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TRANSCRIPTIONAL REPRESSION MEDIATED BY THE
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CONTRIBUTION OF CtBP AND RPD3

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TRANSCRIPTIONAL REPRESSION MEDIATED BY THE *DROSOPHILA* KNIRPS PROTEIN: CONTRIBUTIONS OF CtBP AND RPD3

By

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ABSTRACT

TRANSCRIPTIONAL REPRESSION MEDIATED BY THE *DROSOPHILA* KNIRPS PROTEIN: CONTRIBUTIONS OF CtBP AND RPD3

By

Paolo Struffi

The *Drosophila* Knirps protein is a short-range transcriptional repressor essential for proper embryonic development. Short-range repressors work over distances of less than 100-150 base pairs to inhibit activators in a local fashion, allowing multiple enhancers to be regulated autonomously. The mechanisms of short-range repression remain poorly understood at the molecular level. Knirps mediates repression in part by recruiting the corepressor CtBP, but it also posses a CtBP-independent repression activity that maps to the N-terminus of the protein. The functional relevance of multiple repression activities is not well understood, but the findings that Knirps does not repress some cis-regulatory elements in the absence of CtBP, suggested that the co-factor may supply a unique function essential to repress certain types of activators. I assayed the CtBP-dependent and –independent repression activities of Knirps in *Drosophila* embryos and found that the requirement for CtBP at certain enhancers appears to reflect the need for overall higher levels of repression, rather than a requirement for an activity unique to CtBP. Thus, CtBP contributes quantitatively, rather than qualitatively to Knirps function.

To investigate whether Knirps interacts with other cofactor/s in addition to CtBP, I generated transgenic flies that express inducible, double-tagged versions of Knirps and performed affinity purification experiments. Recombinant, full-length Knirps could be expressed in embryos at the same time as the endogenous factor and the protein acted as a

functional repressor. Gel filtration chromatography of embryonic extracts expressing full-length Knirps indicate that the recombinant protein is part of a complex of ~450 kDa, suggesting that other factors in addition to CtBP interact with Knirps. In a survey of possible cofactors, we found that the histone deacetylase Rpd3 (HDAC 1) co-immunoprecipitates with Knirps and the two proteins cofractionate during gel filtration. To facilitate characterization of novel Knirps-interacting proteins, we developed a tandem affinity chromatography protocol from embryonic extracts overexpressing Knirps and find that Rpd3 copurifies with full-length Knirps, but not the CtBP-independent repression domain. To test the functional relevance of this association, we carried out dosage interaction assays, and find that the *rpd3* and *knirps* interact genetically. Altogether these results suggest that histone deacetylation plays a role in short-range transcriptional repression mediated by Knirps.

To my father, Giuseppe, and my mother, Elda, for their love, wisdom and encouragement

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KEY TO ABBREVIATIONS

aa:	amino acid
A-P:	anterior-posterior
bp:	base pair(s)
cDNA:	complementary deoxyribonucleic acid
ChIP:	chromatin immunoprecipitation
Co-IP:	co-immunoprecipitation
CtBP:	C-terminal binding protein
dCtBP:	Drosophila C-terminal binding protein
DBD:	DNA binding domain
DNA:	Deoxyribonucleic acid
DTT:	dithiothreitol
E. coli:	*Escherichia coli*
EDTA:	ethylenedinitrilo tetraacetic acid
HAT:	histone acetyltransferase
HDAC:	histone deacetylase
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMT:	histone methyltransferase
hsp:	heat shock promoter
kb:	Kilobase
kDa:	Kilodalton
kni:	*knirps*
NAD:	nicotineamide adenine dinucleotide
NSL:	nuclear localization signal
PAGE:	polyacrylammide gel electrophoresis
PcG:	Polycomb group
PCR:	Polymerase chain reaction
pol II:	polymerase II
Rb:	Retinoblastoma tumor suppressor protein
RNA:	Ribonucleic acid
TBP:	TATA binding protein
SDS:	Sodium dodecyl sulfate
Su(Var):	Suppressor of variegation
TF:	transcription factor

Chapter 1

Introduction

The development of multicellular organisms from a single cell, the fertilized egg, is an amazing achievement and a fascinating subject to study. In multicellular eukaryotes, all cells possess the same information, yet they give rise during development to hundreds of different cell types, which form structures as complex and varied as eyes, wings or the brain (1). Each cell type is defined by a specific gene expression program that controls which genes are transcribed, when and for how long. Understanding the molecular basis that underlies cell differentiation remains a formidable challenge. In eukaryotes there are tens of thousands of protein-coding genes, each of which has its own specific program of transcriptional control. Much of the specificity of these programs is affected by sequence-specific DNA-binding transcription factors, which bind to proximal promoter and distal transcriptional regulatory regions and function as a key interface between genetic information and the transcriptional machinery (2). Regulation of transcription, especially the initiation of transcription, is a pivotal step in the control of gene expression. An enormous body of work generated over the past three decades has revealed that eukaryotic gene transcription is a remarkably intricate biochemical process that is tightly regulated at many levels. Despite many advances, surprisingly little is known about the detailed mechanisms by which individual genes are turned on or off in a cell (3). Knowledge of these basic mechanisms will impact several fields, from cell biology to medicine.

In this work, I describe studies that shed light on the mechanisms of transcriptional regulation mediated by one of these sequence-specific transcription factors: the *Drosophila* short-range repressor Knirps. I studied the contribution of a conserved corepressor, the C-terminal binding protein (CtBP), to Knirps-mediated repression, and found that the requirement for CtBP for effective Knirps repression depends on the nature of the target regulatory element (chapter 2 and 3). I provide evidence that supports the hypothesis that CtBP contributes quantitatively rather than qualitatively to Knirps repression (chapter 3). In addition, I developed a biochemical purification scheme suitable to look *in vivo* for Knirps-interacting proteins and found that the histone deacetylase Rpd3 (HDAC1) biochemically and genetically interacts with Knirps, providing experimental evidence that links chromatin modifications to transcriptional repression by Knirps (chapter 4).

Eukaryotic transcriptional repression

Much of the initial efforts in the field of eukaryotic transcriptional regulation have been devoted to two related issues: how the general transcription machinery is assembled to initiate transcription and how activators and coactivators facilitate this process. A third issue, how eukaryotic transcriptional repressors work, did not receive until recently the attention given to the two other topics (4). However, it is becoming increasingly clear that transcriptional repression is just as important as transcriptional activation for establishing cell- and tissue-specific patterns of gene expression. Transcriptional repression has been implicated in a variety of developmental processes including the specification of mating type in yeast (5), segmentation patterning in the *Drosophila* embryo (6), tissue-specific

patterns of gene expression in sea urchins (7), mice (8) and lineage-restricted expression in mammalian lymphocytes (9, 10). During development, boundaries of gene expression are often determined by the spatially-restricted localization or activity of transcriptional repressors (6, 11-12).

Although gene expression can be achieved at several different levels, transcriptional initiation is believed to be the principal regulatory step for many, if not most, genes (13). Transcription initiation of protein-encoding genes by RNA polymerase II (pol II) requires the assembly of a pre-initiation complex (PIC), which involves over one hundred polypeptides (14). Repressors can prevent recruitment of some of these factors to a target promoter, they may prevent the isomerization of the PIC to form an open complex, or they may act at a later stage (promoter escape or elongation).

Considering possible mechanisms of transcriptional repression, we need to remember that *in vivo* the transcription template is organized into condensed, heterogeneous chromatin fibers. Although it is still unclear whether transcription occurs on nucleosomal DNA or on higher order (30 nm) structures, chromatin seems to be inherently repressive for transcription (15, 16). Therefore, factors that promote or stabilize the formation of higher order chromatin structures are thought in most cases to interfere with transcription. An additional level of complexity is provided by the fact that transcription does not occur independently from other cellular processes, such as RNA processing or protein degradation, but is tightly integrated with these events and regulated at many different levels.

Mechanisms of transcriptional repression

Transcriptional repression can be achieved in several different ways (Fig. 1-1). Cellular processes that modify, destroy, or remove from the nucleus positively acting transcription factors (activators or parts of the general transcriptional machinery) can prevent transcription by eliminating an activating signal (Fig. 1-1A). In these cases, negatively acting factors are not required to directly interact with the target gene to repress (17, 18). These cellular activities may affect transcription in alternative ways. For example, the ubiquitin-mediated proteolysis of a transcription factor such as the *Herpes simplex* factor VP16 would be expected to reduce its transcription ability by targeting the activator to destruction. However, recent studies have demonstrated that ubiquination of the VP16 activator is essential for its activity (19), suggesting that "de-stabilization" may be a pre-requisite for transcriptional activity (20).

Transcriptional repression is also mediated by cis-acting elements termed silencers or boundary elements (Fig. 1-1B), which prevent transcription of a gene when located between the core promoter and an upstream enhancer (21, 22). This type of repression does not depend on the particular enhancer or core promoter elements, and seems to involve the formation of a loop in the chromatin structure that isolates enhancers and promoters to functionally independent chromosomal domains, thus preventing communication between an enhancer and the promoter it regulates (23, 24).

Repression is also achieved by preventing an activator from binding to its targets, either by altering the chromatin structure (Fig. 1-1C) or by competing for common or overlapping DNA binding sites (Fig. 1-1D1). Chromatin modifications that render the

Figure 1-1: Mechanisms of transcriptional repression.

Transcriptional repression can be achieved in a number of different ways, and in some cases multiple mechanisms may be employed simultaneously. Repression can be indirectly caused by the inability of positively-acting factors to reach their targets on the promoter (A, C, and D1), or be the direct consequence of protein-protein interactions between repressors and either activators or the basal transcriptional machinery (B and D2-3). Sequence-specific repressors are depicted as dark gray rectangles, activators as gray circles. Arrow depicts the transcription start site.

A. Cellular processes that results in the modification (1), sequestration (2), degradation (3) or removal from the nucleus (4) of positively acting, sequence-specific transcriptional activators will indirectly cause transcriptional repression. **B.** A boundary element or insulator (black bar) positioned between an enhancer and the core promoter will prevent the enhancer from activating the downstream gene. However, if the boundary element is located upstream of the enhancer, the enhancer retains the ability to activate the gene. The repressive activity brought by boundary elements does not depend on the nature of the enhancer element. **C.** Alteration of the chromatin structure caused either by the utilization of particular histone variants to assemble non-canonical nucleosomes (1), by covalent modifications of histones tails or DNA (2), or by the particular spacing of nucleosomes over a regulatory sequence (3) can results in local or general repression.
D. Gene-specific repression by DNA-binding proteins can be caused by competition between the activator and the repressor for the same or an overlapping DNA-binding site (1), by direct interaction between the repressor and the basal transcriptional machinery (2), or by quenching of the activator (3). Figure modified from 18.

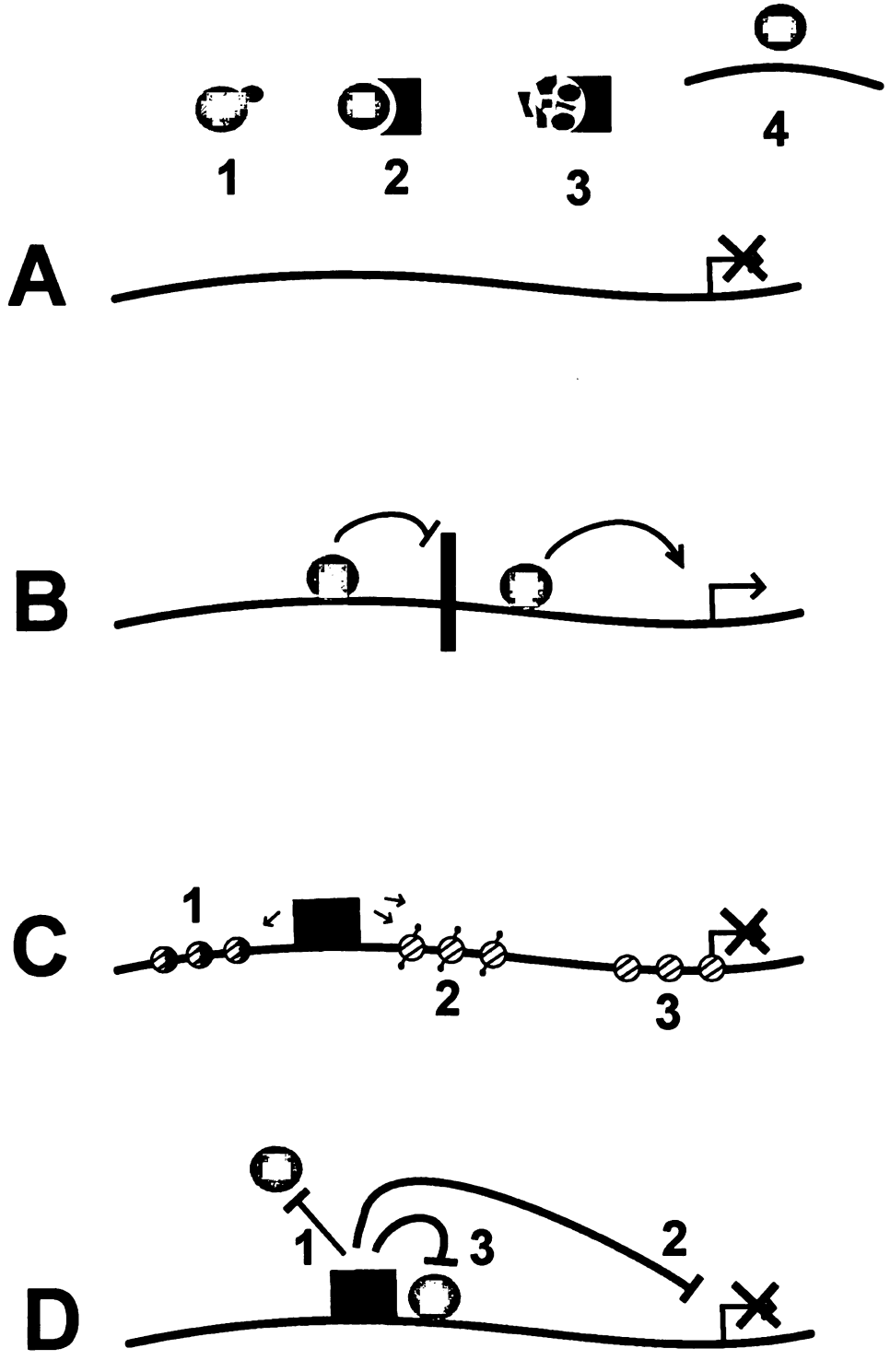

Figure 1-1: Mechanisms of transcriptional repression.

