SK RNA, was already known to be a critical component of a regulatory complex that inhibits transcription elongation by positive-acting transcription elongation factor b (P-TEFb). In this review, we describe the important features of the Bin3/MePCE protein from *Drosophila* and humans, its major substrate (7SK RNA), functional partners (HEXIMs, LARPs, P-TEFb, Bcd), the structure of its catalytic domain, and the role of Bin3/MePCE in gene regulation and embryonic development. Finally, we speculate on additional roles of Bin3/MePCE and mention some major challenges that lie ahead.

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Bin3 IS A CONSERVED RNA METHYLTRANSFERASE

Discovery of Bin3

Bin3 was discovered in a custom yeast two-hybrid screen using *Drosophila melanogaster* Bcd as a bait protein.[4] Bin3 also interacts directly with Bcd *in vitro* in a GST pull-down assay.[4] The *bin3* gene is expressed during *Drosophila* oogenesis and in early embryos from maternal and zygotic promoters, respectively, and *bin3* mRNA is distributed uniformly in embryos with a peak of expression at about 2 h of development, coincident with Bcd.[2,4] *bin3* is also expressed during larval and pupal development, and in adults.[5] Both transcripts encode the same 1368 residue protein. The protein is highly conserved, and contains an *S*-adenosyl-L-methionine (SAM or AdoMet) binding domain, characteristic of DNA, RNA, and protein (Arg/Lys) methyltransferases.[6] Attempts to demonstrate protein methyltransferase activity were negative using histones, cytochromes, and Bcd as substrates.[1]

To understand Bin3, it is useful to introduce Bcd. Bcd is sequence-specific DNA binding (homeodomain) protein that is present in early *Drosophila* embryos in a steep anterior-to-posterior (A-P) concentration gradient.[7] Bcd determines cell fate by stimulating the transcription of target genes at discrete positions along the A-P axis.[8,9] Remarkably, Bcd also binds a specific RNA sequence, the Bcd response element (BRE), in the 3′-untranslated region (UTR) of *caudal* mRNA to repress its translation.[10,11] Bin3 is required for Bcd to prevent Caudal protein from being expressed in the anterior of the embryo, a prerequisite for proper head development.[12,13] We will return to Bin3's function in *Drosophila* development later in the review.

Conservation of Bin3 in Eukaryotes

Putative Bin3 orthologs can be found in a wide range of eukaryotic organisms from *Schizosaccharomyces pombe* to humans.[3,4,14] Phylogenetic analysis suggests an early evolutionary origin, with strong constraints on divergence limited to the AdoMet-binding domain (data not shown). A schematic of selected Bin3 orthologs and a sequence alignment of the conserved AdoMet-binding domain, with signature motifs indicated are shown in Figure 1(a) and (b). No additional conserved domains have been found. The previously noted 'Bin3 domain'[3,15] is actually the second part of the bipartite AdoMet-binding (SAM) domain characteristic of these enzymes and slightly more conserved in Bin3-family members. The *Drosophila* Bin3 protein is much longer than Bin3 in other organisms; the function of the additional sequences is not known. No ortholog of Bin3/MePCE appears to be present in *Saccharomyces cerevisiae*. It should be noted that there is another Bin3 protein in the literature, Bridging-Integrator-3 (also known as Tcp2), originally identified in *S. cerevisiae*,[16] but this is an unrelated protein. Orthologs of the *Drosophila* Bin3 protein (MePCE) are defined by the presence of the AdoMet-binding domain and are all putative methyltransferases.

Bin3 is the 5′-γ-Phosphate Capping Enzyme

Although Bin3 was first reported in 2000,[4] it was not recognized as an *RNA* methyltransferase. In a tour-de-force proteomic study of transcription factor networks, Jeronimo et al.[3] identified the human ortholog of Bin3 (BCDIN3) and showed it had RNA methyltransferase activity. In their study, BCDIN3 copurified with components of a regulatory complex that inhibits P-TEFb, an RNAPII elongation factor. P-TEFb consists of a cyclin-dependent kinase, CDK9 and CyclinT1 or CyclinT2, and is downregulated by sequestration into an RNA–protein complex (snRNP) containing 7SK RNA, hexamethylene bisacetamide-inducible protein (HEXIM)1/2, and La-related protein 7 (LARP7) (Table 1). Jeronimo et al. found BCDIN3 in a protein network that included all of these proteins, and made the critical connection that BCDIN3 was probably the long sought-after 7SK RNA 5′ methyltransferase[17,18] (Box 1). The sequence of human BCDIN3 contained the AdoMet-binding domain originally found in *Drosophila* Bin3, and indeed they showed that BCDIN3 directly and specifically mono-methylates 7SK RNA on its 5′γ-phosphate (Figure 2). Moreover, depletion of BCDIN3 from cultured human embryonic kidney cells (HEK293) using shRNAs significantly lowered 7SK RNA levels, consistent with the idea that uncapped 7SK RNA, like uncapped U6 RNA is sensitive to exonucleolytic degradation.[19]

BOX 1

THE UNUSUAL METHYLATED 5′ – γ-PHOSPHATE CAP STRUCTURE

There are three types of cap structures that characterize eukaryotic RNAs, each of which requires the activity of a distinct AdoMet-dependent methyltransferase.[21] The 5′-cap of most RNA polymerase II (RNAPII) products such as mRNAs and many viral RNAs consists of a 7-methylguanosine (m^7G) attached to the

