Report

An Ancient Anterior Patterning System Promotes *Caudal* Repression and Head Formation in Ecdysozoa

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Summary

Posterior expression of Caudal is required for early embryonic development in nematodes, arthropods, and vertebrates [1–9]. In *Drosophila*, ectopic Caudal in anterior cells can induce head defects, and in *Caenorhabditis* the absence of Caudal in anterior embryonic cells is required for proper development [6, 10]. Anterior Caudal repression in these species is achieved through unrelated translational repressors, the homeodomain protein Bicoid [11] and the KH domain factor Mex-3 [6, 12], respectively. Here we report that the Mex-3 ortholog in the flour beetle *Tribolium* plays a crucial role in head formation and that Caudal in this species is repressed by the combined activities of Mex-3 and Zen-2, a protein sharing common ancestry with the dipteran morphogen Bicoid. We propose that Mex-3 represents an ancient "anterior" promoting factor common to all Ecdysozoa (and maybe all Bilateria), whose role has been usurped in higher dipterans by Bicoid.

Results and Discussion

In *Drosophila*, *cad* mRNA is uniformly distributed whereas CAD protein is present in a posterior-to-anterior gradient as a result of translational repression by BCD [11, 13, 14]. *Drosophila* CAD is involved in posterior blastoderm patterning and hindgut formation [1,2], and *caudal* genes of other insects play even more prominent roles during formation of the segmented trunk [3–5]. Indeed, this crucial function of *caudal* genes in posterior patterning is highly conserved throughout the Bilateria, given that it is found also in *C. elegans* and in mammals [3–9]. No *bcd* gene has been isolated from species other than Diptera [15, 16]. However, the *cad* homolog in *C. elegans*, *Pal-1*, is known to be translationally regulated by the KH-domain protein MEX-3 [6, 12]. KH domains were first described in hnRNP K, a pre-mRNA-binding protein, and have subsequently been found in several proteins known to interact with RNA [17].

To elucidate the mechanisms of CAD gradient formation in a nondipteran insect, we cloned and functionally tested the *Tribolium* ortholog of MEX-3 (see Figure S1 available online). In early blastoderm embryos, *Tc-Mex-3* is expressed in a central domain (Figures 1A and 1B). With the formation of the extraembryonic serosa, *Tc-Mex-3* becomes restricted to a wedge-shaped domain just posterior to the serosa, covering the future head region (Figures 1C–1E). In addition, a domain at

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the posterior pole appears at this stage. Later in the germ rudiment, head expression becomes restricted to a stripe in the mandibular segment (Figure 1F). The posterior domain remains expressed throughout germband growth (Figure S2).

Depletion of *Tc-Mex-3* function by parental RNA interference (RNAi) [18] results in a head phenotype. Weak *Tc-Mex-3* RNAi phenotypes display a deletion of the pregnatal region, including labrum and antennae (Figure 2B; Table S1). Strong RNAi phenotypes exhibit more severe aberrations ultimately resulting in the complete deletion of the entire head (Figure 2C; Table S1). Thus, both the pregnathal and the gnathal segments depend on *Tc-Mex-3* activity (Figures 2B and 2C). To determine whether the *Tc-Mex-3* RNAi phenotype reflects an early patterning or a subsequent maintenance function, we analyzed the expression of the segmentation genes *wingless* (*Tc-wg*, Figures 3A–3F) and *even-skipped* (*Tc-eve*, Figures 3G–3L). In wild-type blastoderm stages, *Tc-wg* is expressed in bilateral anterior domains corresponding to the future ocular region, as well as in a posterior domain. With the formation of the gnathal segments, *Tc-wg* becomes additionally expressed in a segmental fashion. In *Tc-Mex-3* RNAi embryos, both the ocular *Tc-wg* domain and the gnathal *wingless* stripes are absent or strongly reduced (Figures 3D–3F). In wild-type embryos, *Tc-eve* is initially expressed in a double segmental pattern that later resolves into segmental stripes (Figures 3G–3I). In *Tc-Mex-3* RNAi embryos, the first *Tc-eve* stripe is absent whereas the second *Tc-eve* stripe is severely reduced (Figures 3J–3L). Thus, the deletion of gnathal segments is reflected by the *Tc-eve* expression pattern. These results confirm that *Tc-Mex-3* functions early in development and is necessary to pattern the anlagen of the head.