DNA template less accessible to transcription factors may include the use of particular histone variants to assemble different types of nucleosomes, covalent modifications of histone proteins or DNA, and mobilization of nucleosomes relative to the DNA. Histone variants may regulate transcription by creating new chromatin structures. For instance, in mammalian cells the histone variant macroH2A (mH2A) is enriched in the inactive X chromosome (25). *In vitro* studies with reconstituted chromatin have shown that mH2A interferes with transcription factor binding and nucleosome remodeling and may be important for establishing or maintaining the repressive status of large chromatin domains rather than single genes (26). A diverse array of post-translational modifications that often occurs on the N-terminal histone "tails" has been proposed to act in a combinatorial way as an epigenetic code (27). For example, the removal of acetyl groups from lysine residues of the histone proteins, mediated by histone deacetylases (HDACs), has usually an inhibitory effect on gene expression (28). HDACs may mediate repression by increasing the affinity of nucleosomes for their DNA, therefore creating a chromatin structure less accessible to transcription factors. This modification is readily reversible and can be targeted to single nucleosomes, providing a means to control genes in a dynamic and specific manner (29, 30). HDACs have been found to directly interact with a number of corepressors (31-33) including the mammalian homolog of the Knirps corepressor CtBP (34). DNA methylation and histone methylation at specific residues in the histone H3 N-terminal tails (K9 and K27) have also been associated with repression (35-37). Unlike acetylation/deacetylation, methylation is not believed to be readily reversible and therefore it may be important to confer stable gene repression required for maintaining cell identity (38). The mammalian corepressor CtBP 1 has also been shown

to interact with the histone methyltransferases G9a and Eu-HMTase1 (34), although it is not clear whether this interaction could play a role in the transient repression mediated by short range repressors. ATP-dependent nucleosome remodeling complexes change the physical association between DNA and nucleosomes (39-41). *In vitro*, these enzymes weaken the tight wrapping of DNA around the histone octamers, thereby facilitating the sliding of nucleosomes to neighboring DNA segments, their displacement to unlinked DNA, and the accumulation of patches of accessible DNA on the surface of nucleosomes (reviewed in 42). The activity of these complexes *in vivo* has been mainly associated with transcriptional activation, but they can also lead to nucleosome positioning which has been shown to govern the repressed state of the *PHO 5* promoter in yeast (43).

Direct competition for the same or overlapping binding sites can also prevent an activator from reaching its target/s on a promoter and therefore lead to repression (Fig. 1-1D1). Analysis of *Drosophila* cis-regulatory elements have identified several examples where binding sites for an activator and a repressor overlap, leading to the hypothesis that competition for DNA binding might be an important way to control gene expression (11). In some cases, *in vitro* and *in vivo* overexpression studies support this hypothesis (44). However, under physiological levels of protein expression, repression was also observed from non-overlapping sites (45, 46). Moreover, in the case of the *even-skipped* [1] (*eve*) stripe enhancers, where DNA-binding sites for Knirps overlap in many instances with binding sites for activators, *in vivo* overexpression of the Knirps DNA binding domain alone was unable to mediate repression (see chapter 3).

[1] Note the use of new *Drosophila* genetic nomenclature throughout the entire document. All gene names are italicized and written in all lower case letters. Protein names are in regular font and start with an upper case letter.

Transcriptional repressors have also been suggested to function through direct protein-protein interactions with the basal transcriptional machinery (Fig. 1-1D2). According to this model, referred to as direct repression, the repressor would interfere with the formation or activity of the PIC, inhibiting transcription initiation or elongation. Direct repression may occur not only when the repressor binds close to the core promoter, but also when it is brought close to it by looping of the DNA double helix between the start of transcription and the binding site for the repressor. Cell-culture and *in vitro* studies have identified several targets of transcriptional repressors within the basal machinery, including TFIIA, TFIIB, TFIID, RNA polymerase, and the holoenzyme subunits Srb10/11 (reviewed in 47). In the case of *Drosophila* short-range repressors there are no convincing biochemical data supporting possible direct interactions with the basal machinery. However, the *Drosophila* transcriptional repressor *even-skipped* has been shown to inhibit transcription by directly binding to the TATA binding protein (TBP) and preventing in this way the recruitment of TFIID to the TATA box (48-50). In *Drosophila*, Polycomb Group (PcG) proteins are involved in the long-term repression necessary to maintain cell identity (38). Chromatin immunoprecipitation analysis of several repressed genes, indicate that binding of PcG proteins to repressed promoters does not exclude general transcription factors and RNA pol II, suggesting that PcG repression may be at a stage subsequent to PIC formation (51).

Transcriptional repression can also result from direct interactions between activator and repressor (Fig. 1-1D3). According to this mechanism, sometimes referred to as quenching or masking, repressor and activator bind to distinct sites on the DNA and interact with each other in a manner that prevents the activator to function. For instance,

the yeast Gal80 protein represses the *GAL1* promoter by binding to and obstructing the activation domain of the Gal4 activator protein, thus preventing the recruitment of histone acetyltransferase complexes (52). However, because many *Drosophila* repressors including Knirps, have been found to inhibit a wide variety of activators, some of which likely to activate transcription through different pathways, it is likely that they do so without making specific activator-repressor contacts (46, 53).

Consistent with the variety of inhibitory processes involved in establishing transcriptional repression, the effects of repression are also diverse. Repression can be transient and limited to the time when negative factors are found at a gene, or the repressor can leave an epigenetic mark so that transcription is blocked for the life on an organism (38). Repression can be limited to a single gene by the activity of a dedicated repressor, or many genes can be regulated by modifications of the general transcription machinery or the chromatin structure.

Short-range and long-range repression

Studies on transcriptional repression in the *Drosophila* embryo suggest that there are two basic forms of repression, namely long-range and short-range repression (Fig. 1-2; 6, 11-12). The range of activity of repressors from other organisms has not been extensively characterized; however, it is likely that basic mechanisms underlying these modes of repression are conserved between metazoans. Long-range repressors function in a dominant fashion to block multiple enhancers, even when these are located thousands of base pairs from the repressor binding site (Fig. 1-2A; 54). This kind of repression is

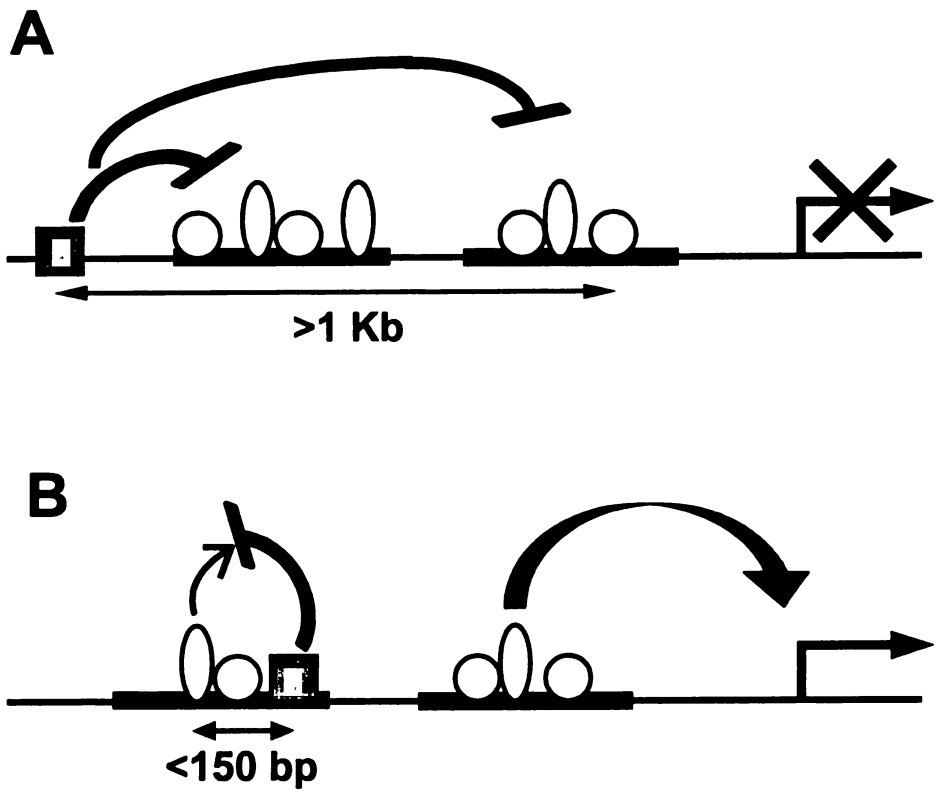

Figure 1-2: Long-range and short-range repression.

In *Drosophila*, repressors have been classified according to the range of their activity. **A.** Long-range repressors such as Hairy, Engrailed and in some contexts Dorsal are able to mediate repression of multiple enhancers over distances of > 1000 bp. These repressors function in a dominant way to silence an entire locus. **B.** Short-range repressors, such as Knirps, Krüppel, Giant and Snail are able to repress the activity of enhancers elements when bound within 100-150 bp from key activator binding sites, or basal promoter elements when cognate sites are introduced close to the start site of transcription. Short-range repressors allow multiple enhancers in a complex promoter to function autonomously, so that repressors acting on one enhancer do not interfere with activators present on a near by enhancer.

Activators are depicted as ovals or circles, repressors as squares. Enhancers are indicated by black rectangles.

often referred to as silencing because an entire chromosomal locus is inactivated (12). Short-range repressors, such as Knirps, Giant, Krüppel and Snail, work over distances of less than 100-150 bp to inhibit upstream activators in a local fashion (55-57). Rather than silencing the gene, they block the function of nearby DNA-bound activators, while the activity of more distant enhancers is intact. Short-range repression represents a more flexible way to regulate gene expression in complex modular promoters, because it allows enhancers to work independently from one another in an autonomous fashion (45). The precise distance over which a short-range repressor is able to work depends to some extent on the repressor concentration. Thus, short-range repressors may provide a sensitive means of responding to a transcription factor concentration gradient (58).

Drosophila early embryogenesis

Embryonic development in *Drosophila melanogaster* is a remarkably rapid process (59-61). It starts immediately following egg deposition and within only one day leads to a larva able to hatch. Following fertilization, the embryo undergoes a series of rapid nuclear replications with cell cycle durations of 8-15 minutes and no intervening cytokinesis, leading to the formation of a syncytium. The first seven zygotic divisions are synchronous (stage 1-2), leading to a syncytium of 128 nuclei distributed in the central region of the embryo. During the course of the next three divisions most of the nuclei, each with a surrounding islet of cytoplasm, migrate outwards as they continue to divide and approach the surface (stage 3). After three additional divisions (stage 4), there is a pause in the cell cycle and cellularization, a morphogenetic program with a duration of

about 50 minutes, starts to enclose the nuclei in newly emerging cell membranes (stage 5). At the beginning of this stage, most nuclei are near the surface of the embryo forming a monolayer, the blastoderm. During cellularization the plasma membrane extends centripetally into the embryo and between the nuclei, eventually cutting in under them to leave, for a time, channels between the nascent cells and the internal yolk. The blastoderm stage is of particular importance because it marks the transition from a syncytial to a cellular environment and the initially homogeneous blastoderm becomes divided up into diverse cell groups, each with a defined role. The result is a ground plan of the embryo, laid out in a two-dimensional cell sheet. The possibility of free diffusion of proteins during these early stages of development is extremely important for the generation of morphogenetic gradients that control embryonic pattern formation. During gastrulation, which last only about 20 minutes (stage 6-7), the newly formed cells change their shape and dramatic morphogenetic movements rearrange their positions respective to one another. Gastrulation is a universal step in animal development and occurs when a ventral subset of the blastoderm cells roll in to create a two-layered embryo. The outer germ layer (ectoderm) specifies the epidermis and the nervous system. The inner layer (mesoderm) will form most of the internal organs, such as muscles. In the meantime, segmentation divides the body into periodically repeated units. Segmentation begins at the level of gene expression at the syncytial blastoderm stage, but becomes morphologically visible only after germ band extension (stage 11). By this stage, the outer ectodermal epithelium displays evenly spaced grooves, which demarcate 14 parasegments, and the internal mesoderm is arranged in corresponding bulges. These

parasegments roughly correspond to the three mouth parts, three thoracic and eight abdominal segments present in the adult fly.

Genes controlling the body pattern in *Drosophila*

Systematic genetic screens have led to the discovery of at least 100 genes that control the organization of the *Drosophila* body plan during embryogenesis (62, 63). These studies demonstrated that early embryonic development in *Drosophila* is controlled by a hierarchy of transcription factors and is initiated by preformed mRNAs and proteins that are synthesized by the mother fly and laid down in the egg. Maternal gene products establish the antero-posterior (A-P) and dorso-ventral (D-V) axes and set up regional differences along each axis in the form of spatially distributed mRNAs and proteins. These maternal proteins act as morphogens that activate or repress zygotic genes at particular positions along both axes for the next round of patterning (64). Sequential activity of maternal and zygotic genes pattern the embryo in series of steps: broad regional differences are established first, and these are then refined to produce a large number of smaller developmental domains, each characterized by a unique gene expression profile and destiny. Developmental genes act in a strict temporal sequence. They form a hierarchy of gene activity in which the action of one set of genes is essential for another set of genes to be activated, and thus for the next stage of development to occur (65-67).

Segmentation is initiated by maternally derived morphogenetic gradients that emanate from sources localized at each end of the embryo (Fig 1-3). These maternal gradients directly or indirectly regulate the expression of the gap class of segmentation

Figure 1-3: Early development in *Drosophila* is controlled by a hierarchy of transcription factors.

Anterior-posterior (AP) and dorsal-ventral (DV) patterning is already established in the late oocyte by the localized accumulation of specific mRNAs. Soon after fertilization maternal gene products laid down in the egg, such as *bicoid* (*bcd*) and *nanos* (*nos*) mRNAs, are translated and the corresponding proteins freely diffuse in the syncytial environment creating morphogenetic gradients. These gradients provide positional informations that activate the zygotic genes in a temporal cascade. The first zygotic genes to be activated are the gap genes which include *hunchback, giant, krüppel, knirps* and *tailless*. Each of the gap genes is expressed in one or two broad domains along the AP axis and together with the maternally derived transcription factors regulate the expression pattern of pair-rule genes. Pair-rule genes are expressed in a periodic pattern of seven transverse stripes along the AP axis, which define the parasegments and foreshadow segmentation. Gap and pair-rule genes regulate the expression of the segment-polarity genes which that define the borders of the future segmental compartments. Segment identity is than determined by selector genes (not shown).

Panels on the left-side represent *in situ* hybridizations with digoxigenin-labled antisense mRNA for the indicated gene. Embryos are oriented with the anterior pole towards the left, dorsal side upwards.

Images in this dissertation are presented in color.

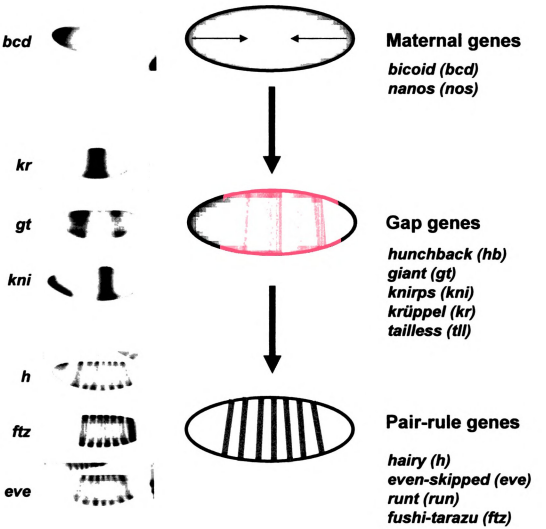

Figure 1-3: Early development in *Drosophila* is controlled by a hierarchy of transcription factors.

genes. Gap genes are the first zygotic genes to be expressed along the A-P axis and include *hunchback* (*hb*), *giant* (*gt*), *krüppel* (*kr*), *knirps* (*kni*), and *tailless* (*tl*). Gap genes are expressed in a simple pattern made up of one or two broad expression domains and perform two major functions. First, they control expression of the pair-rule class of segmentation genes, each of which is expressed in a series of seven transverse stripes along the A-P axis. These striped patterns are the first evidence of a reiterated body plan in early development, and precisely define the segmental compartments of the embryo. The borders of these compartments are then set by the segment polarity class of genes, which are expressed in a series of 14 stripes about one cell wide along the A-P axis. The second function of the gap genes in setting up the body plan is to limit the expression of the homeotic selector genes, which are involved in the specification of structures that are unique to each segment (67-68).