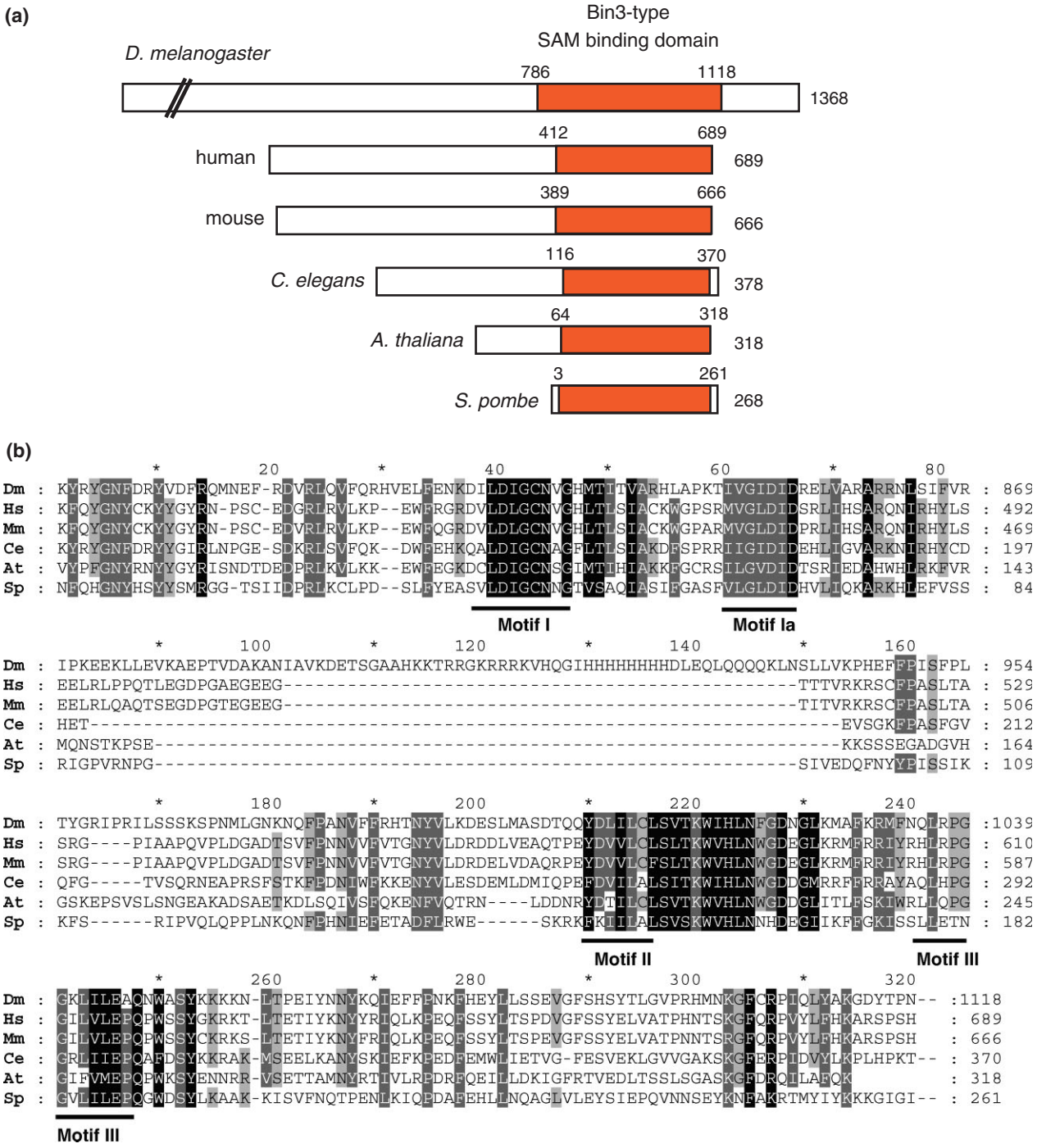

FIGURE 1 | (a) Alignment of Bin3-related proteins from selected species. The conserved catalytic domain, AdoMet (SAM)-binding domain is indicated. We extended the region based on sequence similarities in these Bin3/MePCE orthologs to the indicated residues. (b) Sequence alignment of conserved AdoMet (SAM)-binding domain. Note the extended region present in the *Drosophila* protein that is absent in the other orthologs. Motifs I, II, and III are generic motifs characteristic of all SAM-dependent methyltransferases including RNA, DNA, and protein methyltransferases.[6] Sequences are from *Drosophila melanogaster* (NP_ 724468.1; aa786-1118), *Homo sapiens* (NP_062552.2; aa412-689), *Mus musculus* (NP_659162.3; aa389-666), *Caenorhabditis elegans* (NP_496573.1; aa116-370), *Arabidopsis thaliana* (NP_568752; aa64-318), and *Schizosaccharomyces pombe* (NP_596220.1; aa3-261). The alignment was performed by using ClustalW (http://www.ch.embnet.org/software/ClustalW.html) with default parameters for all settings and formatted using GeneDoc (http://www.psc.edu/biomed/genedoc).

TABLE 1 | Bin3/MePCE-associated co-factors

Name	Notable motifs/Homology	Function	Interacts w/Bin3/MePCE[1]
Bin3/BCDIN3 (MePCE)	SAM (AdoMet) binding domain	RNA methyltransferase, targets 7SK RNA	—
7SK RNA	(RNAPIII transcript; 330–440 nt in length)	Non-coding scaffold RNA; forms snRNP that inhibits P-TEFb	Yes[3]
Transcription regulation			
CDK9 (human)	Kinase (yeast Bur1)	P-TEFb kinase; stimulates transcription elongation	No
CyclinT 1/2 (human)	Cyclin box (yeast Bur2)	P-TEFb regulatory subunit	No
HEXIM 1/2 (human)	Arg-rich motif (ARM)	Binds 7SK RNA; inhibits P-TEFb	?
LARP7 (human)	La-related protein 7; RNA recognition motif (RRM)	Binds 7SK RNA; inhibits P-TEFb	Yes[20]
Translation regulation			
Bicoid (*Drosophila*)	Homeodomain	Anterior morphogen directs head development, activates transcription and represses translation	Yes[4]
dLarp1 (*Drosophila*)	La-related protein 1; RNA recognition motif (RRM)	RNA binding protein, translation repression	?
AGO2	Argonaut, PAZ domain	RISC complex, RNA silencing; translation repression	?
PABP	RNA recognition motif (RRM)	Poly(A)-binding protein; stimulates (or inhibits) translation initiation	?
eIF4E		Translation initiation factor	?

AGO2, Argonaut 2;Bin3, Bicoid-interacting protein 3; HEXIM, hexamethylene bisacetamide-inducible protein; LARP7, La-related protein 7; MePCE, methyl phosphate capping enzyme; PABP, poly(A) binding protein; P-TEFb, positive-acting transcription elongation factor b; RNAPIII, RNA polymerase III; SAM, *S*-adenosyl-L-methionine; snRNP, sequestration into an RNA–protein complex.
[1]Direct interaction documented.

