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Expression of *Drosophila* Cabut during early embryogenesis, dorsal closure and nervous system development

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ABSTRACT

cabut (*cbt*) encodes a transcription factor involved in Drosophila dorsal closure (DC), and it is expressed in embryonic epithelial sheets and yolk cell during this process upon activation of the Jun N-terminal kinase (JNK) signaling pathway. Additional studies suggest that *cbt* may have a role in multiple developmental processes. To analyze Cbt localization through embryogenesis, we generated a Cbt specific antibody that has allowed detecting new Cbt expression patterns. Immunohistochemical analyses on syncytial embryos and S2 cells reveal that Cbt is localized on the surface of mitotic chromosomes at all mitotic phases. During DC, Cbt is expressed in the yolk cell, in epidermal cells and in the hindgut, but also in amnioserosal cells, which also contribute to the process, albeit *cbt* transcripts were not detected in that tissue. At later embryonic stages, Cbt is expressed in neurons and glial cells in the central nervous system, and is detected in axons of the central and peripheral nervous systems. Most of these expression patterns are recapitulated by GFP reporter gene constructs driven by different *cbt* genomic regions. Moreover, they have been further validated by immunostainings of embryos from other Drosophila species, thus suggesting that Cbt function during embryogenesis appears to be conserved in evolution.

cabut (*cbt*) is a gene involved in epidermal dorsal closure (DC) during Drosophila embryogenesis (Muñoz-Descalzo et al., 2005). DC is a morphogenetic process that starts halfway through embryogenesis when the epidermis exhibits a dorsal hole covered by the amnioserosa (AS), a squamous epithelium continuous with the epidermis that does not contribute to the larva (Martinez-Arias, 1993). During DC, contraction of the AS coupled to convergence of the lateral epidermis towards the dorsal midline progressively reduces and eliminates the epidermal discontinuity, closing the hole (Gorfinkiel et al., 2009; Jacinto et al., 2002). It has been shown that epidermal cells elongate in the dorsoventral axis due to the formation of an actomyosin cable at their dorsal-most edge (or leading edge, LE), and that actin dynamics is preceded by planar polarization of the dorsal-most epidermal cells (DMC) associated with a reorganization of the cytoskeleton (Kaltschmidt et al., 2002). Moreover, laser ablation experiments and time-lapse analyses of DC mutants have revealed that contraction of the AS is a major force driving the epidermal movement and that this contraction is patterned in space and in time (Fernández et al., 2007; Gorfinkiel et al., 2009; Hutson et al., 2003; Kiehart et al., 2000; Solon et al., 2009). Indeed, AS cells pulsate and constrict gradually during DC, reducing their apical surface and leading to the contraction of the AS (Fernández et al., 2007; Kiehart et al., 2000). In addition, the yolk cell, which lies underneath the AS, also contributes to DC and it is essential for AS survival (Narasimha and Brown, 2004; Reed et al., 2004). In such a scenario, *cbt* mutant alleles affect several events during DC including cell shape changes in the epidermis, JNK signaling, cytoskeleton activity, and occasionally polarity of the DMC. As a result of this, *cbt* mutants die as embryos with DC defects (Muñoz-Descalzo et al., 2005). We found that *cbt*

Abbreviations: AS, amnioserosa; CNS, central nervous system; DC, dorsal closure; DMC, dorsal-most epidermal cells; GBR, germ band retraction; JNK, Jun N-terminal kinase; LE, leading edge; PNS, peripheral nervous system; VNC, ventral nerve cord.

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transcripts are detected in the yolk cell nuclei and in the lateral epidermis, and that this gene functions downstream of the JNK cascade regulating *decapentaplegic* (*dpp*) expression at the LE cells. Two different mRNA isoforms were associated with the *cbt* gene, which encode two proteins Cbt-RA (428 amino acids) and Cbt-RB (lacking the 81 amino-terminal residues of Cbt-RA). Both proteins contain a serine-rich region at the amino terminus and three C_2H_2-type zinc fingers, which suggests that they function as transcription factors. Cbt orthologs have been identified in Drosophilidae and other invertebrates, but also in vertebrates. Analyses of the expression patterns of these orthologs in other Drosophila species suggested that they may have similar functions during DC (Muñoz-Descalzo et al., 2007). In vertebrates, we found that Cbt is the Drosophila ortholog of the mammalian TGFβ Inducible Early Growth (TIEG) response proteins, which regulate cell growth and play antiproliferative and apoptosis-inducing roles in humans (Cook et al., 1999; Ellenrieder et al., 2002).

Although *cbt* was initially described as a gene involved in DC, several reports indicate that it could participate in multiple developmental processes, such as neuroendocrine cell remodeling and ecdysone response (Beckstead et al., 2005; Zhao et al., 2008), circadian rhythm (Kadener et al., 2007), axon guidance and synaptogenesis (Kraut et al., 2001; Mindorff et al., 2005), pole cell regulation (Yatsu et al., 2008), cell growth (Guertin et al., 2006), autophagic cell death (Gorski et al., 2003) and cell cycle (A.J. Katzaroff, B.A. Edgar, personal communication). Thus, for further investigating Cbt expression during *Drosophila* development, we have raised and validated an anti-Cbt antibody. Here, we report Cbt protein expression studies in *Drosophila* during embryogenesis, but also in embryonic S2 cells, that support our previous observations and also suggest new functions for this gene. We detect Cbt localization on the surface of mitotic chromosomes at early stages of embryogenesis. During germ band retraction (GBR) and DC, Cbt is not only detected the epidermis and yolk cell but also in the AS. Moreover, it is also expressed in the central nervous system (CNS) and peripheral nervous system (PNS) during embryogenesis. These new Cbt localizations have been confirmed by the identification of genomic regions which drive *cbt* expression to those tissues. Moreover, analysis of the distribution of the Cbt protein in other *Drosophila* species confirm that the corresponding orthologs show similar expression patterns, thus validating our results and suggesting that the new Cbt functions may be evolutionary conserved.