A textbook example of how pair-rule expression patterns are generated is provided by the regulation of the *even-skipped* (*eve*) gene (Fig. 1-4). In the syncytial blastoderm embryo, *eve* is expressed in a pattern of seven stripes along the A-P axis (69-72). The striped expression pattern appears gradually. Initially the gene is expressed at low level in most of the nuclei forming a broad stripe of expression which then is restricted to certain nuclei. Each stripe is initially fuzzy, but eventually acquires sharp margins. The *eve* locus (Fig. 1-4D) contains five non-overlapping enhancers that control the expression of individual or pairs of stripes (73, 74). Each stripe is specified independently, but a similar general mechanism is employed: the stripe is potentially expressed in a broad region by the activity of widely distributed activators and the final borders of each stripe are set by the combined activity of localized repressors. The best-

Figure 1-4: Regulation of the pair-rule gene *even-skipped* (*eve*).

At the blastoderm stage, the pair-rule gene *even-skipped* (*eve*) is expressed in seven transverse stripes along the anterior-posterior axis (A and top panel). The expression of *eve* was visualized in the embryo showed at the top by *in situ* hybridization using digoxigenin-labeled, antisense *eve* mRNA probe (the embryo is oriented anterior towards the left, dorsal upwards). The seven blastoderm stripes of *eve* expression are regulated by five independent enhancer elements (D) that are located within a 16-kbp regulatory locus. Three enhancers drive expression of single stripes (*eve* 1, *eve* 2 and *eve* 5), and the remaining two drive expression of pairs of stripes (*eve* 3+7 and *eve* 4+6). The minimal *eve* stripe 2 enhancer (E) is a ~480 bp cis-regulatory element which contains binding sites for the maternally contributed transcriptional activators Bicoid (Bcd) and Hunchback (Hb), and for the short-range repressors Giant (Gt) and Krüppel (Kr). A model for the regulation of *eve* stripe 2 is shown in panel B. The maternal Bcd and Hb (not shown) proteins form steep anterior to posterior gradients, activating *eve* stripe 2 expression in a broad anterior region of the embryo. Giant is expressed in a broad band in the anterior region of the embryo (blue curve) and represses the expression of *eve* stripe 2 in this region, setting the anterior border of the stripe. Similarly, Krüppel, which is expressed in the central region of the embryo (purple curve), sets up the posterior border of *eve* stripe 2 expression pattern. Regulations of other *eve* stripes follow a similar general mechanism although the activators and repressors involved are different. For instance, in the case of *eve* 3+7 and *eve* 4+6 regulation (panel C), the short-range repressor Knirps (Kni) sets up the inner borders for these stripes, whereas Hunchback (Hb) established the outer borders.

Panels A, B, and C are reproduced, with permission, from Dimitri Papatsenko (http://homepages.nyu.edu/~dap).

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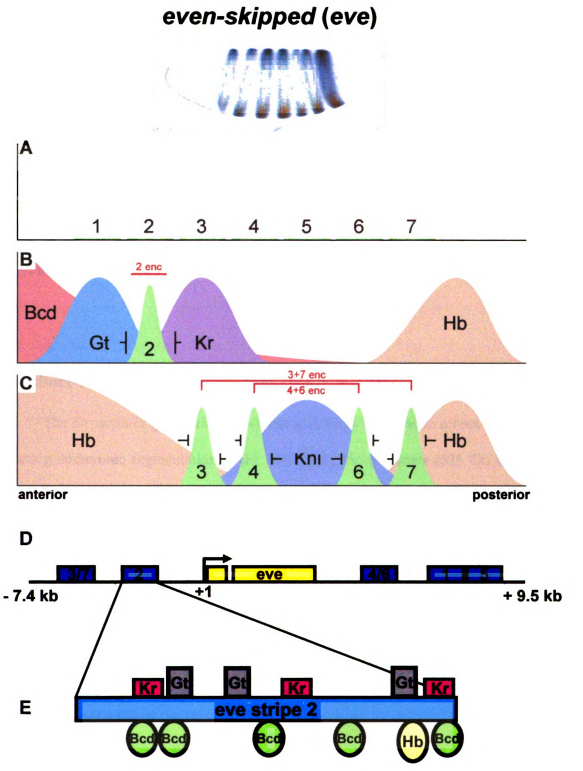

Figure 1-4: Regulation of the pair-rule gene *even-skipped* (*eve*).

characterized *eve* enhancer drives expression of stripe 2 (75-77), which is activated in a broad anterior domain by the maternal morphogens Bicoid (Bcd) and Hunchback (Hb). Borders of the stripe are formed by repressive interactions involving the gap protein Giant (Gt) Krüppel (Kr) and Sloppy Paired, which are expressed in gradients anterior and posterior to the stripe (Fig. 1-4B). Activation and repression are mediated by the direct binding of these proteins to discrete sites in the enhancer (Fig. 1-4E; 75, 77-79). Thus, this enhancer acts as a transcriptional switch that senses activator/repressor ratios in individual nuclei.

The short-range transcriptional repressor Knirps

The *Drosophila* gene *knirps* (*kni*) was identified in a genetic screen for mutations affecting embryonic segmentation in the fruit fly *D. melanogaster* (62). On the basis of its mutant phenotype it was classified as a gap gene, a class of mutants where large sections of the body pattern along the antero-posterior axis are missing. Mutations in the *kni* gene are embryonic lethal and show deletion of adjacent segments in the abdominal region (62, 80). In *kni* lack of function mutant embryos, out of the normal eight abdominal segments (A1-8) only one (A8) is properly formed. In place of the others, a single undifferentiated field is found (80-82). Chromosomal rearrangements associated with *kni* mutants enabled the localization of the *kni* locus to region 77E in the left arm of the third chromosome and the gene was cloned by a chromosome walk approach (80). The *kni* gene encodes a 429 aa basic protein (estimated pI= 10.2) with a calculated relative molecular mass of 45.6 kDa. The N-terminus of the protein shows significant

homology (about 50% identity) with members of the nuclear hormone receptor superfamily (80). This region corresponds to the DNA binding motif (aa 1-74), which encodes a Cys_2/Cys_2 type of zinc finger. Knirps is capable of binding as a monomer to its target DNA (83). The C-terminal region does not resemble the canonical hormone receptor ligand binding domain, however, and Knirps is not known to interact with small molecule ligands.

knirps expression is exclusively zygotic and the transcript is first detected after the 11^{th} nuclear division (stage 4, ~1.5-2 hours after fertilization), forming a broad band in the posterior region of the embryo (84 and Fig. 1-5A). Soon after, *kni* transcripts are also observed in the ventral region at the anterior tip (Fig. 1-5B). During cellularization (stage 5, 2-4 hours after fertilization), a third domain appears as a stripe posteriorly adjacent to the anterior domain (Fig. 1-5C). The posterior domain of *kni* expression gets weaker during gastrulation (Fig. 1-5D) and it cannot be detected during germ band extension (Fig. 1-5E, stage 6-7). In contrast, the anterior domains of expression remain detectable both during germ band extension and thereafter, when the pattern becomes highly complex (Fig. 1-5F, stage 8). The spatial distribution of Knirps protein closely follows the mRNA expression pattern and the protein appears to be nuclear (85 and Fig. 1-5G-L).

At the blastoderm stage, molecular targets of Knirps protein include the *even-skipped* (*eve*), *hairy*, *hunckback* and *runt* genes (53, 88-89). Knirps functions outside the blastoderm stage include tracheal formation during late embryogenesis and vein formation in the wing (90, 91). In transgenic embryo assays, Knirps represses heterologous enhancers and promoters over a short range. This repression function is

Figure 1-5: Knirps mRNA and protein expression patterns.

A-F. *knirps* (*kni*) mRNA expression pattern visualized by *in situ* hybridization using digoxigenin-UTP-labeled antisense *kni* mRNA probe. Embryos were fixed and stained according to standard protocols (77, 86) and are oriented anterior towards the left, dorsal side upwards. *knirps* expression at syncytial blastoderm stage appears first in the posterior domain (A) and slightly thereafter in an antero-ventral domain (B). At cellular blastoderm stage a third expression domain appears posteriorly to the antero-ventral domain (C), whereas the posterior domain gets weaker (D) and cannot be detected during germ band extension (E). At extended germ band stage a complex segmental pattern can be seen (F).
G-L. Knirps protein expression pattern visualized by *in situ* antibody staining. Knirps antibody was kindly provided by John Reinitz and used at 1:100 dilution. Embryos were fixed and stained according to ref. 87, using the ABC Elite kit (Vector Labs, Burlingame, CA) as described by the manufacturer. The spatial distribution of Knirps protein closely follows the mRNA expression pattern.

All embryos were collected from *yellow-white* flies. A and G: stage 4 embryos. B and H: early stage 5 embryos. C and I: late stage 5 embryos. D: stage 6 embryo. E: stage 8 embryo. F and L: stage 9 embryos.

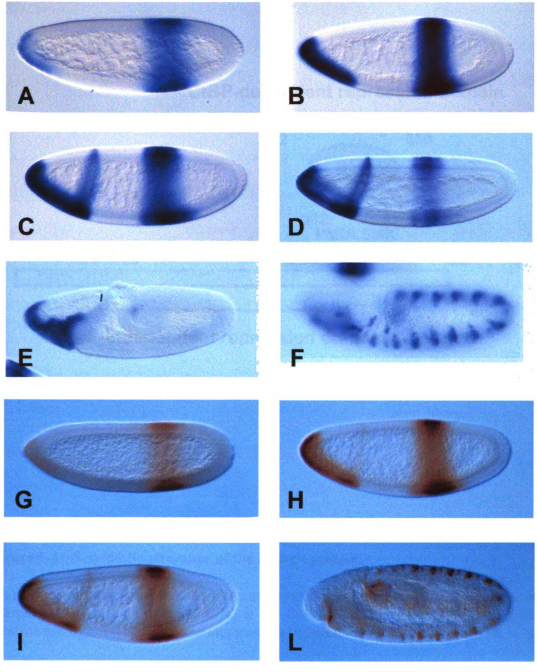

Figure 1-5: Knirps mRNA and protein expression patterns.

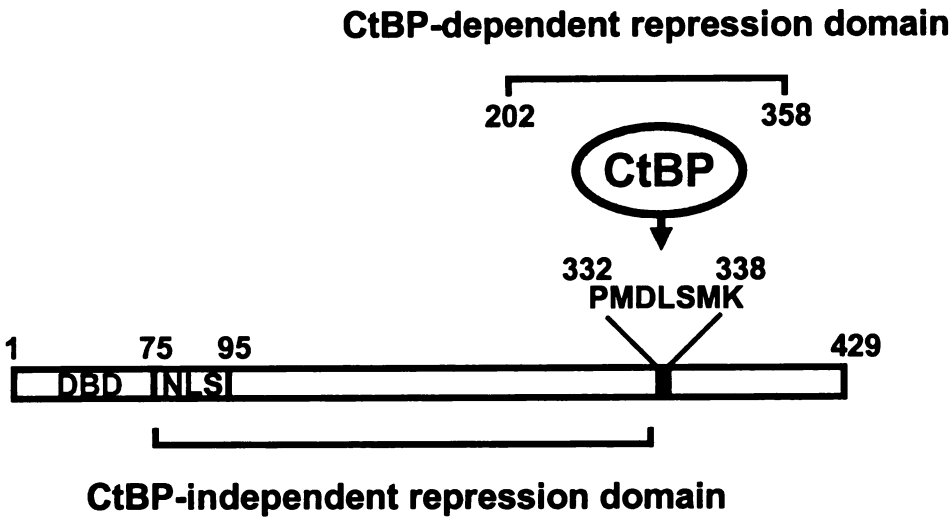

Figure 1-6: Schematic structure of the Knirps protein.

Knirps possess two functionally distinct repression domains: CtBP-dependent and –independent repression domain. DBD is the DNA-binding domain. NSL is the nuclear localization signal. The CtBP-binding motif is between amino acid 332 and 338.

modular and can be transferred to a heterologous DNA binding domain (46). Using an *in vivo* repression assay, Keller and colleagues (92) carried out a detailed structure-function analysis of Knirps and identified two distinct repression domains (Fig. 1-6). The first repression domain maps to the central region of Knirps (minimally between aa 211-358) and includes the CtBP binding motif, PMDLSMK (aa 332-338). This repression activity depends on CtBP, because deletions or mutations in the CtBP binding motif that abrogate CtBP binding also result in loss of repression activity (92-94). This repression function is here referred to as CtBP-dependent repression domain. The second repression domain is localized to the N-terminal region of Knirps (minimally between aa139-330). This domain of Knirps does not bind CtBP *in vitro* and is able to function in the absence of maternal CtBP (92 and Chapter 2). This repression function is here referred to as CtBP-independent repression domain.

To understand the mechanisms of Knirps-mediated repression, it is critical to identify the factors that functionally interact with this repressor. A genetic approach aimed to identify factors involved in *kni* regulation was impaired by the existence of *kni*-cognate gene, *knirps-related* (*knrl*) that can compensate for loss of *kni* activity when the cell cycle is delayed (95). Even though the expression pattern and biochemical functions of *kni* and *knrl* are apparently identical, *knrl* does not function in abdominal segmentation because its large primary transcript cannot be fully transcribed during the syncytial blastoderm stage due to the short duration of mitotic cycles (85). Zygotic suppressors of a *kni* mutant (*Resurrector* and *Godzilla*) were found to cause mitotic cycle delays at metaphase during the blastoderm stage. These mitotic cycle delays result in precocious expression of the *knrl* gene, which compensates for loss of *kni* activity (95).

Yeast two hybrid assay using Knirps as bait led to the identification of the corepressor CtBP (see below), but this assay did not identify additional Knirps-interacting factors (Keller and Arnosti, unpublished; 96). Therefore, I decided to use a biochemical approach to test whether Knirps interacts with additional factors (described in Chapter 4).

C-terminal Binding Protein (CtBP)

The C-terminal Binding Protein (CtBP) was originally identified in human cells during a search for cellular proteins that associate with the C-terminal region of adenovirus E1A oncoprotein and negatively modulate its transforming activity (97-98; reviewed in 99-100). CtBP was first linked to transcriptional repression by its functional interaction with the *Drosophila* repressors Knirps, Snail and Hairy (93, 101), and in subsequent studies has been found to contribute to the repression activity of Giant, Brinker and Hairless (102-104). A short sequence motif in these repressors, similar to the PLDLS motif present in E1A, is important for recruiting CtBP to promoter elements (105) and mutations that abrogate CtBP binding severely impair repression activity (93, 94, 101). CtBP is a *bona fide* transcriptional corepressor because it is able to directly repress a target gene when brought to the gene via a heterologous DNA-binding domain (94). CtBP homologs are present in vertebrates (mouse and *Xenopus laevis*), invertebrates (*Drosophila melanogaster* and *Caenorhabditis elegans*) and plants (106-109). CtBP proteins are homologous to NAD-dependent D-hydroxyacid dehydrogenases, and possess very similar overall structures to other dehydrogenases, as revealed by crystallographic studies (110, 111). CtBP proteins, like the homologous dehydrogenases, are dimeric

proteins containing an NAD/NADH binding domain and a substrate-binding domain (99, 111). Residues involved in NAD/NADH binding and dehydrogenase activity are absolutely conserved in CtBP proteins, suggesting an important function. Recent studies have shown that CtBP has a weak dehydrogenase activity in vitro, although the physiological substrates of CtBP as well as the significance of this enzymatic activity in transcriptional repression remain unknown (110, 112).