5′-nucleoside via a 'reverse' (5′–5′) triphosphate linkage (m^7GpppN). The methyl group is added to the N7 of guanine by a guanylytransferase. The capping reactions occur cotranscriptionally, and the final structure is permissive for translation by the ribosome. For non-coding RNAPII products including most small nuclear and nucleolar RNAs (e.g., U1, U2, U4, U5 snRNAs), this m^7G cap is further methylated by the enzyme Tgs1 to form a trimethylguanosine (TMG) cap.[22] Finally, some RNA polymerase III (RNAPIII) products including U6 snRNA, 7SK RNA, a mouse transposon-associated RNA called B2, and plant U3 snRNA, carry an unusual cap structure that does not utilize a 5′–5′-linked guanosine.[23] Instead, the cap simply consists of a methyl group added to the γ-phosphate of the terminally encoded guanosine residue (Figure 2).[17,24] This structure is not permissive for translation, and only occurs on small non-coding RNAs. Purification of the enzyme activity responsible for γ-methylation of U6 snRNA has been reported,[25] but the enzyme that methylates 7SK RNA had been more elusive until the discovery of the human Bin3 ortholog, BCDIN3, which has since been renamed MePCE for methyl phosphate capping enzyme.[3]

As a result of this work, BCDIN3 was renamed as MePCE. Although we will refer to the original fly protein as Bin3, the human and other orthologs are most often referred to as MePCE. Interestingly, both 7SK and U6 RNAs associate with BCDIN3/MePCE *in vivo*, but the level of U6 snRNA was not reduced in BCDIN3/MePCE knockdown cells.[3] Thus, it is not certain whether BCDIN3/MePCE targets only 7SK RNA or whether it carries out other 5′-γ-methylations in the cell.

Structure of the Human Bin3/MePCE Methyltransferase Domain

The three-dimensional X-ray structure of the human MePCE methyltransferase domain has been determined (PDB ID# 3G07) by the Structural Genomics Consortium in Toronto at 2.65 Å resolution.[26] The

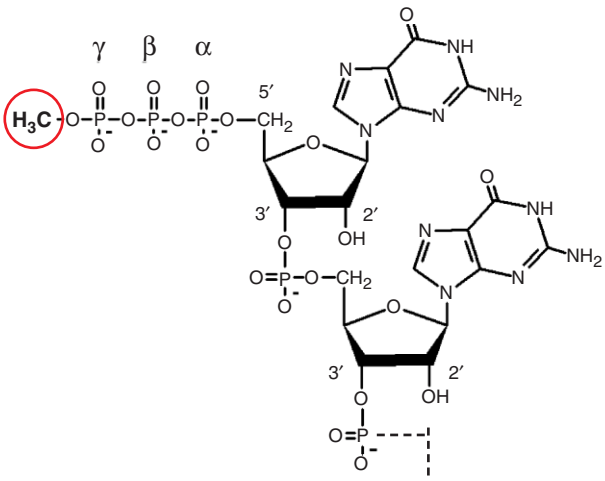

FIGURE 2 | Structure of the mono 5′γ-monomethyl guanosine triphosphate cap of 7SK RNA. The terminal and penultimate residues (GG) of 7SK RNA are shown with the methyl group added to the 5′-γ-phosphate (circled). This is added by Bin3/MePCE from using *S*-adenosyl-L-methionine (SAM) as a donor. This cap structure is different than the canonical m^7G cap of most eukaryotic mRNAs (Box 1).

model contains amino acids residues 431–487, corresponding to conserved motifs I and Ia; and residues 539–685, containing conserved motifs II and III. Fifty-two of the less conserved residues between motifs I and II were disordered and not modeled. In addition, amino acids 665–675 were not modeled.

The structure contains a classical α/β methyltransferase fold, or 'Rossman' fold, with a central parallel β-sheet containing a topological switch point in the center (Figure 3a). Like other nucleotide binding domains, SAM is bound in the cleft formed at the topological switch point that is lined with conserved residues from motifs I and II (Figure 3b). Binding is stabilized by numerous side chain and main chain hydrogen bonds (Figure 3c). Motif I interactions include a bidentate interaction between the carboxylate of D474 and the 2′- and 3′ hydroxyl atoms from the ribose moiety of AdoMet. Coenzyme binding is further stabilized by hydrogen bonds from the side chains of N453 and N559, and from the main chain carbonyls of G451 and L581 (Figure 3c). Residues I475, L583, and W586 form a hydrophobic pocket that surrounds the adenine moiety of SAM and the activated methyl group is oriented toward residues in motifs II and III. We speculate that residues Q618 and E616 from motif III and K585 from motif II might bind 7SK RNA, positioning the γ-phosphate moiety of the 5′ guanosine of 7SK for nucleophilic attack. The side chains of these residues are within 5–7 Å of the activated methyl group of AdoMet and could hydrogen bond or form charge-charge interactions with the oxygen of the γ-phosphate. However, it is also possible that the active site is rearranged upon RNA binding and other residues may perform this function.

On the basis of the structure of human Bin3/MePCE (Figure 3d) and the conservation of amino acids between human and fly orthologs, we created a homology model of *D. melanogaster* Bin3 using the Swiss Model server.[27] (Figure 3e). The model is missing amino acid residues 864–967 (*D. melanogaster* numbering) which corresponds to the *Drosophila*-specific insert (Figure 1b, and red dashes in Figure 3e), the function of which is unknown. Superposition of human Bin3/MePCE with the *Drosophila* homology model of Bin3 (Figure 3d), indicates the overall fold is similar. Most of the conserved residues in the putative active site map to similar positions in the *Drosophila* homology model, suggesting that they will have similar functions. For example, substitutions in *Drosophila* residues L1010, D826, G828, N830, and D851 (corresponding to human MePCE residues L581, D449, G451, N453, and D474, respectively) should compromise AdoMet binding, while subsitutions in residues Q1047, E1045, K1014 (corresponding to residues Q618, E616, and K585 in humans) might affect RNA binding. The *in vivo* importance of these residues could be tested using transgenic *Drosophila*.

