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Role of Cka in imaginal disc growth and differentiation.

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The c-Jun N-terminal kinase (JNK) signaling transduction pathway was initially identified in mammalian cells as a mediator of the cellular response to environmental stress (Hibi, Lin *et al.*, 1993; Derijard, Hibi *et al.*, 1994). Genetic studies in *Drosophila* have revealed that the conserved JNK signaling pathway regulates dorsal closure during embryonic development (Glise, Bourbon *et al.*, 1995; Riesgo-Escovar, Jenni *et al.*, 1996; Sluss, Han *et al.*, 1996; Zeitlinger, Kockel *et al.*, 1997). Dorsal closure is a process that begins at stage 13 of *Drosophila* embryogenesis. During dorsal closure, the lateral epidermal cells elongate and move dorsally to enclose the entire embryo. Mutations that block JNK pathway lead to a failure to express *dpp* (reviewed by Noselli and Agnes, 1999), a TGF-β family member that mediates concerted cell elongation during DC (Glise and Noselli, 1997; Riesgo-Escovar and Hafen, 1997; Riesgo-Escovar and Hafen, 1997; Sluss and Davis, 1997; Zeitlinger, Kockel *et al.*, 1997) and lead to a dorsal closure defect (reviewed by Knust, 1997).

In addition to its role in directing the embryonic dorsal closure, JNK pathway is also required later in development for imaginal disc morphogenesis. Imaginal discs are specialized small epithelial cell sacs that initiate during embryogenesis. They proliferate and grow extensively during larval stages and undergo profound morphological changes at pupal stages to form the external adult structures (Cohen, 1993). One of the changes during metamorphosis is the fusion of the two lateral wing discs, giving rise to the dorsal thorax structure of the adult (Fristrom and Fristrom, 1993). This process, so-called "thorax closure", is similar to the "dorsal closure" at embryo stage, which involves epithelial cell spreading. Loss of JNK pathway activity leads to severe defects in disc morphogenesis, including small and malformed imaginal discs at larval stage and the absence or aberrant fusion of the two lateral wing discs during pupal stage (Agnes, Suzanne *et al.*, 1999; Zeitlinger and Bohmann, 1999; Martin-Blanco, Pastor-Pareja *et al.*, 2000). The regulatory molecules required for thorax closure are similar to the ones directing dorsal closure in the embryo, indicating that the JNK signaling pathway may be widely used for controlling tissue closures during animal development. The small imaginal disc phenotype indicates a growth defect in these tissues (Agnes, Suzanne *et al.*, 1999). However, the underlying mechanism is still unknown.

Recently, *Drosophila cka* (connector of kinase to AP-1) was reported to function in the DJNK pathway (Chen *et al.*, 2002). *cka* deficient embryos display the typical dorsal-open phenotype associated with JNK pathway mutations (Chen *et al.*, 2002). Here, we present a brief description of the characterization of the imaginal disc phenotypes of *cka* mutants. We show that the *cka* mutants exhibit a small imaginal disc phenotype and a defect in neuronal differentiation. Our study suggested that in addition to regulating dorsal closure at embryonic stage, CKA may also plays an important role in controlling the growth and cell differentiation during imaginal disc development.

In order to detect the expression of CKA protein, peptide corresponding to the C-terminal sequence of CKA was used to produce polyclonal antibodies. Western blot experiments using fly