The mechanisms of repression mediated by CtBP are not currently understood. CtBP itself interacts with chromatin-modifying factors, including histone deacetylases and histone metyltransferases (113, 114, and 34). Thus, CtBP might repress transcription by recruiting chromatin- or protein-modifying factors to promoter regions. According to this model, the co-repressor simply acts as a bridging factor and the repression activity is caused by the specific factor/s recruited. Alternatively, or in addition, the dehydrogenase activity observed *in vitro* might also play an important, yet unclear, role in repression. CtBP binding to transcriptional repressors might be regulated by the nuclear NAD^+/NADH ratio, with NADH being two to three orders of magnitude more effective (115), however, others have failed to reproduce this finding. Agents capable of increasing NADH levels were shown to stimulate CtBP binding to its partners *in vivo* and to potentiate CtBP-mediated repression (115).

In addition to its roles in transcriptional repression, CtBP is also found in the cytoplasm, where is thought to participate in other cellular processes. CtBP is reported to possess an acylation activity, which increases membrane curvature during Golgi fission (116, 117). Subcellular localization of mammalian CtBP can be influenced by expression

of CtBP binding partners as well as post-translationally via modifications such as sumoylation or phosphorylation (118-120).

Drosophila has a single CtBP gene (*dCtBP*) mapped to region 87 D8-9 on the right arm of the third chromosome. The transcript is alternatively spliced to give at least three different mRNAs, predicted to produce proteins of 479, 386 and 383 amino acids in length (101). CtBP protein is maternally contributed and ubiquitously expressed throughout embryogenesis. Western blotting using a polyclonal antibody raised against the longer version of dCtBP ($dCtBP_L$) detects three proteins, a doublet that runs at approximately 42 kDa and a polypeptide of approximately 50 kDa. All three forms share the highly conserved dehydrogenase domain also found in vertebrate CtBP proteins and are present throughout embryogenesis (see Appendix A). $CtBP_L$ and $CtBP_S$ (the 383 aa-long isoform) demonstrate similar repression activities when assayed as Gal4-fusion proteins in cell-culture experiments, as well as on integrated reporters in transgenic embryos (121, 122), indicating that the C-terminal extension present in $CtBP_L$ does not affect repression activity. NAD binding to CtBP, but not the conserved histidine in the predicted catalytic site, was found to be critical for the repression activity mediated by Gal4-CtBP *in vivo*, suggesting that NAD may play an important role in short-range repression (122).

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Chapter 2

CtBP-independent repression activity of the *Drosophila* Knirps protein

2.1 Introduction

The identification of CtBP as a common corepressor for several *Drosophila* repressors including Knirps (Nibu et al., 1998a; Poortinga et al., 1998) was an important advance in our understanding of short-range transcriptional repression. By looking at the expression pattern of both synthetic and endogenous targets for *knirps* (*kni*), *krüppel* (*kr*) and *snail* (*sna*) in a maternal *CtBP* mutant, Nibu and colleagues concluded that CtBP was very important, if not essential, for the repression activity of short-range repressors. They also proposed the hypothesis that the functional distinction between short- and long-range repression depended on the type of corepressor recruited, such that short-range repressors utilized CtBP whereas long-range repressors employed Groucho (Nibu et al. 1998b).

A detailed structural-functional study carried out from our laboratory and aimed to map the Knirps minimal repression domain using an *in vivo* repression assay identified two functionally distinct repression domains (Fig. 1-6; Keller et al., 2000). The first repression domain includes the previously identified CtBP-binding domain (P-DLS-K, between amino acids 332-338) and comprises minimally amino acids 202-358. This repression domain is totally dependent upon the presence of an intact CtBP binding motif. Deletions or point mutations that abrogate binding also result in loss of repression *in vivo* (Keller et al. 2000). We refer to this Knirps repression domain as CtBP-dependent repression domain. The second repression domain, which maps to the N-terminal region

of Knirps (minimally between amino acids 139-330) does not contain a recognizable CtBP-binding motif and does not bind CtBP *in vitro* (Keller et al., 2000). These results suggest that the endogenous Knirps protein is potentially able to repress in the absence of the corepressor. Alternatively, the repression activity observed with the N-terminus of Knirps may also be CtBP-dependent, with the corepressor binding to non-canonical binding motif/s.

To test these two hypotheses I determined: 1) whether forms of Knirps containing the putative CtBP-independent activity, namely Gal4-Knirps 75-330 and Gal4-Knirps 75-429ΔPMDLS, would repress a synthetic Knirps target in the absence of maternal CtBP and 2) whether the endogenous Knirps protein was able to repress a native target without the contribution of CtBP. The results showed that both Gal4-Knirps chimeric proteins were able to repress an *eve* stripe 2-*lac Z* reporter gene in a maternal *CtBP*$^{-}$ background, suggesting that endogenous Knirps has the potential to function without the contribution of CtBP. More importantly, endogenous Knirps was able to repress a native target, the *eve* stripe 3 enhancer, in the absence of maternal CtBP, indicating that CtBP is dispensable at least for some targets. These results were included in Keller et al. (2000) and I include here only the portions of that paper that describe these experiments (section 2.2; Figures 2-1 and 2-2).

The short-range repressor Giant does not have a canonical CtBP-binding motif and previous studies suggested that Giant does not require CtBP for repression of the *eve* stripe 2 enhancer (Nibu et al., 1998b). To determine the contributions of CtBP to Giant mediated repression, I tested the expression patterns of endogenous and artificial Giant targets in a *CtBP*$^{-}$ maternal mutant. The results indicate that CtBP is required for Giant

repression of some, but not all, genes and that the Giant-CtBP interaction is likely to be direct. These results were included in Strunk et al. (2001), which I include here in its entirety (Section 2.3), as my experiments contributed to a major portion of this paper (Figures 3-3, 3-4 and 3-5). A parallel study carried out at the same time, also concluded that Giant might interact with the corepressor CtBP (Nibu, Y. and Levine, M. (2001). CtBP-dependent activities of the short-range Giant repressor in the *Drosophila* embryo. Proc. Natl. Acad. Sci. USA **98**: 6204-6208).

2.2 Functional identification of the CtBP-independent repression domain of Knirps[2]

Materials and Methods

Analysis of gene expression in embryos lacking maternal dCtBP. *CtBP*$^{-}$ germ line clones were produced using the autosomal FLP-DFS technique (Chou and Perrimon, 1996). Females carrying an *eve* stripe 2-upstream activation sequence (UAS)-*lacZ* reporter gene on chromosome 1 (Arnosti et al. 1996) were crossed to *D*/TM3, *Sb* males. Male progeny carrying the *lacZ* reporter and *D* were crossed to females carrying a balanced, P-element insertional mutation of *CtBP* (*CtBP*^{03463}/TM3, *Sb*; Bloomington stock no. P1590).

FRT, *ovo*^{D1}/TM3, *Sb* males (Bloomington stock no. 2149) were mated to females carrying the *Saccharomyces cerevisiae* FLP recombinase gene under the control of the *hsp70* promoter (hsFLP; *D*/TM3, *Sb*; Bloomington stock no. 1970) to generate males with hsFLP on chromosome 1 and *ovo*^{D1} over *D* on chromosome 3. Females carrying the *lacZ* reporter and the *CtBP* mutant allele over *D* were crossed to these hsFLP; *ovo*^{D1}/*D* males. Embryos from this cross were collected for 24 h, aged for 48 h and heat shocked for 2 h in a 37°C water bath. The heat shock was repeated 24 h after the first treatment. Females lacking the *D* marker (hsFLP; FRT, *ovo*^{D1}/*CtBP*^{03463}) were mated to males carrying the

[2] The results in this section were included in the following publication: **Keller S.A., Mao Y., Struffi P., Margulies C., Yurk C.E., Anderson A.R., Amey R.L., Moore S., Ebels J.M., Foley K., Corado M., and Arnosti D.N.** (2000). dCtBP-dependent and -independent repression activities of the *Drosophila* Knirps protein. Molecular and Cellular Biology **20**: 7247-7258.

appropriate Gal4-Knirps transgene. To assay for expression driven by the *eve* stripe 3 enhancer in a *CtBP* mutant background, males carrying an *eve-lacZ* fusion gene containing a 500-bp (–3.8 to –3.3 kbp) or an 800-bp (–3.8 to –3.0 kbp) portion of the *eve* stripe 3 enhancer (Small et al., 1996) were crossed to the females producing oocytes deficient in dCtBP. Embryos were collected, fixed, and stained as described (Small et al., 1992). As expected for *CtBP* mutants, embryos derived from these crosses died before hatching.

Results

Repression by Knirps proteins in *dCtBP* mutant embryos. We tested whether the N-terminal region of the Knirps protein, which does not directly interact with dCtBP protein, would mediate transcriptional repression in embryos lacking maternal dCtBP. Such embryos have been shown to be defective for repression by other dCtBP-binding repressor proteins, such as Snail (Nibu et al., 1998a), and the embryos show patterning defects reflective of disruption in pair-rule gene expression (Poortinga et al., 1998). The *CtBP* gene is expressed during oogenesis, and the message is deposited in the egg prior to fertilization (Poortinga et al., 1998). Therefore, we generated embryos that lacked a maternal contribution of dCtBP by the dominant female sterile FLP-DFS method (Perrimon et al., 1996). An *eve* stripe 2 *lacZ* reporter (Fig. 2-1A) was crossed into the *dCtBP* background, and somatic recombination was induced in females heterozygous for ovo^{D1} and the *CtBP* mutant allele to generate oocytes lacking dCtBP. Females were mated to males carrying Gal4-Knirps transgenes, and embryos were analyzed by *in situ* hybridization. The pattern of expression of the *eve* stripe 2 transgene is noticeably altered

Figure 2-1: Knirps N-terminal repression activity functions in *dCtBP* mutant embryos.

(A) *eve* stripe 2 *lacZ* reporter in a wild-type embryo. **(B)** *eve* stripe 2 *lacZ* reporter in a mutant embryo lacking maternal dCtBP. Anterior and posterior boundaries of the stripe are less well defined. **(C)** Repression by Gal4-Knirps 75-332 in a *dCtBP* mutant. Ventral expression of the transgene is repressed. **(D)** Repression by Gal4-Knirps 75-364mut, lacking the dCtBP binding motif. **(E)** Repression by Gal4-Knirps 75-429mut, lacking the dCtBP binding motif. Embryos are oriented with anterior being to the left and dorsal being to the top. Lateral views are shown, except for the ventrolateral view in panels C and E. *CtBP* embryos were generally shorter and broader than wild-type embryos. In the absence of repressor, 4% of embryos showed loss of ventral expression of the *eve* stripe 2 *lacZ* stripe in the mutant embryos (versus less than 1% in wild-type embryos). For the repressor shown in panel C, 31% of embryos showed loss of ventral staining (n = 140); for panels D and E, 31% (n = 39) and 75% (n = 65) of embryos, respectively, showed loss of ventral staining.

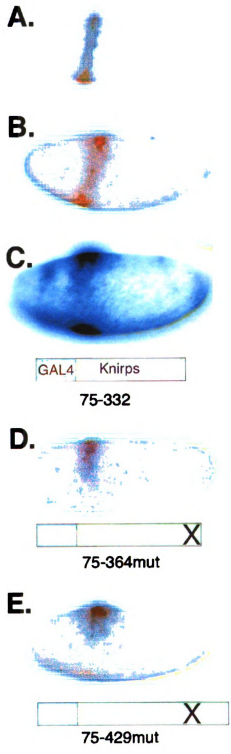

Figure 2-1: Knirps N-terminal repression activity functions in *dCtBP* mutant embryos.

in such a genetic background (Fig. 2-1B), changing from the normal narrow stripe to a broader band, consistent with the loss of Krüppel repression activity in posterior regions (Nibu et al., 1998) and possibly loss of Giant activity in anterior regions (B. Strunk, unpublished observations). The Gal4-Knirps 75-332 transgene was able to repress reporter gene expression in a significant number of embryos (Fig. 2-1C), while very few control embryos showed loss of ventral expression. Repression of the transgene in the *dCtBP* mutant background was also observed when we assayed Gal4-Knirps 75-364mut and 75-429mut proteins that lack the dCtBP interaction motif (Fig. 2-1D and E). Overall levels of repression were higher than those observed in wild-type embryos, possibly due to changes in Gal4-Knirps protein stability or transcription complex stability.

The endogenous Knirps target *eve* stripe 3 is repressed in a *dCtBP* mutant. To test whether endogenous targets of the *knirps* gene might also show repression in a *dCtBP* mutant embryo, we examined expression of a *lacZ* reporter gene derived from *eve*, which is a direct target of Knirps. Five binding sites for the Knirps protein have been identified within the 500-bp *eve* stripe 3 enhancer (Small et al., 1996). Consistent with this picture, a *lacZ* transgene driven by the *eve* stripe 3 enhancer shows broad posterior derepression in a *kni* mutant embryo (Fig. 2-2A and B) (Small et al., 1996). To test whether a similar pattern of derepression would be observed in the absence of dCtBP, we crossed males carrying a 500-bp *eve* stripe 3 *lacZ* transgene (Small et al., 1996) to females producing dCtBP-deficient oocytes. Expression of the *eve* stripe 3 transgene was not derepressed in posterior regions of the embryo, suggesting that Knirps is still able to repress this element in the absence of maternal dCtBP (Fig. 2-2C). The mutant embryos did show other alterations in *lacZ* expression, including ectopic expression in anterior regions and a

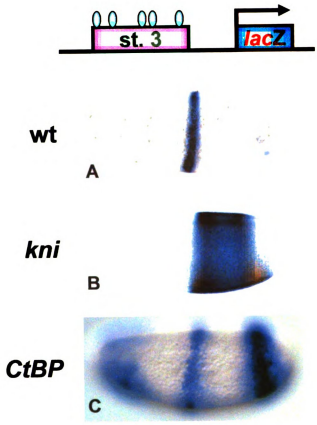

Figure 2-2: *eve* stripe 3 lacZ reporter gene is repressed normally in posterior regions in a *CtBP* mutant embryo.

(A) Expression pattern of a 500-bp *eve* stripe 3 reporter gene in a wild-type embryo. **(B)** Posterior derepression of the reporter gene in a *knirps* mutant embryo. **(C)** Expression pattern of *eve* stripe 3 *lacZ* in a *CtBP* mutant embryo. Embryos did not show derepression in the posterior region of the embryo but did show consistently stronger staining in *eve* stripe 7 regions (an activity partially contained within *eve* stripe 3 enhancer sequence [Small et al., 1996]) and ectopic activation in the anterior regions of the embryo. (A and B) Parasagittal view; (C) surface view. Embryos are oriented with anterior at the left and dorsal at the top.

broadening and intensifying of the posterior stripe (Fig. 2-2C). A similar pattern of repression was observed with a stripe 3 *lacZ* reporter carrying an 800-bp enhancer (–3.8 to –3 kbp) (data not shown). The stronger derepression phenotype of the *kni* mutant compared to the *CtBP* mutant suggests that Knirps contains a repression activity separate from dCtBP, consistent with our identification of an additional N-terminal repression region.