7SK RNA IS THE MAJOR TARGET OF Bin3/MePCE

Basics of 7SK RNA

7SK is a small non-coding RNA discovered in human cells[28] and transcribed by RNAPIII.[29] 7SK gene transcription is directed from an upstream promoter element, which at the time, was unusual for an RNAPIII product.[30] In humans, 7SK is a 331 nucleotide GC-rich RNA. that is very abundant in cells (50–100,00 copies per cell), and localizes primarily to the nucleus, mostly within nuclear speckles, which contain mRNA processing factors.[31,32] In addition to its 5′-γ-methylation, 7SK RNA is adenylated at its 3′-end, as are other RNAPIII products such as ribosomal 5S and U6 RNA.[33,34] In 7SK, a single adenosine is added 3′ to the terminal UUUU sequence.[34] 7SK RNA is highly conserved[35], and although initially thought to exist primarily in vertebrates, detailed sequence analyses revealed 7SK genes to be widespread in metazoans, including *D. melanogaster* and *Caenorhabditis elegans*.[14,36,37] Interestingly, organisms with 7SK RNA have almost always coevolved with recognizable HEXIM, LARP7, and Bin3/MePCE orthologs.[14] Exceptions include *S. pombe*, which has Bin3/MePCE but no recognizable

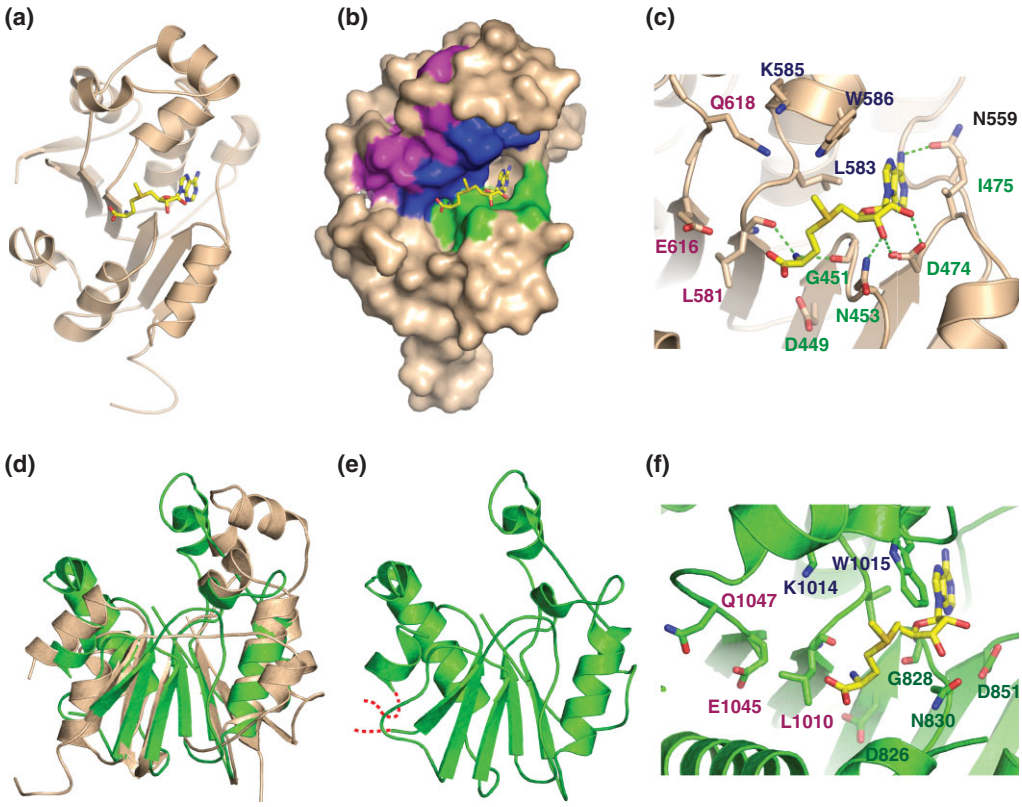

FIGURE 3 | (a) Cartoon diagram of the X-ray crystal structure of the catalytic domain of human Bcd-interacting protein 3/methyl phosphate capping enzyme (Bin3/MePCE; residues 431–685) bound to *S*-adenosyl-L-methionine (SAM or AdoMet) (Yellow). The structure was drawn with PDB coordinates 3G07 using PyMol. (b) Surface representation of human MePCE showing the locations of the consensus AdoMet-binding motifs I (green), II (blue), and III (purple).[6] (c) Active site of human MePCE highlighting hydrogen bond contacts (green dashed lines) between conserved MePCE residues and AdoMet. The position of the donor methyl group of AdoMet suggests that residues from motifs II and III may be involved in orienting the 7SK RNA substrate for methylation. (d) Superposition of human MePCE and *Drosophila* Bin3. (e) The *Drosophila* structure (green) is a homology model based on based on the human structure and refined with the Swiss Model server.[27] Residues modeled include aa806–864 and 981–1092. The modeled residues are 47% identical between human and *Drosophila* Bin3. The RMS deviation for Cα atom positions is 3.5 Å. (f) Putative active site of *Drosophila* Bin3 based on homology modeling. Shown are residues that could potentially interact with AdoMet and RNA as in (c).

7SK RNA or HEXIMs, and nematodes, which have 7SK RNA, HEXIM, and Bin3/MePCE but no LARP7.[14] Several excellent reviews about 7SK RNA have been published.[38–41]

Structure of 7SK RNA

Two major themes have emerged from computational and structural studies of 7SK RNA. First, 7SK RNA is highly structured and the motifs formed as a result of folding serve as binding sites for HEXIMs, LARP7, and other proteins in the P-TEFb inhibitory complex.[14,31,42,43] Second, the structure is dynamic, and as part of P-TEFb regulation, structural changes occur in 7SK RNA that accompany protein exchange.[44,45] Most predicted structures display four major stem-loop regions, with alternate substructures forming as a result of protein interactions (see Peterlin et al. al for review).[40] With respect to binding and 5′-γ-methylation by Bin3/MePCE, it is not known what sequences within 7SK RNA are required, although most models have the 5′ and 3′ regions of the RNA in close proximity. For U6 snRNA, the terminal guanine and a short stem-loop followed by the sequence AUAUAC was necessary and sufficient for γ-methylation by HeLa cell extracts[24], although it is not known whether Bin3/MePCE was the enzyme responsible.