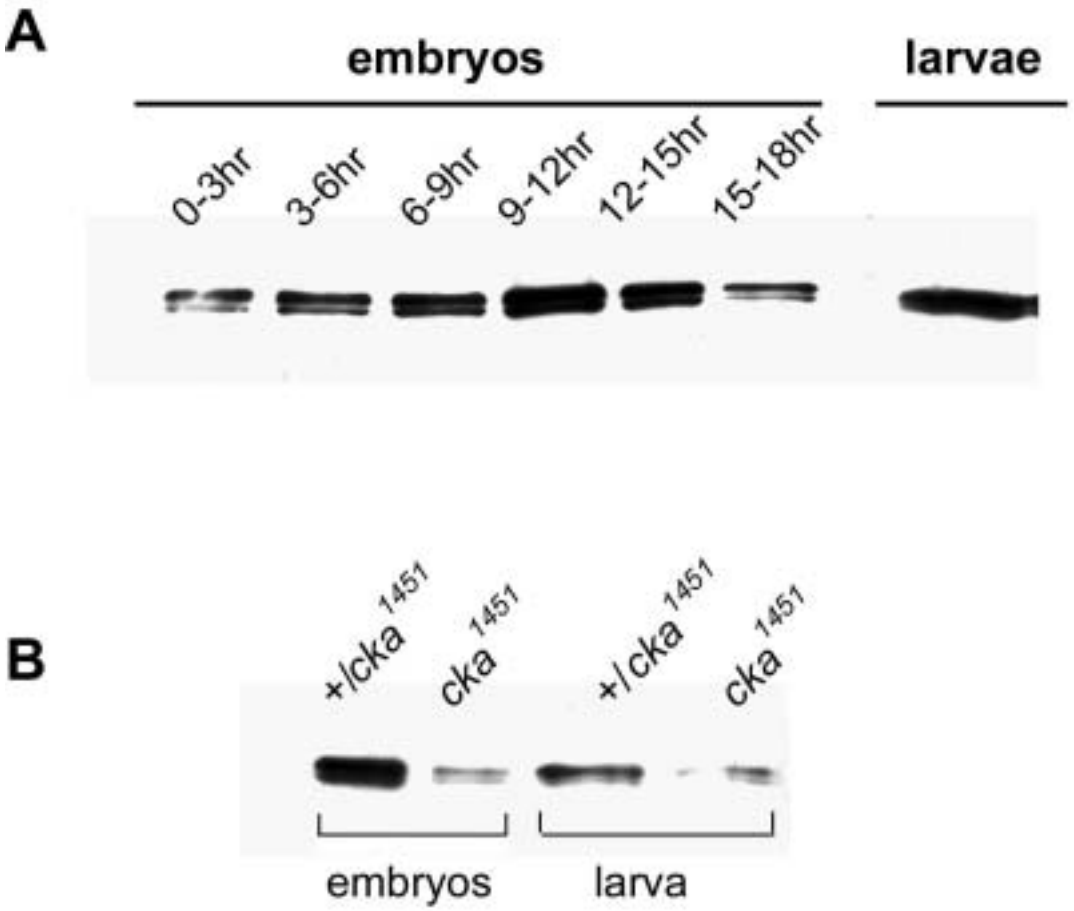

Figure 1. Western blot analysis of CKA expression. (A), Wild type *Drosophila* embryos and larval extract were resolved by SDS-PAGE and analyzed by Western blot using anti-CKA antibodies. The age of the embryos is indicated as number of hours after egg deposit and is labeled on top of each lane. (B), Expression of CKA is disrupted in *cka* mutant embryos and larvae. Genotypes are as indicated above each lane.

embryonic and larval extracts showed that CKA is expressed in all developmental stages. The presence of CKA protein in early embryos (0-2 hr) suggests that this protein is maternally expressed (Figure 1A)

Two independent P element insertion lines, referred to as cka^{1451} and cka^{2039}, were obtained from the *Drosophila* stock center. PCR amplification and sequencing analysis revealed that both alleles have a P element inserted in the 5' untranslated region of the CKA gene, about 1 kb upstream of the ATG site. The cka^{1451} insertion disrupts the expression of the CKA gene, as shown by the Western blot assays in Figure 1B. The protein level is greatly reduced but not completely removed in the zygotically homozygous mutant flies (Figure 1B). It is possible the *cka* mutants are not null alleles or the maternal protein persists till late developmental stages.

Animals zygotically homozygous or trans-heterozygous for these two alleles survive to pupal stages. The pupal lethality results from disruption of the *cka* locus by P element insertion, as precise excision of the P element completely reversed the lethality. Furthermore, ubiquitous expression of a transgene containing the full-length *cka* cDNA driven by a tublin promoter fully rescued the lethality of the homozygous or transheterozygous *cka* mutant flies. These results demonstrated that the lethality

associated with these two mutant alleles is due to disruption of CKA gene expression by P element insertions.