2.3 Role of CtBP in transcriptional repression by the *Drosophila* Giant protein[3]

Abstract

The Giant protein is a short-range transcriptional repressor that refines the expression pattern of gap and pair-rule genes in the *Drosophila* blastoderm embryo. Short-range repressors including Knirps, Krüppel, and Snail utilize the CtBP cofactor for repression, but it is not known whether a functional interaction with CtBP is a general property of all short-range repressors. We studied giant repression activity in a *CtBP* mutant and find that this cofactor is required for Giant repression of some, but not all, genes. While targets of Giant such as the *even-skipped* stripe 2 enhancer and a synthetic *lacZ* reporter show clear derepression in the *CtBP* mutant, another Giant target, the *hunchback* gene, is expressed normally. A more complex situation is seen with regulation of the *krüppel* gene, in which one enhancer is repressed by Giant in a CtBP-dependent manner, while another is repressed in a CtBP-independent manner. These results demonstrate that Giant can repress both via CtBP-dependent and CtBP-independent pathways, and that promoter context is critical for determining Giant-CtBP functional interaction. To initiate mechanistic studies of the Giant repression activity, we have identified a minimal repression domain within Giant that encompasses residues 89-205, including an evolutionarily conserved region bearing a putative CtBP binding motif.

[3] This section was published as the following manuscript: **Strunk B., Struffi P., Wright K., Pabst B., Thomas J., Qin L., and Arnosti D.N.** (2001). Role of CtBP in transcriptional repression by the *Drosophila* giant protein. Developmental Biology **239**: 229-240.

Introduction

The precise expression of developmentally regulated genes often reflects the coordinate activity of both transcriptional activators and repressors acting on complex regulatory elements (Arnone and Davidson, 1997; Ghazi and VijayRaghavan, 2000). Transcriptional repressors involved in early gene expression in *Drosophila* embryogenesis include the products of gap genes, pair-rule genes, and mesoderm-specific genes. A major advance in understanding the action of some of these proteins came in the recognition that some of these factors, including Krüppel, Knirps, Snail, and Giant, are "short-range' repressors, able to act over distances of 100-150 bp to interfere with the activity of enhancers and basal promoter elements (Gray et al., 1994). Other "long-range" repressor proteins such as Hairy are able to interfere with enhancers and promoters over distances of >1kb, and can block the activity of multiple enhancers simultaneously (Cai et al., 1996).

The mechanisms by which short-range and long-range *Drosophila* repressors inhibit transcription are poorly understood, although a variety of potential pathways have been described, including competitive binding with activators or elements of the basal machinery, "quenching" of nearby activators, and chromatin remodeling (Stanojevic et al., 1991; Hoch et al., 1992; Gray et al., 1994; Chen and Courey, 2000). Differences in cofactor requirement suggest that these proteins are likely to utilize distinct pathways to effect transcriptional repression. Long-range repression complexes involving Dorsal protein and the Hairy protein have been shown to bind to the Groucho corepressor, which is thought to act in turn through histone deacetylases (Jimenez et al., 1997; Chen and Courey, 2000). Several short-range repressors have been shown to interact with the CtBP

corepressor, although it is not known if this is a general characteristic of all short-range repressors (Nibu et al., 1998a, 1998b). It has been suggested that Giant, in particular, does not require CtBP for repression of the *eve* stripe 2 enhancer (Nibu et al., 1998b). In addition, CtBP has been shown to interact with Hairy, although in this case the cofactor appears to inhibit, rather than potentiate, repression (Poortinga et al., 1998; Zhang and Levine, 1999).

Previous work has established that the Giant protein functions in a number of embryonic transcriptional circuits to regulate the expression of gap and pair rule genes, including *even-skipped* (*eve*), *hunchback* (*hb*), and *Krüppel* (*Kr*) (Stanojevic et al., 1991; Kraut and Levine, 1991; Capovilla et al., 1992; Wu et al., 1998). Recent work has also identified the functional interaction of Giant with the *iab-2* enhancer of the *abd-A* homeotic selector gene (Shimell et al., 2000). Several lines of evidence suggest that *eve*, *Kr*, and *abd-A* are direct targets of Giant: their expression is derepressed in a *giant* (*gt*) mutant background, and these genes' regulatory elements contain binding sites for giant protein. In the cases of *eve* and *abd-A*, the sites have been mutated to verify that *giant* repression is lost in vivo (Small et al. 1992; Arnosti et al., 1996; Shimell et al., 2000). In addition, ectopic expression of Giant, either via a heatshock inducible promoter or an ectopic *eve* stripe 2 enhancer, represses *Kr* and *eve* expression in the blastoderm embryo (Kraut and Levine, 1991; Capovilla et al., 1992; Wu et al., 1998). Acting within these regulatory regions, the short-range repression activity of Giant prevents regulatory "cross-talk", so that Giant repression of one enhancer does not interfere with the activity of another (Small et al. 1993; Hewitt et al., 1999). The short range of Giant activity can be used to produce genetic switches which are finely "tuned" to respond to small

differences in Giant protein concentration (Hewitt et al., 1999). Such fine adjustments in repression activity appear to have been used during the evolutionary modification of the *eve* stripe 2 enhancer, where a Giant binding site has been repositioned to compensate for increased activation activity due to acquisition of a novel Bicoid activator binding site (Ludwig et al., 1998; Hewitt et al., 1999; Ludwig et al., 2000).

While much is known about the action of Giant in native regulatory circuits, we do not understand the molecular details of repression by the Giant protein. In particular, it is not known whether Giant functionally interacts with the CtBP cofactor. Furthermore, it is not known whether the ultimate target of Giant is the transcriptional machinery, activator proteins, or chromatin, although Giant, like other short-range repressors, is capable of repressing from within enhancers or when situated proximal to basal promoter elements (Small et al., 1992; Hewitt et al. 1999, Shimell et al., 2000). To determine whether CtBP is required for Giant's short-range repression activity, we have studied the activity of endogenous and chimeric repressors in wild-type and *CtBP* mutant embryos, and we have identified an evolutionarily conserved minimal repression region that is sufficient to mediate transcriptional repression in transgenic embryos.

Materials and Methods

Plasmids. The following oligonucleotides were used in construction of Gal4-giant chimeric constructs:

5'-GATCCGCCGATTACAAGGATGACGATGACAAGTAGTAATTAGTTAGT-3' (a), 5'-CTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCGGCG-3' (b), 5'-GATCCCGCCGATTACAAGGATGACGATGACAAGTAGTAATTAGTTAG T-3' (c), 5'-CTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCGG CGG-3' (d), 5'-GGCCGCCGATTACAAGGATGACGATGACAAGTAGTAATTAGTT AGT-3' (e), 5'-CTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCG GC-3' (f), 5'-ATGAAGCTACTGTCTTCTATC-3' (g), 5'-GGGGTCTAGACTAACTA AT TACTACTTGTCATCGTCATCCTTGTAATCGGCGTAAAAAGCGGGATACAG GGAGGC-3' (h), 5'-GGGGTCTAGACTAACTAATTACTACTTGTCATCGTCATCC TTGTAATCGGCTTGGGCGGCATACAGAAGATTGCT-3' (i), 5'-GGCCGATTACA AGGATGACGATGACAAGTAGTAATTAGTTAGT-3' (j), 5'-CTAGACTAACTA ATTACTACTTGTCATCGTCATCCTTGTAATCGGCCTGCA-3' (k), 5'-CGCAGCT GCA-3' (l), 5'-GCTGCGGTAC-3' (m), 5'-GGGTCGGTAACCGCAGCCCAACAGCA GCAACATCAG-3' (n), 5'-GGGTCGGTACCGCAGCCGCTGCCGCCTCTGCTGCG-3' (o), 5'-CGCCGCAGC CG-3' (p), 5'-GATCCGGCTGCGGCGGTAC-3' (q), 5'-GGGGTACCGCCGCAGCGC AGCAGCAGCATACCTCCTCTGCA-3' (r), 5'-GGGGTCTAGACTAACTAATTACTACTTGTCATCGTCATCCTTGTAATCGGC GTTAGCGGTTGGTGTGACCTTGGG–3' (s), 5'-GGGGTCTAGACTAACTAATT ACTACTTGTCATCGTCATCCTTGTAATCGGCGTAAAAAGCGGGATACAGGGA

GGC-3' (t), 5'-GGGTCGGTACCGCCGCAGCGAGCGTAGAGACGCCCAGGAAGA CT-3' (u), 5'-GGGTCGGTACCGCCGCAG CGAATCTTCTGTATGCCGCCCAA ATG-3' (v), 5'-CTCTGTAGGTAGTTTGTCC-3' (SV40 3'UTR) (w).

To generate construct 2, construct 1 (Gal4-giant1-389, described in Hewitt et al., 1999) was digested first with *XbaI* and partially with *HindIII*. The digested plasmid was then ligated with oligonucleotides (a) and (b) to generate Gal4-giant (1-322). Construct 3 was made the same way, using the linearized vector containing Giant codons 1-265 and ligating oligos (c) and (d). Construct 4 was made by digesting construct 1 with *NotI* and *XbaI* and ligating with oligos (e) and (f). Constructs 7, 8, and 11 were made in a similar fashion digesting construct 1 with *PstI* and *XbaI* and ligating oligos (j) and (k) for construct 7, digesting construct 1 with *KpnI* and *PstI* and ligating oligos (l) and (m) for construct 8, and digesting construct 1 with *KpnI* and *BamHI* and ligating oligos (p) and (q) for construct 11. Constructs 5, 6, 9, and 10 were made by PCR amplifying the appropriate portion of the gene, digesting with *KpnI* and *XbaI*, and ligating the fragment into pTwiggy (Hewitt et al., 1999). Oligos (g) and (h) were used to generate construct 5, (g) and (i) were used for construct 6, (n) and (w) were used for construct 9, (o) and (w) were used for construct 10, (r) and (w) for construct 12, (r) and (t) for construct 14, (u) and (w) for construct 15, and (v) and (w) for construct 16, and (r) and (s) for construct 13.

Isolation of *giant* homolog from *Drosophila hydei*. *D. hydei*, originally derived from a parent stock collected in 1993 at the South Coast Agricultural Research Station, California, was obtained from the Scott Pitnick laboratory, Syracuse University.

Genomic DNA was prepared from adult flies using the Promega Wizard Genomic Prep Kit (cat. #A1120). Degenerate oligos:

DA-190 5'-AAAAGAATTCATGCAYCAYCAYCARTAYCARC-3' and DA-191 5'-AAAAGAATTCNGCNGCGAARTTNGCNGCHAT-3' were used to amplify a region of the gene corresponding to *D. melanogaster giant* codons 23-274 using 35 one minute cycles of 95°C, 50°C, and 72°C. Visible bands of approximately the correct size were isolated, digested with *EcoRI*, subcloned into pBluescript SK(+) and individual clones were sequenced. Sequence information was used to generate nondegenerate oligonucleotides DA-441 5'-AAAAGAATTCCAGCAGCAGCAAGCATCGCAT-3', DA-443 5'-AAAAGAATTCCACGAGCGGATCACGCGGAAAG-3' and DA-444 5'-AAAAGAATTCGGAAAGGCCTTAAACGGGCGCG-3' corresponding to *D. melanogaster* codons 32 to 267. These oligonucleotides were used in genomic amplifications, and resulting products were directly sequenced without subcloning to reconfirm sequences. (GenBank accession number AF356543).

P-element transformation, whole-mount *in situ* hybridization of embryos, and crosses to *lacZ* reporter lines. P-element mediated germline transformation and *in situ* hybridization was carried out as described, except that during the hybridization procedure, embryos were not fixed again with formaldehyde and not treated with proteinase K (Small et al., 1992). Probes for *eve* and *Kr* staining were prepared by subcloning a 2.6 kbp *EcoRI/XbaI eve* fragment or 1.9 kbp *EcoRI/XbaI Kr* fragment from bacterial expression vector pAR3040 (S. Small) into pBluescript II KS(+) and performing *in vitro* transcription reactions of template linearized with *XbaI* with T3 RNA polymerase

in the presence of digoxigenin UTP as described (Small et al., 1992). Quantitative assays of percent repression by Gal4-giant fusions were performed as previously described, using *eve* stripe 2 *lacZ* and *eve* stripe 2/*eve* stripe 3 *lacZ* transgenes as reporters (Keller et al., 2000). Levels of repression never exceed 50% because of heterozygosity of the Gal4-giant lines.

Analysis of gene expression in embryos lacking maternal *CtBP*. CtBP germline clones were produced using the autosomal FLP-DFS technique (Chou and Perrimon, 1996). Single reporter transgenes were assayed in the mutant embryo background by crossing males carrying the transgene to females producing *CtBP* embryos. To test the activity of Gal4-giant in a *CtBP* mutant background, the *eve* stripe 2 *lacZ* reporter gene was crossed into the *CtBP* mutant stock as described previously (Keller et al., 2000).

Results

Giant repression is compromised in a *CtBP* mutant background.

To determine if repression activity by the Giant protein was affected in a *CtBP* mutant background, we studied the expression pattern of *eve* and synthetic *lacZ* reporter genes that are direct targets of Giant, using *in situ* hybridization. Giant protein helps to set the anterior border of *eve* stripe 2, and binding sites for the Giant protein have been identified in the stripe 2 enhancer (Stanojevic et al., 1991; Small et al., 1992). Loss of *gt* activity or disruption of Giant binding sites within the stripe 2 enhancer causes anterior expansion of expression of an *eve* stripe 2 *lacZ* reporter gene (Small et al., 1992; Arnosti et al., 1996). The expression pattern of endogenous *eve* shows complex changes in a *CtBP* mutant (Poortinga et al., 1998; Nibu et al., 1998b; Fig. 2-3B), including a possible anterior expansion of stripe 2, but the presence of multiple enhancers in the endogenous gene makes it difficult to determine specifically how the stripe 2 enhancer activity is affected. We therefore examined the expression pattern of an *eve* stripe 2 *lacZ* reporter gene and found, in contrast to an earlier report (Nibu et al., 1998b) that in most embryos there is a significant anterior expansion of the *eve* stripe 2 expression pattern in the *CtBP* mutant background, as well as the posterior expansion previously noted. The expression pattern changes from the wild-type pattern of 56-62% egg length (S.D. 1.5%, n=25) to 52-67% egg length (S.D. 3%, n=34) in the mutant (Fig. 2-3C-F). Posterior expansion results from loss of Krüppel activity (Nibu et al., 1998b), while anterior expansion mimics that seen in a *gt* mutant (Stanojevic et al., 1991; Small et al., 1992; Wu et al., 1998). This result is consistent with CtBP participating in establishment of the anterior border of expression

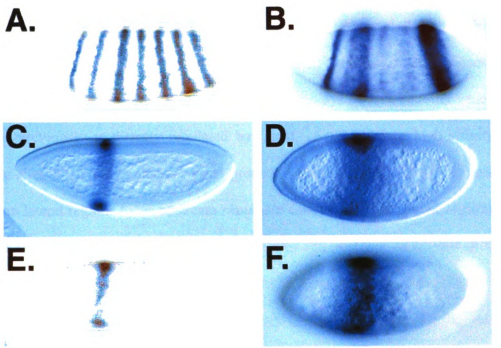

Figure 2-3: Derepression of *eve* stripe 2 expression in a *CtBP* mutant background.