To obtain evidence for the involvement of Tc-MEX-3 in translational repression of *Tc-cad*, we searched the *caudal* 3' untranslated region (3'UTR) for potential Tc-MEX-3 binding sites. Previously, a conserved motif was identified in the 3'UTRs of KH-domain target genes [19]. We were able to identify putative KH-domain RNA interaction sites in the 3'UTRs of *Tribolium*, *Gryllus*, and *C. elegans caudal* homologs (Figure S4), suggesting that Tc-MEX-3 could mediate translational repression of *Tc-cad* through these sites. Interestingly, we also found such sites in tunicate and mouse *caudal* genes (Figure S4).

To test whether *Tc-Mex-3* functions in the translational regulation of *Tc-cad*, we observed the distribution of Tc-CAD by immunohistochemistry [8]. Tc-CAD is uniformly expressed in the early wild-type blastoderm, but as development proceeds, this expression changes into a posterior-to-anterior protein gradient that spans the anlagen of the gnathal and thoracic segments [3, 8, 20] (Figures 4A and 4B). In *Tc-Mex-3* RNAi embryos, however, Tc-CAD remains uniformly distributed throughout the embryonic tissue and is repressed only in the serosa primordium (Figures 4C and 4D). This ectopic CAD expression is consistent with the extent of the *Tc-Mex-3* domain and suggests that Tc-CAD translation is indeed inhibited by MEX-3. If *Tc-cad* was the sole Tc-MEX-3 target gene and the *Tc-Mex-3* RNAi head phenotype was caused by ectopic Tc-CAD alone, we would expect that the phenotype of *Tc-Mex3 + Tc-cad* double knockdown would be identical
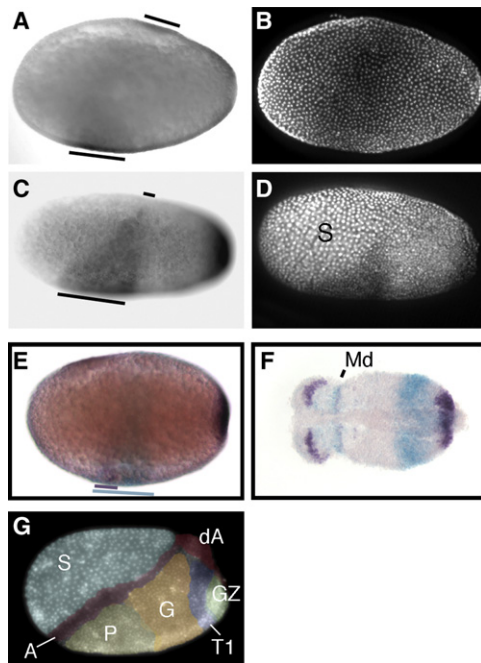
Figure 1. Early Expression of *Tc-Mex-3*

(A–D) Embryos stained for *Tc-Mex-3* mRNA. (B) and (D) were also stained for DNA (Hoechst 33342 epifluorescence).

(A and B) Early in the blastoderm, a central *Tc-Mex-3* expression domain (bars) appears with initially fuzzy boundaries.

(C and D) In the differentiated blastoderm, *Tc-Mex-3* forms a wedge-shaped domain just posterior to the serosa-embryo boundary (serosa in D with large, polyploid nuclei). In addition, *Tc-Mex-3* becomes expressed at the posterior pole.

(E and F) Embryos double-stained for *Tc-Mex-3* (blue) and *Tc-wg* (purple) mRNA.

(E) In the blastoderm, the *Tc-Mex-3* domain (blue bar) overlaps with the ocular *Tc-wg* domain (purple bar).

(F) Flat-mounted early germ rudiment; the blastoderm domain of *Tc-Mex-3* has receded to the mandibular segment (Md) while an additional posterior domain emerges just anterior to the growth zone (which also expresses *Tc-wg*).