Drosophila 7SK RNA

The *Drosophila* 7SK RNA transcript (444 nt) is significantly longer than its mammalian counterpart (330 nt),[36,46] and although the function of these extra nucleotides is not known, they were predicted to form an expanded loop between stems M4 and M5 that occur in the second major hairpin of mammalian 7SK RNA.[14] To better understand *Drosophila* 7SK

RNA, we utilized a computational analysis program known as Sfold[47] to predict its secondary structure (Figure 4). Using Sfold and alignment information and the consensus structure model from Marz et al.[14] we annotated the stems in our predicted structure (Figure 4). We found that M6 is absent as reported by Martz et al.[14] In addition, none of M2a, M2b, or M2c are present, and M4 is missing due to complete single-strandedness of the nucleotide block from U^{120} to C^{157}, which was aligned to a human 7SK RNA sequence block containing the 5′ end nucleotides in M4. A downstream neighboring nucleotide block (C^{168} to G^{190}) forms a new stem upstream of M5 with an extension. We named this new stem M4D (D for *Drosophila*). The stem-loops implicated in protein binding for human 7SK RNA, M1, M3, and M8 are retained in the *Drosophila* 7SK RNA, which in addition to M4D, also has a much larger M5 (Figure 4).

The 5′G that is methylated by Bin3/MePCE is indicated in bold as is a putative recognition motif, AUGUAC (Figure 4), based on the AUAUAC sequence utilized in U6 RNA.[24] Remarkably, this AUGUAC motif is located in the exact position relative to the 5′ G that is required for methylation in U6.[24] Curiously, this sequence does not seem to be well-conserved in human 7SK RNA (AAAUGA). Alternatively, the 3′ stem-loop whose presence (but not sequence) near the 5′G is conserved between *Drosophila* and humans could be important for recognition by Bin3/MePCE. In the long single-stranded 'bowling pin' region, about two thirds of the 89 nucleotides are *Drosophila*-specific, suggesting that this region may contain regulatory sites for *Drosophila*-specific RNA binding proteins.

Bin3/MePCE IN TRANSCRIPTION REGULATION

Regulation of P-TEFb Activity by the Bin3/7SK snRNP

P-TEFb is thought to be important for transcription of most if not all RNAPII-dependent genes (reviewed in Refs 38–40). P-TEFb works by overcoming promoter-proximal pausing of RNAPII, which may be a common regulatory step of transcription in metazoans.[51–53] The CDK9 catalytic subunit of P-TEFb phosphorylates Ser2 within the heptapeptide repeat $(YSPTSPS)_n$ of the carboxy-terminal domain of the large subunit of RNAPII, generating an elongation-competent form of the polymerase. P-TEFb also phosphorylates a negative elongation factor (NELF) leading to its release, and DSIF (DRB-sensitive inducing factor; aka Spt4/5), leading to its conversion into an elongating form. The action of P-TEFb is thus a key step in the conversion of initiated but stalled RNAPII into a form capable transcribing genes.

Where does Bin3/MePCE fit in? 7SK RNA, the target of Bin3/MePCE, was discovered to inhibit the activity of P-TEFb.[54,55] 7SK RNA serves as a scaffold for a snRNP, later shown to contain two RNA-binding proteins, HEXIM (hexamethylene bisacetamide-inducible) proteins 1 and 2,[56–58] LARP7 (La-related protein 7)[34,43,59], as well as Bin3/MePCE.[3] Bin3/MePCE and LARP7 are stably bound to 7SK RNA[34,43], while a HEXIM dimer binds reversibly and is required for inhibition of P-TEFb via interaction with the CyclinT subunits.[57,60] When HEXIM dissociates from 7SK, hnRNPs (hnRNPA1/2, hnRNPQ, hnRNPR) take its place.[61,62] Regulation of P-TEFb therefore occurs by a mechanism of sequestration and release from the 7SK snRNP (Figure 5). Knockdown of 7SK RNA was reported to disrupt the organization of the nuclear speckle, and cause upregulation of reporter genes, most likely because P-TEFb is released from the inhibitory 7SK snRNP.[32]

Bin3/MePCE plays at least two critical roles. First, it covalently modifies 7SK RNA via 5′ methylation, which is likely to protect it from degradation (and from translation). Second, Bin3/MePCE interacts directly with LARP7, and together these proteins bind cooperatively to 7SK RNA, further stabilizing the complex.[20] Interactions were also detected between Bin3/MePCE and both CDK9 and HEXIM1, but it is not known if these were direct or indirect.[3] In the absence of Bin3/MePCE, 7SK RNA levels are drastically reduced.[2,3] Without a functional 7SK snRNP, P-TEFb activity is unleashed and gene expression goes unregulated, which can lead to cancer and other disease states.[34,63–65] .

Several important cellular and viral signaling pathways converge on P-TEFb, resulting in phosphorylation/dephosphorylation of CDK9, CyclinT, HEXIMs, and Brd4, a protein that recruits P-TEFb to genes, thus controlling the activity of this critical regulator.[66–68] P-TEFb is a direct target of HIV Tat protein, which recruits P-TEFb to the TAR RNA element allowing expression of viral genes.[69,70] Regulation of P-TEFb activity is a very active area of research and has been reviewed elsewhere.[38,71] The human MePCE sequence contains 13 potential MAPK/CDK kinase sites (S/T-P).[72] It seems plausible that Bin3/MeCPE is a target for modification and/or regulation that would impact P-TEFb. For example, stimulation of ERK (extracellular-signal-regulated kinase; a MAPK) via T-cell receptor-mediated activation disrupts the 7SK snRNP complex, freeing P-TEFb