The expression of the endogenous *eve* gene (A, B) or an *eve* stripe 2 *lacZ* reporter gene (C-F) was assayed in wild-type (A,C,E) or *CtBP* mutant embryos lacking maternal CtBP (B, D, F) by *in situ* hybridization. Anterior border expansion, consistent with loss of Giant activity, and posterior border expansion, resulting from loss of Krüppel repression, can be seen in D and F. The average position of the pattern generated by the *eve* stripe 2 *lacZ* transgene in wild-type embryos was 56 to 62% egg length (n=25, standard deviation for each border 1.5%), while the average position of the pattern in *CtBP* mutant embryos was 52 to 67% egg length (n=34, standard deviation for each border 3%). Embryos are shown anterior to the left, dorsal side up. (C, D) parasaggital views (to compare age of embryos); (E, F) surface views. *CtBP* embryos are typically shorter than wild-type embryos.

lacZ mRNA and endogenous *eve* mRNA were visualized by in *situ* hybridization.

of *eve* stripe 2, but does not prove that CtBP works through the Giant protein. CtBP may interact with a putative heterodimeric partner of Giant, or it may interact with other repressors that have been proposed to also play a role in setting the anterior border of *eve* stripe 2 (Vasisht, V., Theodosopoulou, K., Small S., Abstract 452A; 40th Annual *Drosophila* Research Conference, Seattle, WA, 1999[4]). Therefore, to study Giant activity in the absence of other putative repressor sites, we employed a *lacZ* reporter gene that we showed previously is directly regulated by Giant, containing two high-affinity Giant binding sites 5' of the P element basal promoter (Hewitt et al., 1999). Expression is driven in lateral regions by an upstream *rhomboid* enhancer, and in ventral regions by the *twist* enhancer (Fig. 2-4A, C). The strong anterior and posterior repression of the *lacZ* transgene is almost completely abolished in the *CtBP* mutant background (Fig. 2-4B, D), leaving weakly attenuated expression in narrow anterior and posterior regions. This pattern is reminiscent of those obtained from *lacZ* reporter derivatives that have the Giant binding sites moved to distal positions at –110 bp or –160 bp, at the limit of giant's range of activity (Hewitt et al., 1999). The *gt* gene is still expressed in the *CtBP* mutant, indicating that the loss of repression is not simply due to loss of *gt* expression, although the area of posterior expression is expanded, as has been previously noted (Nibu et al., 1998b; Fig. 2-4E, F).

[4] Now published as: **Andrioli L.P.M., Vasisht V., Theodosopoulou E., Oberstein A., and Small S.** (2002). Anterior repression of a *Drosophila* stripe enhancer requires three position-specific mechanisms. Development **129**: 49314940.

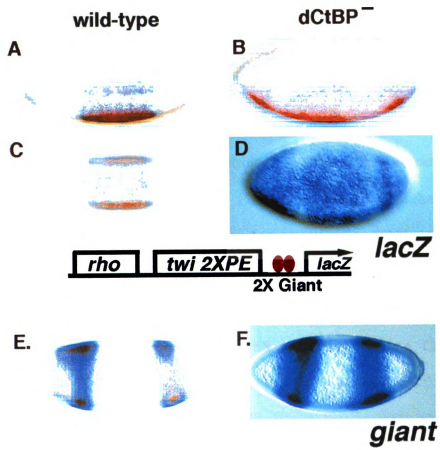

Figure 2-4: Loss of Giant repression activity in a *CtBP* mutant background.

Wild-type (A, C) and *CtBP* mutant (B, D) embryos carrying a *lacZ* reporter gene with tandem Giant binding sites at –55 bp were assayed by *in situ* hybridization. Ventral and ventrolateral expression is driven by *rhomboid* and *twist* enhancer elements, which in the absence of Giant binding sites allow expression of the *lacZ* transgene from anterior to posterior (Hewitt et al., 1999). The strong anterior and posterior repression mediated by Giant (A, C) is greatly attenuated in the *CtBP* mutant embryos (B, D). Expression of *gt* in wild-type (E) and *CtBP* mutant (F) embryos indicates that *CtBP* mutant embryos express *gt* in an almost wild-type pattern. Embryos are shown anterior to the left, dorsal side up (A, B, E, F) or ventral side toward viewer (C, D).

Selective requirement for CtBP in regulation of *Kr*

The loss of repression in the *CtBP* background exhibited by the two different *lacZ* reporter genes strongly suggests that Giant repression can depend on CtBP. Therefore we carefully examined the patterns of *Kr* and *hb*, two endogenous targets of *giant*. The anterior border of *Kr* is highly sensitive to changes in levels of Giant protein (Wu et al., 1998), and two high-affinity binding sites for the Giant protein have been identified within the upstream regulatory region that controls expression of *Kr* in the central domain (CD) of the embryo (Capovilla et al., 1992). Previous studies indicated that the central domain of *Kr* expression is not grossly disrupted in a *CtBP* mutant (Nibu et al., 1998b; Poortinga et al., 1998), but as early blastoderm embryos were shown in these studies, it is unclear whether the later anterior shifts in *Kr* expression caused by loss of *gt* would have been noted. We compared the pattern of *Kr* expression in wild-type, *CtBP*, and *gt* embryos, and did not detect noticeable anterior expansion of the CD in the *CtBP* mutant. However, a striking difference was noted in the *Kr* anterior domain (AD), which is wider and persists later in development in *CtBP* embryos than in wild-type embryos (Fig. 2-5). *gt* embryos show a similar pattern of altered expression in the AD (Fig. 2-5 G, H, I). The AD stripe is expressed in ventral regions in the *CtBP* mutant, while in the wild-type and *gt* embryos, this stripe does not extend into ventral regions. This loss of ventral repression in a *CtBP* mutant is probably due to a loss of *knirps* activity, for *knirps* is expressed in ventral anterior regions, and Knirps protein has been shown to bind to the *Kr* promoter (Hoch et al., 1992). *giant* is also required for repression of *hb* expression in the region of the embryo anterior to the parasegment 4 stripe, and low levels of ectopic Giant protein are sufficient to repress *hb* in this area, suggesting that the element is highly

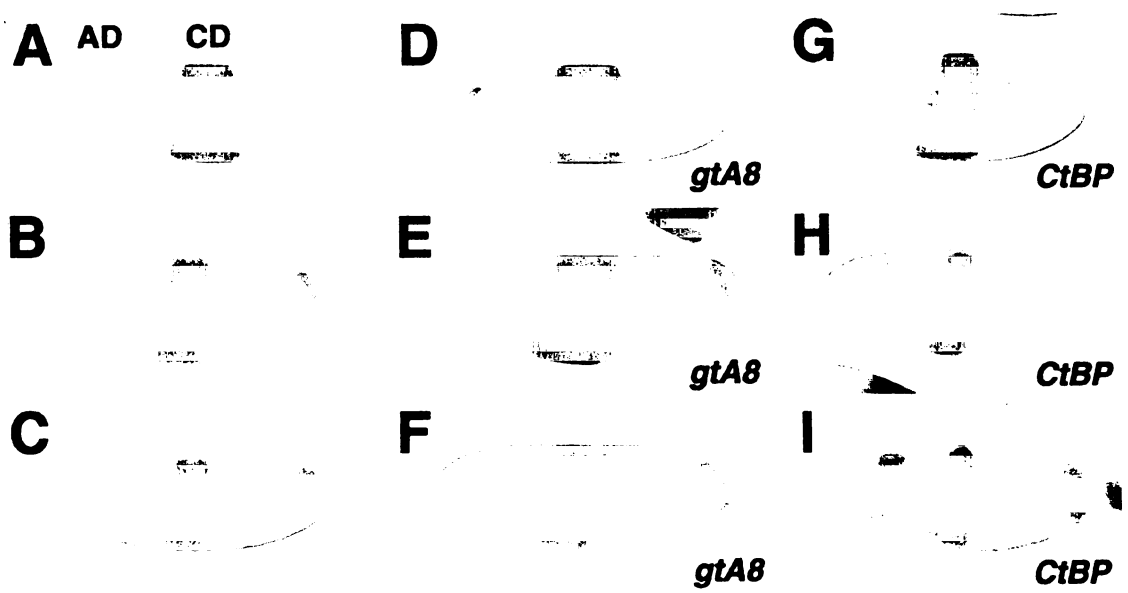

Figure 2-5: Anterior domain (AD) of *Kr* expression is regulated by CtBP and Giant, while central domain (CD) is regulated by Giant but not CtBP.

Kr expression in staged embryos (youngest at top) was examined by *in situ* hybridization. Blastoderm expression is comprised of a small AD stripe, a prominent CD domain, and a posterior domain of expression. (A-C) Wild-type embryos, (D-F) gt^{A8} mutant embryos, and (G-I) *CtBP* embryos. The expression of the AD is expanded in both *gt* and *CtBP* mutant embryos, while the CD is expanded only in *gt* mutant embryos. Abnormal ventral expression of AD in *CtBP* mutant embryos may represent loss of Knirps activity. Embryos are oriented anterior to the left, dorsal side up.

sensitive to Giant (Wu et al., 1998). We did not find any differences in the *hb* expression pattern between wild-type and *CtBP* mutant embryos (data not shown). These results indicate that CtBP is not required for Giant mediated repression of some endogenous genes and enhancers, consistent with earlier suggestions that Giant may function by more than one mechanism (Wu et al., 1998).

Gal4-giant repression domain can function in a *CtBP* mutant embryo

It is not known whether Giant normally acts as a homodimer or a heterodimer, thus assays of endogenous Giant activity might reflect the contribution of a basic zipper partner protein rather than the Giant protein itself (Vavra et al., 1989). The non-DNA binding region of the Giant protein is clearly a bona fide repressor; when tethered to the Gal4 DNA binding domain this protein can mediate repression in the embryo, indicating that another basic-zipper partner protein is not required for activity (Hewitt et al., 1999). We tested whether the Gal4-giant fusion protein used in these assays was capable of repressing in a *CtBP* background. Females producing embryos that lacked maternal CtBP protein and containing the *eve* stripe 2 *lacZ* reporter gene were crossed to males carrying the Gal4-giant repressor gene (Figure 2-6). A high percentage of embryos showed repression in ventral regions, where the Gal4-giant fusion protein is expressed under control of the *twist* promoter. These results indicate that the Giant protein itself contains an activity that is capable of repressing under conditions where the CtBP protein is severely reduced or absent.

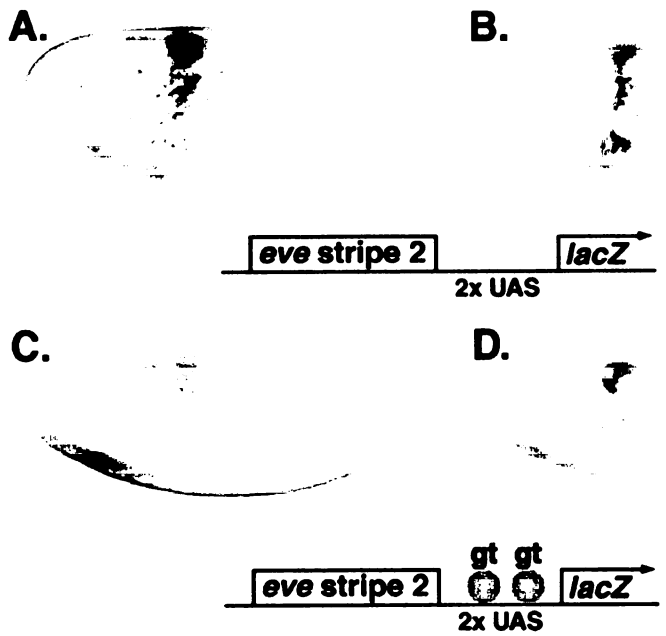

Figure 2-6: Gal4-giant protein is an active repressor in a *CtBP* mutant background.

Expression of an *eve* stripe 2 *lacZ* reporter gene is shown in wild-type (A, B) and a *CtBP* mutant background (C, D). Embryos shown in C, D contains Gal4-giant chimeric protein expressed in ventral regions under control of the *twist* promoter. Embryos are oriented anterior to the left, dorsal side up. Parasagittal views (A, C) shown to compare stage of embryos, and surface views (B, D) to illustrate ventral interruption of stripe. In *CtBP* embryos expressing Gal4-giant, 32 of 81 (40%) embryos scored showed loss of ventral activation, compared with 4 of 132 (3%) in *CtBP* embryos without Gal4-giant. (In wild-type embryos, less than 0.5% of embryos show abnormal stripes).

Identification of a minimal repression domain in Giant

Repression by Giant can be mediated by the N-terminal 389 residues of the protein, independent of its native basic-zipper DNA-binding domain (Hewitt et al., 1999). We tested which residues are sufficient to mediate repression in transgenic embryo assays by preparing and testing transgenic lines expressing chimeric Gal4-giant proteins in ventral regions of the embryo (Fig. 2-7). These repressors were assayed on *eve* stripe 2 *lacZ* and *eve* stripe 2+3 *lacZ* reporter genes and the fraction of embryos showing repression was quantitated (Table 2-1). Fusion proteins containing most of the Giant protein showed robust repression, comparable to levels achieved with Knirps fusion proteins (Keller et al., 2000). C-terminal truncations to residue 205 retained significant, although somewhat reduced levels of repression activity, as did N-terminal deletions to residue 96. A minimal Gal4-giant (89-205) chimeric protein was also active for repression in these assays (Fig. 2-7 and Table 2-1). However, a further N-terminal deletion of this minimal repressor, removing residues 89-106, produced an inactive construct. Within this short deletion is a sequence (residues 98-104, V-DLS-R) that is similar to a high affinity CtBP binding motif (P-DLS-K/R) (Nibu et al., 1998b, Poortinga et al., 1998), therefore it is possible that the N-terminal deletion removes a CtBP interacting site. The first residue of the canonical CtBP-binding motif, a proline, is required for high affinity *in vitro* binding (Molloy et al., 1998), consistent with the lack of measurable direct *in vitro* interaction between Giant and GST-CtBP (data not shown, see Discussion). Constructs truncated after residue 169 (number 5 and 14) had detectable, but significantly reduced activity, while the activity of lines containing a construct truncated after residue 143 (number 6) was close to background levels (Table 2-1). Some lines expressing a Gal4- giant fusion

Figure 2-7: Structure and activity of Gal4-giant chimeras assayed in transgenic embryos.

(A) Genes encoding Gal4 fusions including Giant residues indicated in constructs 1-16 were introduced into *Drosophila* by P-element mediated germline transformation. Repression activity of the chimeric proteins was assayed by crossing Gal4-giant lines to reporter lines containing *eve* stripe 2 *lacZ* and *eve* stripe 2 + stripe 3 *lacZ* reporter genes. Activities are shown as "+" (> 9% repressed), "+/-" (1.5-4% repressed), and "-" (less than 1% repressed). **(B)** Representative embryos showing pattern of the unrepressed reporter gene, and embryos from Gal4-giant (1-389), Gal4-giant (1-322), and Gal4-giant (89-205) crosses, showing ventral interruption of stripe 2 pattern. In comparing embryos showing ventral repression, no significant differences in extent of repression of stripe patterns were noted.