1. Results and discussion

1.1. Generation and validation of a specific anti-Cbt antibody

To characterize in detail *cbt* expression during *Drosophila* embryogenesis, we generated a polyclonal antibody against a truncated form of the Cbt protein (CbtΔZn) fused to GST. This protein lacks 79 amino acids of its carboxy-terminal region and thus the C_2H_2 zinc finger domain (see Section 2). Several experiments were carried out to confirm the validity of the antibody. First, we performed immunostainings of wild-type embryos to determine whether there is a correlation between *cbt* mRNA and protein distribution. It was reported that *cbt* is ubiquitously expressed in the germ plasm and pole cells during early embryogenesis (Muñoz-Descalzo et al., 2005; Yatsu et al., 2008). During GBR it is expressed in the epidermis, yolk cell nuclei and the gut primordium, and during DC *cbt* expression persists in those tissues (Muñoz-Descalzo et al., 2005). Consistently, strong ubiquitous Cbt expression is observed from earliest stages of embryogenesis onwards (Fig. 1A and B, Fig. 3 and Fig. 4). This protein is nuclear (as expected for a transcription factor) and is first detected in the germ plasm and pole cells of syncytial embryos, and subsequently in blastoderm stages and during gastrulation (Fig. 4 and data not shown). Furthermore, the Cbt protein is clearly localized in the yolk cell nuclei, lateral epidermis and gut primordium/hindgut during GBR and DC, but also appears to be present in the AS cells at these stages (Fig. 1A and B, Fig. 3 and data not shown) despite that *cbt* transcripts were not detected in that tissue (Muñoz-Descalzo et al., 2005). Since the AS is a monolayer of cells located over the yolk sac and to distinguish between nuclei of both tissues, we used an enhancer trap line in the *basigin* (*bsg*) gene, which is specifically expressed in the yolk cell (Reed et al., 2004). We also used a *puckered-lacZ* (*puc-lacZ*) enhancer trap line in which β-Gal expression is present in the LE cells and persists in the AS perimeter cells that abut the LE at the onset of DC (Reed et al., 2001). Embryos from both lines were stained with anti-β-Gal and anti-Cbt. We found that although both proteins colocalize in yolk cell nuclei in the *bsg-lacZ* line, Cbt is also detected in the surrounding AS nuclei, which are smaller in size (Fig. 1C). In *puc-lacZ* embryos, both proteins colocalize in AS perimeter cells (Fig. 1D). In addition, we performed anti-Cbt stainings in mechanical transverse sections of wild-type and $cbt^{EP(2)2237E1}$ embryos, which allow a clear distinction between AS and yolk cell nuclei that lie underneath. These stainings showed that in wild-type embryos Cbt is also detected in AS nuclei. Moreover, they showed that in $cbt^{EP(2)2237E1}$ embryos, in which *cbt* mRNAs were hardly detected in the epidermis and yolk cell nuclei (Muñoz-Descalzo et al., 2005), there was a significant reduction of Cbt expression in the epidermis at stage 13 when compared to wild-type embryos processed in parallel and imaged under the same conditions (Fig. 1E–G). A mild but significant reduction of Cbt protein levels in yolk cell nuclei and AS cells was also observed (Fig. 1E–G), although the staining was not completely lost. These results support the idea that $cbt^{EP(2)2237E1}$ is a hypomorphic allele, as previously reported. Moreover, we performed Western blot analysis using protein extracts from 0 to 24 h wild-type embryos with the anti-Cbt antibody and detected a protein of ~43 kDa, and other low molecular weight bands (Fig. 1H and K). To test whether this protein was the Cbt-RA isoform, we used two additional anti-Cbt polyclonal antibodies generated against two Cbt peptides: one only present in Cbt-RA (peptide 1), and another from a common region of both Cbt-RA and Cbt-RB (peptide 2) (see Section 2). We found that the same ~43 kDa band is detected with all three antibodies (Fig. 1H), thus confirming that it corresponds to the Cbt-RA protein and suggesting that Cbt-RB is not expressed. Supporting this hypothesis, we found that the intensity of this band is increased in protein extracts from embryos overexpressing the Cbt-RA isoform with the *armadillo*-GAL4 (*arm*-GAL4) driver (Fig. 1H). The low molecular weight bands observed in these analyses could either represent degradation products or processed forms of the Cbt protein, since their intensity is increased in Cbt-overexpressing embryos (Fig. 1G). Finally, we were also able to detect endogenous Cbt expression as well as Cbt overexpression in S2 cells transiently transfected with a construct encoding a β-Galactosidase-Cbt (β-Gal-Cbt) fusion protein by performing immunostainings of such cells with the anti-Cbt antibody (Fig. 1L).

As Cbt is maternally expressed and $cbt^{EP(2)2237E1}$ is a hypomorphic allele, we decided to further confirm the specificity of the anti-Cbt antibody by performing protein competition assays, a technique that has been efficiently used with antibodies raised against either peptides or proteins (as an example see Sasikumar and Roy (2008) and Wingen et al. (2009)). To this end, the antibody was incubated with different concentrations of the GST-CbtΔZn fusion protein (used for immunization), and with an unrelated GST fusion protein (GST-Sep4; Muñoz-Soriano and Paricio, 2007) or the GST protein alone as a control. Competition assays were also performed by incubating the antibody with a full-length Cbt protein obtained by *in vitro* translation in a cell-free system, and with