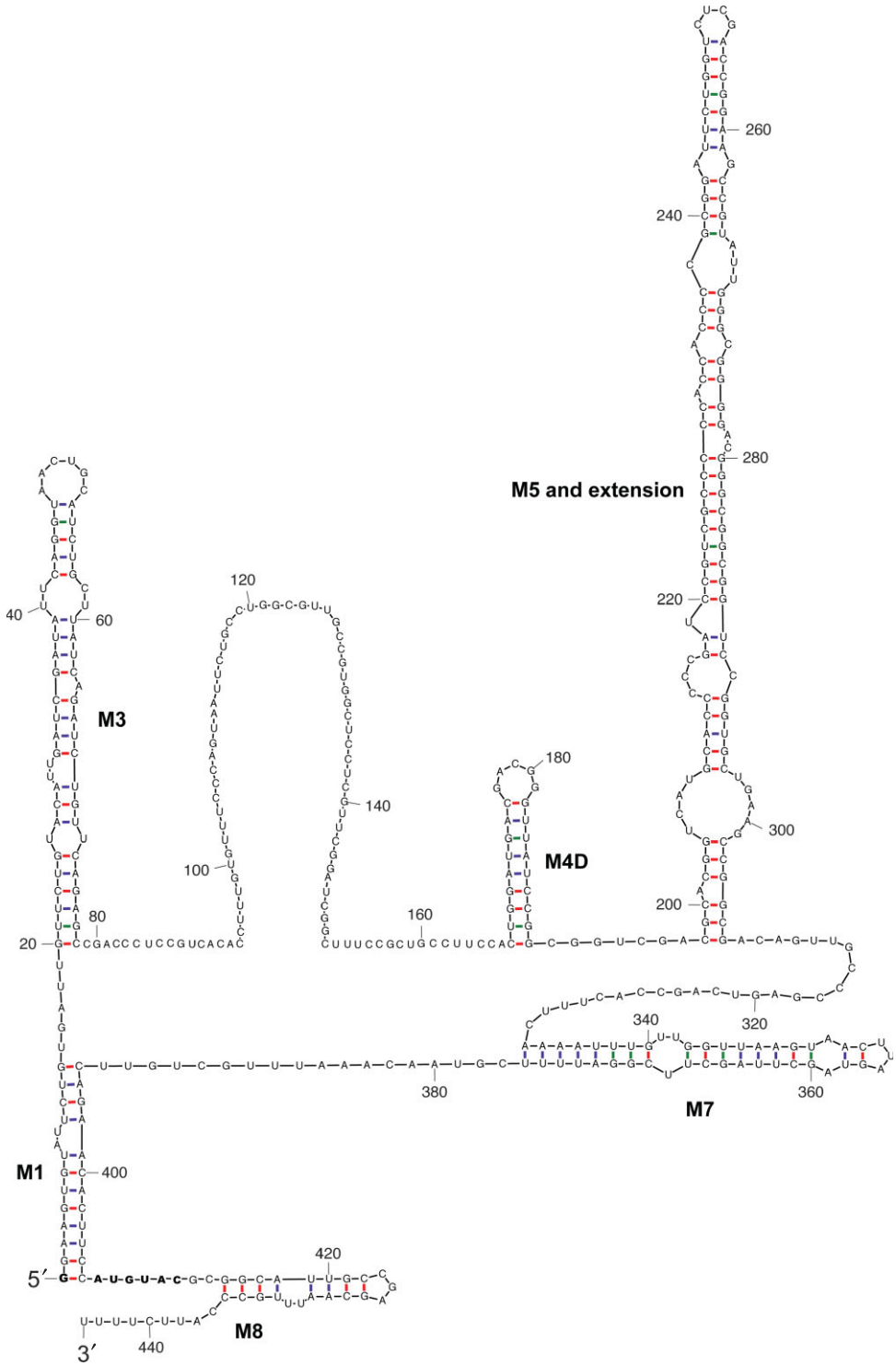

FIGURE 4 | Secondary structure prediction of *Drosophila melanogaster* 7SK RNA. Sfold software is based on structure ensemble sampling, structure clustering, and centroid representation of clusters.[48,49] It has been observed that the centroid structure of one of the clusters can often make an accurate structure prediction.[49] However, the identity of the best performing centroid is unknown without additional information. For human 7SK RNA, the centroid structure for one of four structural clusters closely matched the proposed consensus structure.[14] This indicated that, by using information from the consensus structure, we could identify the best performing centroid for structure prediction of 7SK RNA. For *Drosophila* 7SK RNA, we found that the centroid of one of two clusters closely resembles the consensus structure. This centroid is an informed predictor of the secondary structure for *Drosophila* 7SK RNA. The structure diagram was produced by the Sir_Graph program of the UNAFold package.[50] Red = G-C; blue = A-U, green = G-U base pairing.

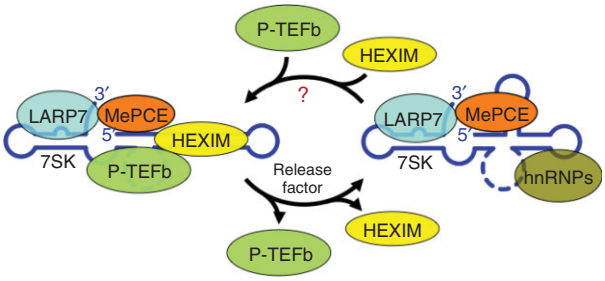

FIGURE 5 | Dynamic association of positive-acting transcription elongation factor b (P-TEFb) with the inhibitory 7SK snRNP. Figure summarizes work from a number of laboratories (reviewed in Refs 38–40). The P-TEFb transcription elongation factor is composed of CDK9 and CyclinT1 or T2. 7SK RNA forms a scaffold for an snRNP containing 7SK, LARP7, MePCE, and hnRNPs. When P-TEFb, along with a HEXIM1/2 dimer is incorporated into the 7SK snRNP, it displaces hnRNPs and inactivates P-TEFb. P-TEFb inactivation involves interaction between HEXIMs and the CyclinT subunit of P-TEFb. 7SK RNA folding is different in the two complexes, as it is known to undergo structural rearrangements. LARP7 and HEXIMs are shown in the approximate positions they bind to 7SK RNA based on chemical protection experiments. See text for details. Figure is from Peterlin et al. (2011).[40]

to enter the nucleus and promote HIV provirus elongation (independent of Tat protein)[67]. It is possible that ERK targets Bin3/MePCE for phosphoryation, as well as other components of the inhibitory snRNP to cause release and activation of P-TEFb.