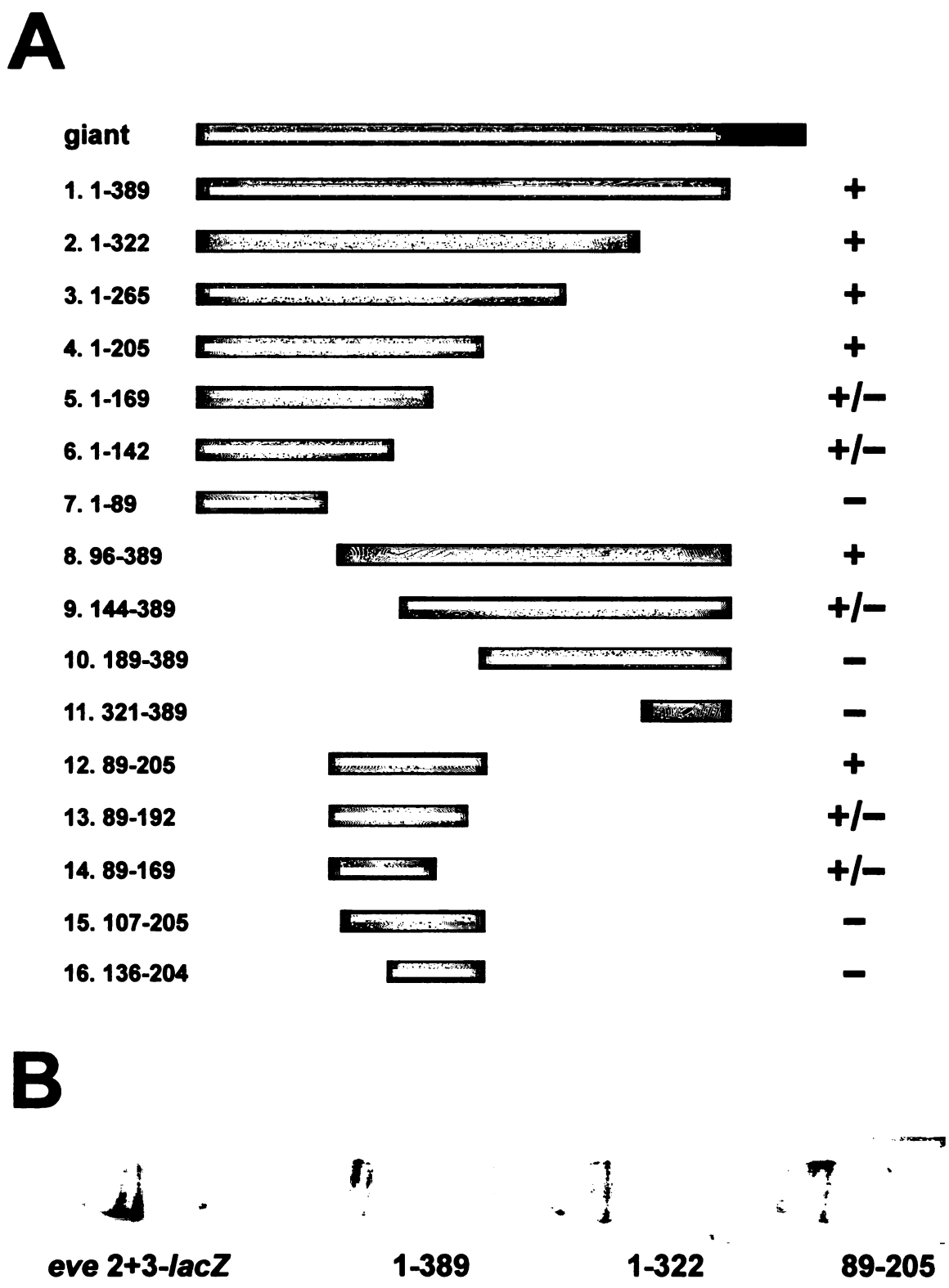

Figure 2-7: Structure and activity of Gal4-giant chimeras assayed in transgenic embryos.

Table 2-1: Activity of chimeric Gal4-giant repressor proteins in transgenic embryos.

Construct number	Residues from giant protein	% Repressed[a]	Number of embryos scored	Number of lines analyzed
1	1-389	26 ± 9	708	4
2	1-322	39 ± 5	579	3
3	1-265	18 ± 1	177	2
4	1-205	9 ± 1	199	3
5	1-169	3.5 ± 2	1202	5
6	1-142	2 ± 2	1010	5
7	1-89	0	471	4
8	96-389	13 ± 1	641	3
9	144-389	1 – 9[b]	1107	3
10	198-389	1	338	1
11	321-389	0	936	3
12	89-205	9 ± 3	1674	7
13	89-192	1.5 ± 1	1514	4
14	89-169	3	262	1
15	107-205	0.6± 0.6	1522	3
16	136-204	0	475	3

[a] embryos from individual lines were scored for repression. Average % repression and standard deviations were calculated for constructs where multiple lines were tested. No embryos showing repression were found for constructs 7, 11, and 16.

[b] activities of the three lines tested were 1%, 3%, and 9%.

including residues 144-389 showed activity, suggesting that the putative CtBP binding motif is apparently not strictly required for repression activity. The transgenes were not expressed at high enough levels for us to quantitate expression by antibody staining (data not shown). Therefore, it is possible that the inactive constructs are simply not well expressed or are unstable.

giant homolog from *Drosophila hydei* contains regions of conserved residues

To identify regions of the Giant protein that have been evolutionarily conserved, and hence of possible functional importance, we sought the homologous gene from a related *Drosophila* species, *Drosophila hydei*, which is thought to have shared a last common ancestor with *D. melanogaster* approximately 60-80 million years ago (Beverley and Wilson, 1984). No close homolog to *giant* has yet been reported, aside from genes that encode similar basic-leucine zipper dimerization/DNA-binding domains. Therefore we designed degenerate oligonucleotides corresponding to several regions of the repression domain and carried out PCR reactions under conditions of low stringency. Southern blotting was used to analyze PCR products, and primer pairs that yielded products of similar size to the *D. melanogaster* clone were used in further rounds of PCR. A degenerate primer pair that amplified a region corresponding to codons 30 to 268 of *D. melanogaster* was found to give optimal results, and several clones of the corresponding PCR products were sequenced. A 774 bp fragment encompassing 258 codons was recovered, including the entire minimal repression domain (Figure 2-8). This portion of

Figure 2-8: Peptide sequence of *giant* homolog isolated from *D. hydei* aligned with *D. melanogaster* sequence.

Minimal repression domain spanning residues 89-205 in *D. melanogaster* shown in box, conserved putative CtBP binding motif underlined. Vertical arrows indicate N- and C-terminal deletions in minimal repression region that abolish repression activity. A *D. hydei* sequence corresponding to *D. melanogaster* residues 30 – 268 was isolated from genomic DNA by degenerate PCR. Sequences were obtained from multiple isolates of independently generated PCR products.

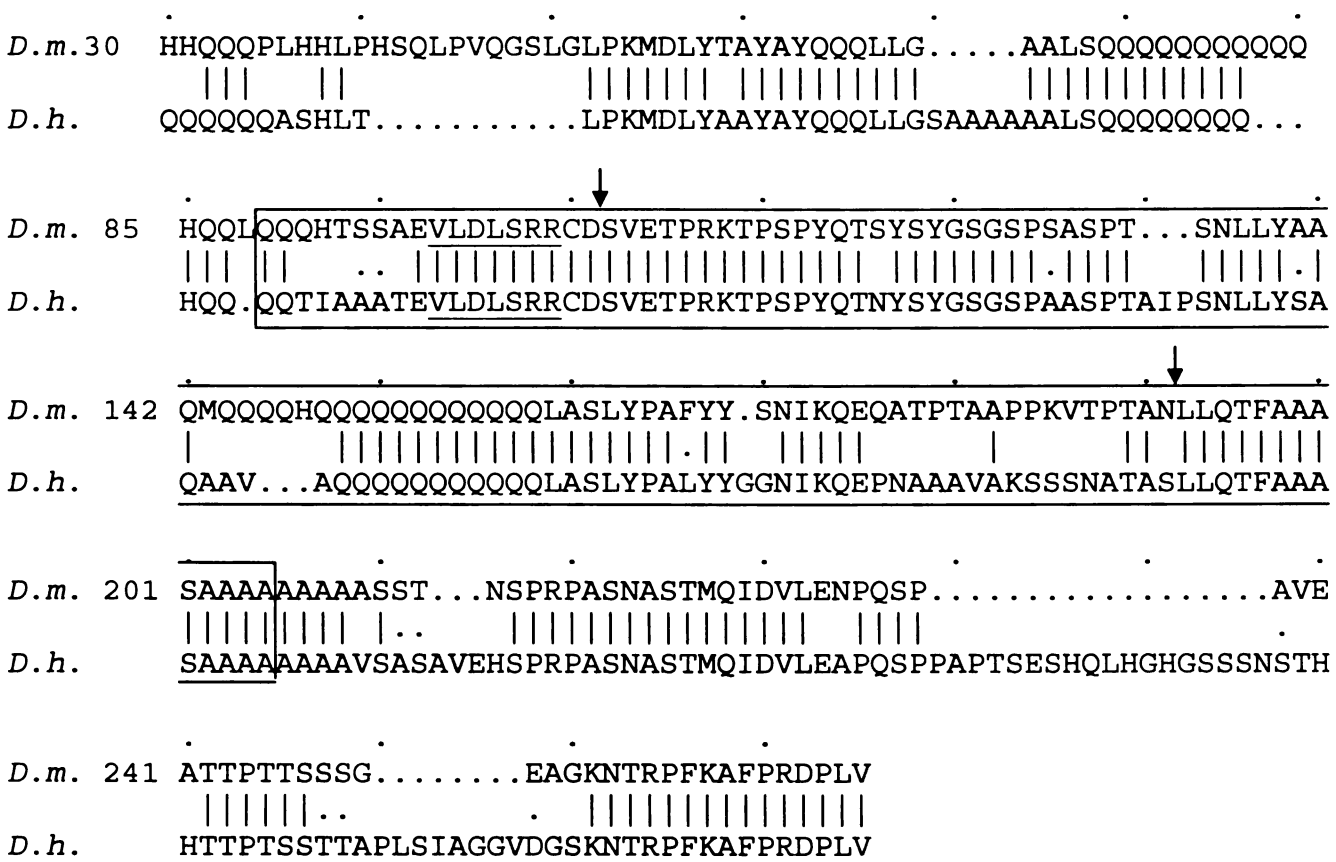

Figure 2-8: Peptide sequence of giant homolog isolated from *D. hydei* aligned with *D. melanogaster* sequence.

the gene had 66% identity (70% similarity) at the amino acid level and 65% identity at the nucleic acid level. The minimal repression domain was overall somewhat more conserved, including three large blocks of identical residues, with overall 73% identity (78% similarity) at the amino acid level (Fig. 2-8). These levels of identity with *D. melanogaster* genes are similar to those of the *D. hydei fushi tarazu* (68%) and *Hairless* genes (69%) (Jost et al., 1995; Marquart et al., 1999). The putative CtBP binding motif spanning residues 98-104 is absolutely conserved, as are other blocks of residues throughout the predicted protein sequence.

Discussion

Giant represses through CtBP-dependent and –independent mechanisms

Previous work suggested that Giant, in contrast to other short-range repressors, does not require CtBP activity to mediate repression, based on the expression pattern of an *eve* stripe 2 *lacZ* transgene (Nibu et al., 1998). Our studies of the same enhancer element in a *CtBP* mutant background indicates that although there is a range of phenotypes, with a small percentage of embryos showing a sharp anterior border, the border of the stripe is clearly expanded in most embryos, consistent with loss of *giant* function (Fig. 2-3). Additional evidence for CtBP dependence comes from analysis of a reporter gene that is directly repressed through tandem Giant binding sites at –55 bp. This gene shows strong, but not complete, derepression in a *CtBP* mutant background indicating that CtBP is required for full activity of Giant on this gene (Fig. 2-4).

Repression by Giant of the *eve* stripe 5 enhancer is also reportedly compromised in a *CtBP* mutant background (M. Levine and Y. Nibu, personal communication). Clear evidence for Giant- and CtBP-dependent repression of an endogenous target gene comes from expression in the anterior domain (AD) of the *Krüppel* gene, where marked derepression is observed in both *giant* and *CtBP* mutant backgrounds (Fig. 2-5). Although the roles of individual Giant binding sites in the *Kr* promoter are less well characterized than with *eve* stripe 2 and the synthetic *lacZ* reporters shown in Figs. 2-3 and 2-4, Giant and CtBP most likely work through common DNA elements on the *Kr* promoter because Giant protein is present in the region of AD expression and there are identified high-affinity binding sites for Giant within the AD enhancer region (Capovilla et al., 1992).

Our results clearly indicate that Giant repression can be CtBP dependent, but Giant also appears to act through CtBP-independent pathways. A direct indication of such activity is that the Gal4-giant repressor is still active in a *CtBP* mutant background (Fig. 2-6). In addition, Giant repression of the *Kr* CD enhancer elements is unaffected by loss of CtBP activity (Fig. 2-5). Another endogenous target of the Giant repressor, *hb*, is not derepressed in a *CtBP* mutant, suggesting that CtBP dependence of Giant activity can vary on a gene-to-gene as well as enhancer-to-enhancer basis. Ironically, while this study consolidates the view that a characteristic property of short-range repressors is functional interaction with CtBP, our results also indicate that providing a binding platform for CtBP is not the only activity of short-range repressors. A growing body of evidence demonstrates that many, or perhaps all, short-range repressor proteins also exhibit CtBP-independent activity: Knirps can repress the *eve* stripe 3 enhancer in a *CtBP* mutant

background (Keller et al., 2000), Krüppel can repress the *hairy* stripe 7 enhancer in the absence of CtBP (La Rosée-Borggreve et al., 1999), and this study indicates that Giant likewise possesses CtBP-independent repression activity.

The CtBP-independent activity of short-range repressors is still poorly characterized, although this activity must by definition be limited to a short-range of action. The CtBP-independent activity may be mediated in part through direct competition with transcriptional activators. The tight linkage of activators and repressors on the *eve* stripe 2 enhancer has been suggested to be an example of this competitive situation, and experimentally, competition between the Bicoid activator and the Knirps repressor has been demonstrated on the *Kr* promoter (Hoch et al., 1992). Competitive binding between repressors and activators cannot explain all CtBP-independent repression, however; the N-terminus of Knirps contains a CtBP-independent repression activity that can inhibit activators binding to non-overlapping sites (Keller et al., 2000).

Identification of an evolutionarily conserved minimal repression domain

The deletional analysis of Gal4-giant chimeras indicates that Giant repression function can be localized to residues 89-205, an area of the protein that contains several tracts of highly conserved residues (construct 12; Fig. 2-7, Table 2-1). Chimeras containing other portions of the Giant protein (constructs 7, 10, 11) did not exhibit significant repression activity, suggesting that these regions cannot act autonomously to mediate repression, and might instead contribute to protein stability or expression. In particular, residues 266-322, present in constructs 1 and 2, appear to correlate with significantly higher repression activity of these proteins. The low levels of chimeric

protein expression in the embryo precluded direct quantitation of each protein, thus our analysis is based primarily on those that did show significant activity.

We have not detected a significant physical interaction between Giant and CtBP in vitro (A. Kumar, unpublished results), and the Giant protein lacks a perfect match to the consensus CtBP binding motif P-DLS-K/R/H found in the Knirps, Krüppel and Snail proteins. However, a partial match is present: VLDLSRR (residues 98-104). The motif is evolutionarily conserved and is found within the minimal repression domain we have defined (Figs. 2-7 and 2-8), consistent with a possible role in repression. Indeed, deletion of residues 89-107 inactivates the chimeric repressor (Fig. 2-7 and Table 2-1). This region is clearly not sufficient for high-level repression, however, (demonstrated by the weak activity of constructs 5, 6, 13 and 14), suggesting that other portions of the protein play important structural or functional roles.