(G) Schematic representation of the *Tribolium* fate map at the differentiated blastoderm stage. Color shading indicates the following embryonic primordia: S, serosa; A, amnion; dA, dorsal amnion; P, pregnathal segments; G, gnathal segments; T1, first thoracic segment; GZ, growth zone.

to the *Tc-cad* loss-of-function phenotype. Indeed, we find that in *Tc-Mex3 + Tc-cad* double-RNAi embryos, pregnathal structures are reestablished (Figure 5), indicating that the pregnathal defects in *Tc-Mex-3* RNAi are caused by Tc-CAD derepression. Whether additional genes are regulated by Tc-MEX-3 in gnathal segments remains unresolved, however, as these segments also are missing in *Tc-cad* RNAi embryos and therefore cannot be rescued in the double-RNAi situation.

In *Drosophila,* BCD has been shown to directly bind to the *Dm*-cad 3′UTR, and no Dm-CAD gradient is observed in the absence of BCD [11, 13, 14]. Given that a *Mex-3* ortholog is also present in *Drosophila* (Figures S1 and S3), conceivably its involvement in Dm-CAD regulation might have been overlooked. However, we find that *Dm-Mex-3* mRNA is neither maternally expressed in oocytes (data not shown) nor at the blastoderm stage when the Dm-CAD gradient forms (data not shown). We could only detect *Dm-Mex-3* mRNA at later stages, in the embryonic central nervous system (CNS) (Figure S3).
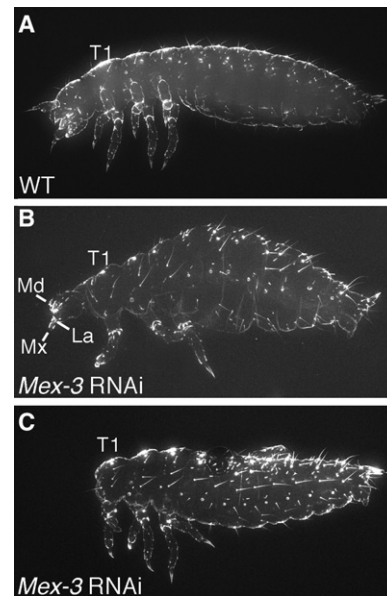

Figure 2. Depletion of *Tc-Mex-3* Causes Loss of the Larval Head

Cuticles of wild-type larva (A) and *Tc-Mex-3* RNAi larvae (B and C).

(A) In wild-type, the segments of anterior head and gnathum are properly formed.

(B) Mildly affected *Tc-Mex-3* RNAi larva. The segments of the anterior head (labrum and antenna) are absent.

(C) Strong *Tc-Mex-3* RNAi phenotype. In addition to the anterior head, all gnathal segments are also deleted. The following abbreviations are used: Md, mandibular segment; Mx, maxillary segment; La, Labium; T1, first thoracic segment.

Moreover, *Drosophila cad* lacks the KH protein recognition sites present in *Gryllus* and *Tribolium cad* mRNAs (Figure S4), suggesting that *Dm-cad* might not be recognized by KH-domain proteins like MEX-3. To test whether *Dm-cad* is susceptible to regulation by *Tc-Mex-3*, we expressed this gene at the posterior pole of transgenic *Drosophila* embryos (Figure S5). We found that, whereas posteriorly localized Dm-BCD noticeably downregulated Dm-CAD protein, Tc-MEX-3 had no detectable effect on *Drosophila* CAD regulation (Figure S6), confirming that in *Drosophila* this function has been taken over entirely by the BCD morphogen.