Conservation of the P-TEFb System in *Drosophila*

While P-TEFb was originally discovered in *Drosophila* cell extracts,[73] it was unclear whether 7SK snRNP control of P-TEF was operational in this organism. In fact, only recently was it even possible to identify a 7SK gene in *Drosophila*. This was done by a novel strategy that took into account the 7SK gene's upstream RNAPIII promoter.[36] Singh et al.[2] used qRT-PCR to show that 7SK RNA is present in ovaries and embryos and is destabilized in *bin3* mutants, consistent with this transcript being a target for 5′-γ-methylation as in human cells.[3] They also found that a 7SK insertion allele was embryonic lethal (Singh and Hanes, unpublished). Nguyen et al.[46] then showed that all the key players in the 7SK RNA/P-TEFb regulatory network are present in *Drosophila* cells. In addition to identifying dHEXIM and dLARP7 orthologs, they showed these proteins bind to 7SK RNA, and are present in DRB- and flavopiridol-sensitive complexes along with CyclinT, as expected if they regulate P-TEFb by a conserved mechanism. These findings open the door to study of 7SK snRNP regulatory functions in a developmental context. Indeed, a series of UAS-GAL4 RNAi knockdown lines were used to show that loss of dHEXIM caused pronounced mutant phenotypes in the tissues in which it was reduced.[46] Further work using insertion and excision alleles of 7SK snRNP components will be useful for studying maternal and early embryonic functions.

Additional Roles for Bin3/MePCE in Transcription and RNA Processing

RNA splicing occurs cotranscriptionally, and RNAPII elongation rates can effect splicing efficiency and even splice-site choice.[74–77] Not surprisingly, defects in splicing are observed when regulation of P-TEFb by the 7SK snRNP is disrupted.[78] For example, RNAi depletion of Bin3/MePCE or LARP7 in HeLa cells and zebrafish embryos reduced 7SK RNA levels and altered the splicing patterns on selected mRNAs.[79] Interestingly, depletion of Bin3/MePCE in zebrafish lead to developmental defects in the anterior (brain) regions. No splicing defects were observed, however, in early *Drosophila* embryos[2], perhaps because the mRNAs were maternally-derived and elongation might not be subject to P-TEFb regulation during oogenesis.

Another role for 7SK RNA has been proposed.[80] This was based on the observation that 7SK RNA knockdown affected the expression of a larger set of genes than P-TEFb was known to regulate. Eilebrecht et al.[80] used a structural loop (L2) of 7SK RNA that is not involved in binding HEXIMs, LARP7, or MePCE, and biochemically identified HMG (high mobility group) protein A1 as a direct binder of 7SK RNA. HMGA1 normally binds DNA and functions as a chromatin architectural protein affecting the expression of a large number of genes.[81] They found that 7SK RNA competes for DNA binding with HMGA1 and that overexpression of 7SK RNA (L2) altered the expression of HMGA1-dependent genes, separate from P-TEFb effects.[82] For common targets it was proposed that HMGA1 recruits P-TEFb to genes via their interactions with the 7SK snRNP.[82] Specific roles for Bin3/MePCE in this function of the 7SK snRNP have not been described.

Bin3/MePCE IN TRANSLATION REGULATION

Bin3 is Critical for Embryonic Axis Formation

To study Bin3/MePCE's role in *Drosophila* development, Singh et al.[2] used site-specific recombination to generate precise excision alleles in the *bin3* gene that removed most exons including the entire AdoMet

(SAM)-binding domain. The surprise result was that although Bin3 was identified as a Bcd-interacting protein, *bin3* null mutants showed no effect on Bcd-dependent gene transcription. None of the Bcd target genes examined had detectable changes in abundance or spatial/temporal expression patterns. Instead, embryos from *bin3* null mothers failed to repress *caudal* mRNA translation in the anterior region of the embryo, leading to severe head involution defects and lethality (Figure 6a).[2] Notably, 7SK RNA levels were reduced dramatically in *bin3* mutants, 140-fold in ovaries and 50-fold embryos, respectively.[2] As in MePCE knockdowns in human cells, there was no effect on U6 snRNA levels in *bin3* mutants (Singh and Hanes, unpublished). Perhaps a different enzyme methylates U6, or uncapped U6 is not subject to degradation. There are two predicted Bin3-like proteins in *Drosophila* that might have capping activity (CG11342, CG1239).

A model for how Bin3 augments Bcd's function in translation repression is shown in Figure 6b. In this model, Bin3 directly binds to Bcd, which targets the 3′-UTR of *caudal* mRNA at the BRE. Bin3 methylates 7SK RNA and remains bound to it, protecting it from degradation. 7SK RNA functions as a scaffold to stabilize the formation of a repressive snRNP complex that contains poly(A) binding protein (PABP), La-related protein 1 (Larp1), and Argonaut 2 (Ago2), and inhibits initiation by preventing binding of required factors to eIF4E. Each of these proteins has been shown to play roles in translation repression in *Drosophila*.[83–87] The model is supported by double mutant analyses as well as RNA coimmunoprecipitation data.[2] These studies were the first indication that a distinct 7SK snRNP, which includes Bin3/MePCE plays a role outside of transcription.