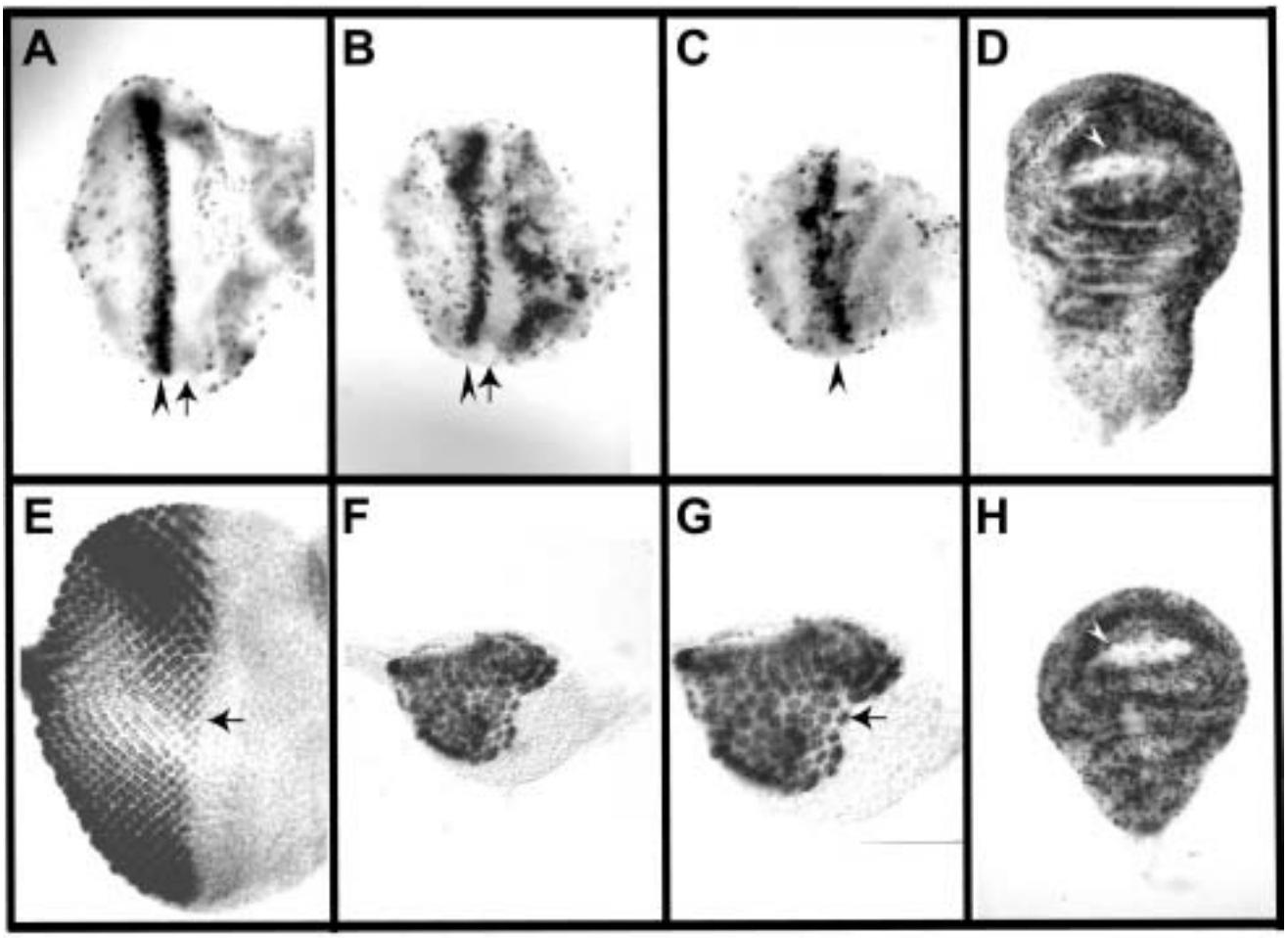

Figure 2. Imaginal disc phenotypes of zygotic *cka* mutant larvae. (A-C), BrdU incorporation assay of a wild-type (A) and two *cka*$^{1451/2039}$ mutant (B and C) third instar eye imaginal discs. Black arrow, morphogenetic furrow; Black arrowhead, second mitotic wave. (D and H), BrdU incorporation of a wild type (D) and a *cka*$^{1451/2039}$ mutant (H) third instar wing discs. White arrowhead, zone of non-proliferating cells (ZNC). (E-G), Anti-Elav staining of wild type (E) and *cka*$^{1451/2039}$. (F-G), third instar eye imaginal phenotypes. Panel G is an enlarged view of panel F. Black arrows point to the anterior most ommatidial pre-clusters expressing Elav.