Even though CAD forms a perfect gradient in *Drosophila*, attempts to demonstrate a function as morphogen gradient in specifying the position of downstream expression domains have failed so far [1, 2, 10, 21]. Because a BCD gradient is lacking in *Tribolium*, one might conceive that the Tc-CAD gradient could provide such a morphogen function. That gnathal segments are deleted in *Tc-Mex-3* and *Tc-cad* RNAi larvae suggests that these segments depend on intermediate Tc-CAD concentrations. A gradient mechanism for CAD has also been proposed for *Gryllus* [4]. To elucidate the role of the Tc-CAD gradient, we analyzed a phenotypic series of *Tc-cad* RNAi embryos and visualized the emergence of gnathal segments by using *Tc-eve* as a marker (Figures 3M–3O). Interestingly, in mildly affected *Tc-cad* RNAi embryos, the first *Tc-eve* stripe is affected first, whereas the other two stripes remain unaffected (Figures 3M–3O). Were the Tc-CAD gradient to provide positional information in a similar way as BCD does in *Drosophila*, we would expect *Tc-eve* stripes 2 and 3 to be more sensitive to the reduction of CAD levels whereas *Tc-eve* stripe 1 should move posteriorly. Our analysis therefore does
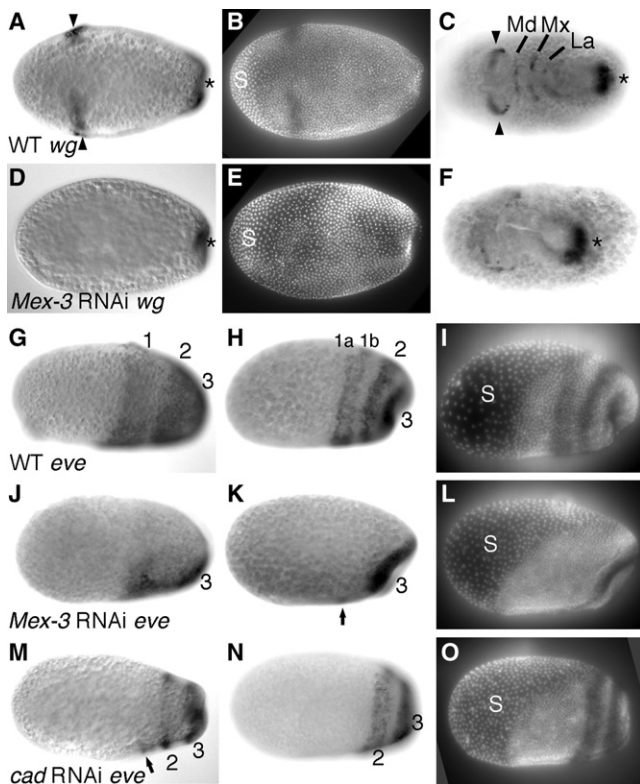
Figure 3. *Tc-Mex-3* RNAi Affects Patterning of Pregnathal and Gnathal Segments

Expression of *Tc-wg* in wild-type (A–C) and *Tc-Mex-3* RNAi embryos (D–F). (G)–(N) depict *Tc-eve* expression in wild-type (G–I), in *Tc-Mex-3* (J–L), and *Tc-cad* RNAi embryos (M–O). Embryos were stained by in situ hybridization and in some cases also for Hoechst 33342 to visualize the embryonic stage (B, E, I, L, and O depict the same embryos as A, D, H, K, and N, respectively).
(A and B) Early expression of *Tc-wg* in wild-type.
(D and E) In a *Tc-Mex-3* RNAi embryo of similar stage as the embryo in (A) and (B), the ocular domain is missing.
(C) In a wild-type germ rudiment, *Tc-wg* is expressed in the three gnathal segments.
(F) In a *Tc-Mex-3* RNAi embryo of similar age, no gnathal expression is detected and the ocular domain is strongly reduced.
(G–I) *Tc-eve* expression in wild-type blastoderm (G) and gastrulating embryos (H and I).
(G) Three primary *Tc-eve* domains (*Tc-eve* stripe 1, *Tc-eve* stripe 2, *Tc-eve* stripe 3) can be detected.
(H and I) *Tc-eve* stripe 1 begins to split into two secondary segmental stripes (1A and 1B) corresponding to the mandibular and maxillary segments.
(J–L) *Tc-Mex-3* RNAi embryos of stages corresponding to (G)–(I).
(J) *Tc-eve* stripe 1 is absent, and *Tc-eve* stripe 2 does not form properly.
(K and L) With beginning gastrulation, the third primary *Tc-eve* stripe forms normally, whereas *Tc-eve* stripe 1 is absent and *Tc-eve* stripe 2 remains severely reduced (arrow).
(M) Weak *Tc-cad* RNAi phenotype; stage corresponds to (H) and (I). The first *Tc-eve* stripe is severely reduced.
(N and O) Slightly older *Tc-cad* RNAi embryo. Only two primary *Tc-eve* stripes are visible, i.e., stripe 1 is missing. The following abbreviations are used: Md, mandibular segment; Mx, maxilliary segment; La, labium; S, serosa.