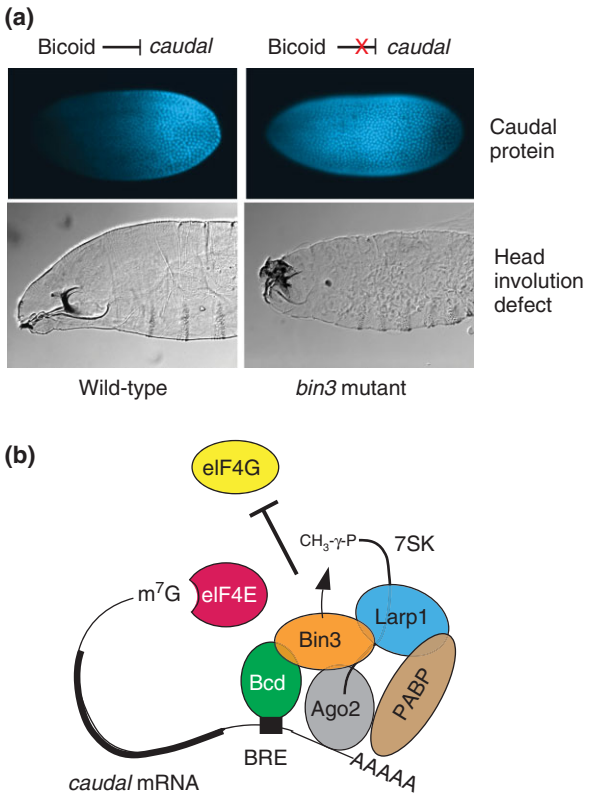

FIGURE 6 | (a) Bin3/MePCE is required for translation repression in early development. Blastoderm-staged embryos (0–2 h) are stained for Caudal protein (upper panels). Embryos and larvae are oriented with the anterior-left, dorsal-up. In wild-type embryos, Bcd represses translation of *caudal* mRNA preventing accumulation of Caudal protein in the anterior (left panel). In *bin3* loss-of-function mutant embryos, Bcd is unable to repress *caudal* translation and Caudal protein accumulates throughout the embryo (right panel), resulting in failure to undergo proper head involution (lower, right panel), as visualized in first instar larvae. Data are from Singh et al. (2011).[2] (b) Model for Bin3-Bcd repression of *caudal* mRNA translation. Bin3 stabilizes Bcd binding to the Bcd response element (BRE) in the *caudal* 3′-UTR. Bin3 does so by methylating and remaining bound to 7SK RNA which serve as scaffold for binding of other proteins, including the La-related protein 1 (Larp1), Argonaut 2 (Ago2) and poly(A) binding protein (PABP) which contribute to negative regulation of initiation. Although Bin3 is drawn as methylating the 5′-end of 7SK RNA as part of this repression complex, this modification may instead take place early, e.g., during transcription of 7SK RNA synthesis.[20] See text for details. Model based on Singh et al. (2011).[2]

A General Role for Bin3/MePCE in Translation Regulation?

It is unlikely that the role of Bin3/MePCE in early embryos is strictly dedicated to Bcd function in A-P axis formation. In fact, oocytes from *bin3* mutant mothers also show dorso-ventral (D-V) defects. Loss of *bin3* results in dorsalized oocytes, whereas overexpression of *bin3* leads to ventralized oocytes.[2] These defects are likely due to faulty repression of *gurken* mRNA translation, which also requires a repression complex to assemble on its 3′-UTR.[88,89] It is possible that Bin3/7SK RNA forms a scaffold that stabilizes this complex. Thus, we suspect that in early *Drosophila* embryos, before cellularization occurs, Bin3/MePCE might play a global role in translation regulation. This is the time of development, prior to the onset of zygotic transcription, that is directed by stores of maternal RNAs and proteins that have been deposited in the oocyte. During this time, transport, localization, and translation of individual mRNAs is carefully regulated to allow spatial and temporal control of protein production within the embryo.[89,90] By contrast, in mammalian embryos, where the influence of maternal gene products is far

less important, Bin3/MePCE may have little or no role(s) in translation regulation.

Additional Roles of Bin3/MePCE during Development

Analysis of *bin3* mutants, and HEXIM and LARP7 knockdown flies clearly indicates additional roles for Bin3/MePCE and the 7SK snRNP in late embryonic, larval, and pupal development.[2,46] During these stages, transcription regulation is even more critical and the 7SK snRNP probably plays a major role in P-TEFb regulation of developmental genes such as those in the HOX cluster.[91] Bin3/MePCE has even been implicated in adult function in sleep regulation.[92] The recent identification of the genes encoding components of the 7SK complex in *Drosophila* provides an excellent opportunity for future studies.

CONCLUSION

Much attention has focused on non-coding RNAs discovered in genome-wide transcriptome analysis, but the biological roles of these RNAs remain poorly understood.[93,94] The 7SK RNA provides a clear example of how a longer non-coding RNA (i.e., not a microRNA) can play critical roles in gene regulation. Bin3, or MePCE as it will probably become known, plays a key role in the metabolism and activity of 7SK RNA in both transcription and translation regulation. Thus, Bin3/MePCE is of central importance for understanding regulatory mechanisms at the heart of embryonic developmental and cellular regulation. Many questions remain, however.

It is currently not understood how Bin3/MePCE can play dual roles in transcription and translation, and how 7SK RNA might serve as a scaffold for distinct complexes. While there is no direct biochemical evidence to indicate which proteins are present in the putative 7SK translation regulatory complex other than Bcd and Bin3 (e.g., are HEXIMS, LARP7 present?), it is clear that Bcd, PABP and Ago2 and Larp1 are not present in the P-TEFb transcription regulatory snRNP. Therefore, how would distinct 7SK RNP complexes form? Perhaps transcription- and translation-specific RNPs assemble at distinct times in development (i.e., are temporally regulated), or their formation is driven by nuclear versus cytoplasmic localization of the respective protein components. For Bin3/MePCE, it is possible that post-translational modifications could direct distinct forms of the enzyme to the nucleus and cytoplasm. It will also be interesting to know if the different complexes contain alternatively-folded forms of 7SK RNA. Additional biochemical and cell biological studies are required to address these important issues.

No mechanistic studies have been done on Bin3/MePCE. The sequence-specificity of Bin3/MePCE and the mechanism of binding to RNA have not been defined, nor has the catalytic mechanism been studied for this class of RNA methyltransferase. The spectrum of substrates is not known. It has not even been rigorously determined whether Bin3/MePCE methylates U6, plant U3 or other RNAPIII products that contain a $5'$-γ-methyl-phosphate cap structure. Only about half of Bin3/MePCE was reported to be associated with P-TEFb[34,43], suggesting alternative activities.

From an evolutionary standpoint it is intriguing that Bin3/MePCE seems to be present in some organisms that lack 7SK RNA, *S. pombe,* for example, suggesting it may have other cellular targets.[14] Assuming the targets are RNAs, might they also serve a scaffold-type function to assemble additional regulatory snRNPs? For *Drosophila* Bin3, it is also not clear why this protein is so much larger (1368 aa) than its human MePCE counterpart (689 aa), nor what the extra 100 or so nucleotides in *Drosophila* 7SK RNA are doing. No information is available on the function or relevance of Bin3-related proteins encoded in many genomes. Finally, given the link between P-TEFb and a number of human diseases, including cancer and HIV infection, its seems reasonable to think that targeting of Bin3/MePCE might have future therapeutic applications. An ample research frontier exists for study of this interesting enzyme.

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