Imaginal discs were dissected from third instar *cka*$^{1451/2039}$ larvae to characterize the role of CKA in imaginal disc development. Interestingly, *cka*$^{1451/2039}$ mutant eye discs display a variable reduction of disc size as compared to wild type, suggesting a growth defect in the mutant imaginal discs (Figure 2). In some of the *cka*$^{1451/2039}$ mutant imaginal discs, the overall morphology is normal, and the pattern of BrdU incorporation appears normal (Figure 2B and H). Malformed and misfolded discs were also

observed in the mutant flies, with a higher frequency in smaller discs (Figure 2C and F). Similar phenotypes have been observed with mutations in DJNK signaling pathways, indicating that CKA may also function through the JNK pathway to regulate imaginal disc morphogenesis and growth during larval development.

The *Drosophila* compound eye is composed of approximately 800 repeating units called ommatidia, which consist of eight photoreceptor cells (R cells) and 12 non-neuronal accessory cells. To determine if the *cka* mutation affects ommatidial development, we examined the expression of the protein Elav, a marker for neuronal cell differentiation (Robinow and White, 1991). During the third larval instar, neuronal differentiation initiates at the morphogenetic furrow (MF), which is marked by a depression in the apical surface of the disc epithelium. In the wild type eye disc, the Elav staining is first visible in the R8 cells immediately posterior to the morphogenetic furrow and continues as other photoreceptors are recruited into the cluster, reflecting a stepwise maturation of the growing cluster (Figure 2E). These clusters of photoreceptor cells are well organized in rows. Such expression pattern was disrupted in the *cka* mutant eye discs. Elav expression was observed in the photoreceptor cells at the posterior portion of the *cka* mutant eye disc (Figure 2G and F). However, the photoreceptor clusters are disorganized throughout the disc. Furthermore, the anterior most Elav positive clusters already contain multiple differentiated photoreceptor cells (Figure 2G), indicating a differentiation defect during eye development in the *cka* mutant flies.

In conclusion, phenotypic characterization of the cka^{1451} and cka^{2039} alleles revealed a novel role of the *cka* gene in regulating imaginal disc development. The small imaginal disc phenotype indicates that CKA may function to regulate organ growth. Furthermore, we observed neuron differentiation defects in the *cka* mutant during eye development. This phenotype could be due to delayed differentiation of early photoreceptor cells (such as R8), or it could be due to precocious differentiation of late photoreceptor cells (such as R1 and R6). Further investigations are needed to elucidate the mechanism by which CKA regulates eye cell differentiation.

References: Agnes, F., M. Suzanne, *et al.* 1999, Development 126(23): 5453-5462; Chen, H.W., M.J. Marinissen, *et al.*, 2002, Mol. Cell Biol. 22(6): 1792-1803; Cohen, S.M., 1993, Imaginal disc development. in *The Development of* Drosophila melanogaster. Arias, M.B., and A.M. Arias, eds.). New York, Cold Spring Harbor Laboratory 2: 747-841; Derijard, B., M. Hibi, *et al.*, 1994, Cell 76(6): 1025-1037; Fristrom, D., and J.W. Fristrom 1993, The metamorphic development of the adult epidermis. in *The Development of* Drosophila melanogaster. (Arias, M.B., and A.M. Arias, eds.). New York, Cold Spring Harbor Laboratory Press. 2: 843-897; Glise, B., H. Bourbon, *et al.*, 1995, Cell 83(3): 451-461; Glise, B. and S. Noselli 1997, Genes Dev. 11(13): 1738-1747; Hibi, M., A. Lin, *et al.*, 1993, Genes Dev. 7(11): 2135-2148; Knust, E., 1997, Curr. Biol. 7(9): R558-561; Martin-Blanco, E., J.C. Pastor-Pareja, *et al.*, 2000, Proc. Natl. Acad. Sci. USA 97(14): 7888-7893; Noselli, S., and F. Agnes 1999, Curr. Opin. Genet. Dev. 9(4): 466-472; Riesgo-Escovar, J.R.m and E. Hafen 1997, Science 278(5338): 669-672; Riesgo-Escovar, J.R., and E. Hafen 1997, Genes Dev. 11(13): 1717-1727; Riesgo-Escovar, J.R., M. Jenni, *et al.*, 1996, Genes Dev. 10(21): 2759-2768; Robinow, S., and K. White 1991, J. Neurobiol. 22(5): 443-461; Sluss, H.K., and R.J. Davis 1997, J. Cell Biochem. 67(1): 1-12; Sluss, H.K., Z. Han, *et al.*, 1996, Genes Dev. 10(21): 2745-2758; Zeitlinger, J., and D. Bohmann 1999, Development 126(17): 3947-3956; Zeitlinger, J., L. Kockel, *et al.*, 1997, EMBO J. 16(24): 7393-7401.