not confirm a role for Tc-CAD comparable to the long-range maternal BCD gradient. Rather, the stripe specificity of weak *Tc-cad* phenotypes is reminiscent of zygotic gap gene phenotypes in *Drosophila*. Gap genes regulate specific pair-rule stripes through overlapping short-range gradients. Such a role at a more downstream position within the segmentation
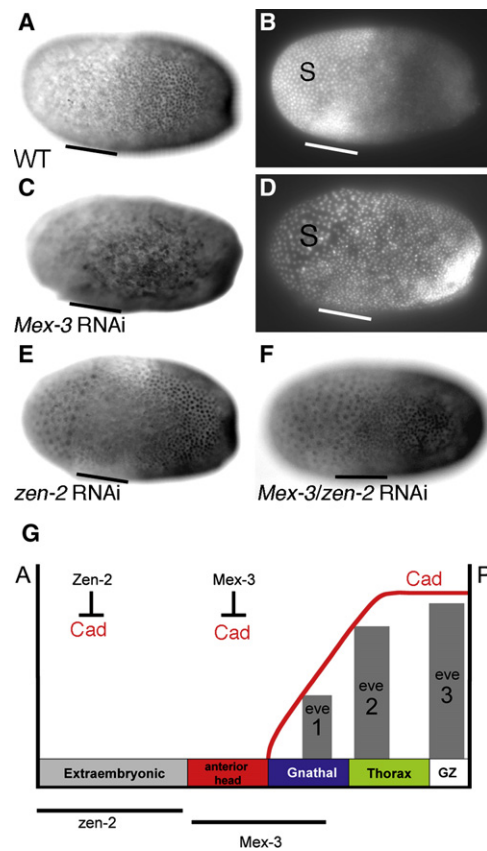

Figure 4. *Tc-Mex-3* and *Tc-zen-2* Both Function in Repression of Caudal Translation

Lateral views of stage-matched embryos stained for Tc-CAD and Hoechst 33342 (B and D depict the same embryos as A and C, respectively). Bars indicate the anterior head region.
(A and B) In wild-type, Tc-CAD forms a posterior-to-anterior gradient, leaving serosa (S) and anterior head (bars) free of expression.
(C and D) *Tc-Mex-3* RNAi results in the expansion of Tc-CAD into the anterior head (bars), but not into the serosa.
(E) In embryos depleted of *Tc-zen-2*, Tc-CAD is expressed in the serosa.
(F) In this embryo lacking *Tc-Mex-3* and *Tc-zen-2*, the Tc-CAD gradient fails to form and Tc-CAD remains expressed in all tissues.
(G) Summary of *caudal* regulation. Schematic representation of the *Tribolium* anterior (A) to posterior (P) axis. *Tc-eve* stripes 1 and 2 require intermediate concentrations of Tc-CAD to form.

cascade would be consistent with our finding that *Tc-cad*— even though it is maternally expressed in the *Tribolium* blastoderm—is spatially regulated in the syncytial blastoderm by zygotic rather than maternal factors.

Given that *Tc-Mex-3* is not expressed near the anterior pole, additional repressors must be involved in Tc-CAD regulation. Possible candidates are the two *Tribolium zen* genes, which are expressed in the serosa [22]. Indeed, after injection of double-stranded RNA (dsRNA) corresponding to *Tc-zen-2*, Tc-CAD becomes expressed in the cells of the serosa (Figure 4E), whereas the embryonic posterior-to-anterior CAD gradient is formed normally. Double knockdown of *Tc-zen-2* and *Tc-Mex-3* results in embryos in which CAD remains expressed in all tissues (Figure 4F). Thus, in *Tribolium* the CAD posterior-to-anterior gradient is established by the combined action of Tc-ZEN-2 and Tc-MEX-3 (Figure 4G), which jointly exclude Tc-CAD from both the prospective serosa and the anterior head.
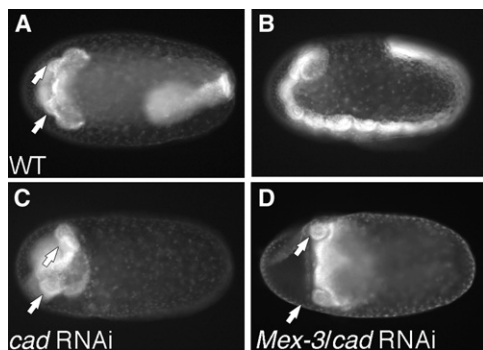
Figure 5. Knockdown of *Tc-cad* in a *Tc-Mex-3* RNAi Background Rescues Pregnathal Segments

(A–D) Embryos stained for DNA (Hoechst 33342 epifluorescence); phenotypes are shown at the germband stage because *Tc-cad* RNAi embryos fail to secrete a cuticle.

(A and B) Wild-type embryo at the extended germband stage, dorsal (A) and lateral (B) aspect.

(C) *Tc-cad* RNAi embryo of similar stage as in (A). The antennae (arrows) are visible anterior to the yolk. All gnathal, thoracic, and abdominal segments are missing.

(D) *Tc-Mex-3* and *Tc-cad* double-RNAi embryos form pregnathal structures (e.g., antennae, arrows) but lack all other segments. In all panels, anterior is to the left. (A), (C), and (D) are ventral views; (B) is a lateral view. Arrows in (A), (C), and (D) point to the antennae.

Our finding that the combined actions of Tc-ZEN-2 and Tc-MEX-3 generate the CAD gradient in *Tribolium* has intriguing implications for the evolution of early pattern formation within Ecdysozoa and the molecular evolution of the BCD morphogen. It has been suggested that *bcd* evolved through duplication of an ancestral *Hox-3/zen* homolog [15] and that one copy, *zen*, retained its function in specifying extraembryonic tissues, whereas the other copy, *bcd*, acquired new features, i.e., mRNA localization signals in the 3′UTR, a new DNA binding specificity resembling that of *orthodenticle*, and the ability to interact with mRNA to translationally repress *cad* [23]. That *Tc-cad* is negatively regulated by ZEN-2 suggests that the ability to regulate translation might have been a feature of *Hox-3/zen* homeodomains preceding the emergence of *bcd*. This would also explain the previous finding that *Tc-cad* translation is inhibited by BCD in transgenic *Drosophila* embryos [20], assuming Tc-ZEN-2 and BCD recognize similar RNA target structures. However, arginine 54, which mediates RNA binding of BCD [24], is not present in the Tc-ZEN-2 homeodomain (methionine at position 54). Moreover, when we expressed Tc-ZEN-2 at the posterior pole of transgenic *Drosophila* embryos, *Dm-cad* mRNA translation was not affected (Figure S6), indicating that Tc-ZEN-2 either has a target specificity different from BCD or requires one or more additional factors or intermediates to regulate *cad* translation. Along these lines, we also found that inactivating the *Tribolium* homolog of 4EHP, an eIF4E-related cap-binding protein that, in *Drosophila*, specifically interacts with Bcd to suppress Cad translation [14], does not affect *Tc-cad* translational repression (data not shown). To elucidate the molecular evolution of BCD, it will be important to see whether Tc-ZEN, despite these molecular differences, also interacts directly with *Tc-cad*, and whether *cad* translation is regulated by Zen proteins in other insects.

Beyond the evolution of *bicoid* within insects, our findings have relevance for understanding the evolution of anterior embryonic patterning in Ecdysozoa and possibly Bilateria in general. We show for the first time that the KH-domain factor MEX-3 is involved in anterior patterning in embryos other than nematodes. The *Tribolium* ortholog of *Mex-3* plays a crucial role in head formation, which might involve target genes in addition to *cad*. The similarity of its function in nematode and beetle embryos indicates that *Mex-3* is a key element of an ancient anterior fate-promoting system. The presence of putative KH-binding sites in ascidian and vertebrate *cad* mRNAs (Figure S4) suggests roles for *Mex-3* orthologs in early embryonic patterning throughout all Bilateria. It appears that, in the lineage leading to *Drosophila*, an evolutionary transition from a MEX-3- to a BCD-dependent head patterning system has occurred. We propose that *Tribolium* represents an intermediate state, given that anterior patterning involves both the translation factor MEX-3 and the ZEN-2 homeodomain protein. It will be of great interest to elucidate the function of *Mex-3* in head patterning of additional bilaterian species.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one table, and six figures and can be found online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01706-0.

Acknowledgments

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