If CtBP directly contacts Giant *in vivo*, the lack of strong interaction *in vitro* may indicate that Giant must be posttranscriptionally modified to facilitate interaction with CtBP, perhaps via phosphorylation (Capovilla et al., 1992). Posttranslational modifications are known to play a role in CtBP binding in some instances; E1A-CtBP interactions have been shown to be regulated by acetylation of a conserved lysine residue in the CtBP binding motif (Zhang et al., 2000). Alternatively, Giant may bind CtBP indirectly through a cofactor, much as BRCA1 has been suggested to bind CtBP through CtIP (Li et al., 1999), or CtBP might be recruited via a heterodimeric basic-zipper partner of Giant. To determine whether CtBP-dependent and CtBP-independent repression activities are mediated by the same or distinct portions of the Giant protein, future studies

will need to focus on identifying mutant proteins that are deficient in each of these activities.

What characteristics of a regulatory region dictate CtBP-dependent or CtBP-independent repression?

In considering which features of a gene determine CtBP-dependence or –independence, the structure of the basal promoter cannot be the deciding factor, for the same *Kr* promoter is regulated by distinct elements, some that exhibit CtBP-dependence and some that show CtBP–independence. Similarly, the *eve* gene is repressed by Knirps via CtBP-dependent and CtBP–independent regulatory elements (Keller et al., 2000). While the *eve* enhancers in question are kilobases apart, the *Kr* regulatory elements driving AD and CD expression are closely intertwined, and appear to share at least some of the same activator binding sites, suggesting that subtle differences in enhancer architecture or differences in levels of regulatory proteins interacting with those elements may dictate CtBP dependence (Hoch et al., 1990; Hoch et al., 1991; Jacob et al., 1991). The Giant binding site in the *Kr* CD2 enhancer site was shown to be of higher affinity than the Gt1 site in the *eve* stripe 2 enhancer (Capovilla et al., 1992). Thus, there may be a correlation between Giant binding site affinity and the requirement for CtBP, with elements containing Giant sites of lower affinity showing CtBP-dependence. We derived a consensus for the Giant protein by aligning binding sites for Giant from *eve*, *Kr*, and the recently identified *abdA iab-2* enhancer site (Fig. 2-9; Shimell et al., 2000). The consensus features an extended half-site inverted repeat TNTTAC, consistent with the dimeric nature of basic zipper proteins, and a central ACGT core common to recognition

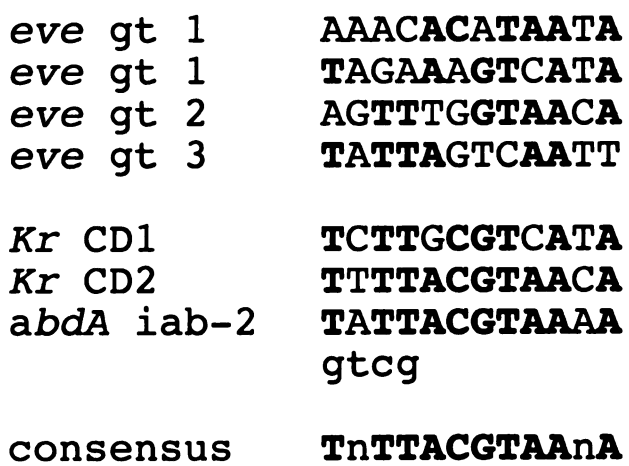

Figure 2-9: Alignment of Giant binding sites and consensus.

Footprinted sites from the *eve* stripe 2 enhancer (Stanojevic et al., 1991), the *Kr* CD1 and CD2 enhancers (Capovilla et al., 1992), and the *abdA iab-2* enhancer (Shimell et al., 2000) were aligned with a sequence derived from the center of the small footprinted regions (13-16 nt) found in the *Kr* and *abd-A* genes. Residues that match the consensus are indicated in bold. The central ACGT cluster is identical to that found in motifs recognized by other basic zipper DNA binding proteins (Dlakic et al., 2001). The sequence from the *eve* Gt3 site is located in the center of a 26 nt footprinted region, and the two sequences from the Gt1 site are adjacent to one another within the 22 nt footprinted region. The sequence from the Gt2 site is in the 3' region of the large 44 nt footprinted site (Stanojevic et al., 1991). The lower case letters below the *iab-2* sequence indicate a mutation that abolishes Giant regulation of the *iab-2* enhancer (Shimell et al., 2000).

motifs for many basic zipper proteins (Capovilla et al., 1992; Dlakic et al., 2001). The higher affinity sequences from the CtBP-independent *Kr* CD element are closer to the consensus than those of the CtBP-dependent *eve* stripe 2 enhancer. Weaker sites may only be partially occupied, resulting in an overall lower level of Giant mediated repression. A loss of CtBP might further depress repression activity below a critical threshold, leading to the derepression we observe in Figures 2-3 and 2-5. Repression of the *lacZ* reporter containing the Giant CD1 site from *Kr* was CtBP dependent, a result that contrasts with the CtBP independence of the CD itself (Fig. 2-4), but this particular site may not be optimal, as it contains two mismatches (Fig. 2-9). Full Giant activity may also be mediated on the native CD element through the additional high-affinity CD2 site.

Other factors besides binding site affinity can affect Giant's activity, and possibly its CtBP-dependence. We have previously demonstrated that small alterations in the location of Giant binding sites are sufficient to strongly affect the ability of Giant to repress in transgenic embryo assays (Hewitt et al., 1999). Thus, we need to consider location and affinity of Giant sites in studying CtBP-dependent repression. We do not believe that differences in the nature of the activators explain CtBP-dependence or -independence, because both AD and CD enhancers of *Kr* are activated by Bicoid protein (Jacob et al., 1991; Hoch et al., 1991), as is the *eve* stripe 2 enhancer. Detailed studies illuminating how the general properties of short-range transcriptional repressors are integrated into the design of promoter elements will promote our understanding of the control of complex developmentally regulated genes.

Note added in proof. Material cited as Nibu and Levine, personal communication, has now appeared as Nibu Y., and Levine M.S. (2001). CtBP-dependent activities of the short-range giant repressor in the *Drosophila* embryo. Proc. Natl. Acad. Sci. USA **98**: 6204-6208.

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Chapter 3

Quantitative contributions of CtBP-dependent and –independent repression activities of Knirps[5]

Abstract

The *Drosophila* Knirps protein is a short-range transcriptional repressor that locally inhibits activators by recruiting the CtBP corepressor. Knirps also possesses CtBP-independent repression activity. The functional importance of multiple repression activities is not well understood, but the finding that Knirps does not repress some cis-regulatory elements in the absence of CtBP suggested that the cofactor may supply a unique function essential to repress certain types of activators. We assayed CtBP-dependent and –independent repression domains of Knirps in *Drosophila* embryos, and found that the CtBP-independent activity, when provided at higher than normal levels, can repress an *eve* regulatory element that normally requires CtBP. Dose response analysis revealed that the activity of Knirps containing both CtBP-dependent and –independent repression activities is higher than that of the CtBP-independent domain alone. The requirement for CtBP at certain enhancers appears to reflect the need for

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overall higher levels of repression, rather than a requirement for an activity unique to CtBP. Thus, CtBP contributes quantitatively, rather than qualitatively, to overall repression function. The finding that both repression activities are simultaneously deployed suggests that the multiple repression activities do not function as cryptic "backup" systems, but that each contributes quantitatively to total repressor output.

Introduction

Dynamic patterns of gene expression in the *Drosophila* embryo are orchestrated by the combined action of transcriptional activators and repressors acting on complex *cis* regulatory elements, or enhancers. In a number of well-studied cases in the *Drosophila* embryo, multiple enhancers act independently on a single promoter, in part due to the action of so-called short-range repressors, proteins whose inhibitory action is restricted to ranges of ~100 bp from the factor binding site (Gray and Levine, 1996a). In cell culture and transgenic embryo assays, short-range repressors can selectively inhibit individual enhancers, or entirely silence a gene if bound close to the basal promoter (Arnosti et al., 1996; Gray and Levine, 1996b; Ryu and Arnosti, 2003). The apparent impuissance of short-range repression actually provides a highly flexible mechanism for specific gene regulation, allowing genes to be "tuned" to respond to subtle differences in repressor protein concentration and by small changes in the positions of factor binding sites (Hewitt et al., 1999). Changes in the spacing of short-range repressor binding sites correlate with functional alterations observed during enhancer evolution (Ludwig and Kreitman, 1998; Ludwig et al., 2000).

The CtBP corepressor is required for full activity of short-range repressors such as Knirps, Krüppel, Giant, and Snail that play important roles in patterning the blastoderm embryo (Nibu et al., 1998a,b). This evolutionarily conserved cofactor also interacts with a number of vertebrate transcriptional regulators, including the adenovirus E1A protein, Net, Ikaros, Zeb, and, indirectly, the Retinoblastoma tumor suppressor protein (reviewed in Chinnadurai 2002). Transcription factors typically bind to CtBP via a short peptide motif similar to the PLDLS sequence originally identified in E1A (Schaeper et al., 1995). CtBP is homologous to α-hydroxyacid dehydrogenases, and contains a conserved NAD binding domain as well as conserved residues in the putative active site (reviewed in Chinnadurai, 2002; Turner and Crossley, 2001). Although not identified in previous studies, recent reports found a weak dehydrogenase activity associated with CtBP (Kumar et al., 2002; Balasubramanian et al., 2003; Shi et al., 2003). CtBP has been found to bind directly to histone deacetylases (HDACs), suggesting that the corepressor may effect repression by chromatin remodeling (reviewed in Turner and Crossley, 2001; Chinnadurai, 2002). A recent biochemical purification of CtBP identified additional proteins in a complex, including histone methyltransferases, the CoREST repressor, and a protein homologous to polyamine oxidases (Shi et al., 2003). This additional complexity suggests that CtBP itself may utilize multiple activities to effect transcriptional repression. *Drosophila* factors functionally characterized as short-range repressors all interact with CtBP, although it has not been established that all factors that bind the cofactor are necessarily short-range repressors. The long-range repressor protein Hairy, in particular, is thought to interact with CtBP, although this might be in an antagonistic mode (Poortinga et al., 1998; Zhang and Levine, 1999).

CtBP-mediated repression is critical for full activity of short-range repressors; however, *Drosophila* short-range repressors also possess CtBP-independent repression activities (La Rosee-Borggreve et al., 1999; Keller et al., 2000; Strunk et al., 2001; Nibu et al., 2003). In the case of Knirps, a form of the protein that lacks the CtBP binding motif exhibits weak activity when overexpressed (Nibu et al., 1998b). The CtBP independent activity has been mapped to an N terminal repression domain that lacks a CtBP-binding motif and is able to repress in the absence of CtBP (Keller et al., 2000). Although many transcriptional repressors have been found to possess multiple activities, the functional relevance of such activities is not well understood. Previous studies suggest that multiple repression activities underlie both gene specific and activator specific effects. In the case of the Zeb repressor, a protein with CtBP-dependent and -independent activities, it was found that specific repression activities are directed at distinct classes of transcriptional activators (Postigo and Dean, 1999). In another case, distinct mechanisms are used at different promoters: the NRSF repressor mediates HDAC and DNA methylation-dependent repression of the *NaCh II* gene and a distinct form of repression of the *SCG10* gene (Lunyak et al., 2002).

Previous studies also hint that the possession of CtBP-dependent and –independent activities may confer important quantitative effects. For example, the *Krüppel* promoter is activated by Bicoid in both anterior and central regions of the blastoderm embryo, and is repressed by Giant in either CtBP-independent or CtBP-dependent manners, depending on the region of the embryo (Strunk et al., 2001). The higher levels of Bicoid activator in anterior regions of the embryo might necessitate additional repression activities beyond those afforded by CtBP-independent pathways,

suggesting that CtBP might contribute quantitatively to overall repressor output. In cell culture studies, CtBP-dependent and CtBP-independent repression activities of Knirps possess similar functional attributes, including distance dependence, trichostatin A insensitivity, and activator specificity, suggesting that their quantitative effects might be mediated through similar pathways (Ryu and Arnosti, 2003).

The Knirps protein is able to regulate at least one known target, the stripe 3 enhancer of the *even-skipped* (*eve*) gene in a CtBP-independent fashion, yet this CtBP-independent activity is not sufficient to supply the full biological function of Knirps (Keller et al. 2000; Nibu et al., 1998b). For instance, transheterozygous *knirps* and *CtBP* embryos have disruptions in *eve* expression, suggesting that Knirps function is partially impaired, and a frameshift mutation in *knirps* encoding a protein lacking the CtBP binding motif is a strong hypomorph (Gerwin et al., 1994; Nibu et al. 1998a). Furthermore, a point mutation in the CtBP binding motif results in a protein that lacks the dominant phenotype of the wild-type protein when misexpressed in a pattern of *eve* stripe 2 (Nibu et al., 1998b). These results suggest that Knirps requires CtBP for effective regulation of at least some of its targets. Here, we examine the regulation of several enhancers targeted by Knirps to test the possibility that the CtBP-dependent and CtBP-independent repression activities of Knirps might be deployed to achieve qualitatively or quantitatively distinct effects. Our results suggest that in the case of the *eve* gene, the two activities are both required to achieve quantitatively sufficient levels of repression.

Materials and Methods

Plasmid construction. To generate transgenic flies that carry inducible, double tagged Knirps genes, the P-element transformation vector pCaSpeR-hs (Pirrotta, 1988) was modified to incorporate an N-terminal hexahistidine tag and a C-terminal double FLAG tag in frame with a *KpnI –XbaI* insert (reading frame commencing with GGT). First, an oligonucleotide containing the ribosome binding site (Kozac) consensus sequence for *Drosophila* (Cavener and Ray, 1991) and an N-terminal sequence encoding MARGS$(his)_6$ was introduced into the unique *Eco*RI site of pCaSpeR-hs. This fragment was generated by annealing and extending the following primers: 5'CCG CGG AAT TCA CAA CCA AAA TGG CGA GAG GAT CGC ATC 3' and 5' GGC CGA ATT CGG TAC CGT GAT GGT GAT GGT GAT GCG ATC C 3', to generate an *Eco*RI-Kozac-MARGS$(his)_6$-*Kpn*I-*Eco*RI-containing oligonucleotide, which was restricted with *Eco*RI, PAGE-purified and cloned into pCaSpeR-hs. Two FLAG epitope sequences were introduced in two successive steps using annealed oligonucleotides. To introduce the first FLAG epitope tag the vector containing the hexahistidine tag was cut with *Kpn*I and *Stu*I and ligated with two annealed oligonucleotides (5' CGA TCG ATC GTC TAG **AGA TTA CAA GGA TGA CGA TGA CAA G**GC GGC CGC TTA GTA ATT AGT TAG 3' and 5' CTA ACT AAT TAC TAA GCG GCC GC**C TTG TCA TCG TCA TCC TTG TAA TC**T CTA GAC GAT CGA TCG GTA C 3', FLAG-codons in bold) to generate a *Kpn*I-(9bp)-*Xba*I-FLAG-(stop)s fragment. A second FLAG epitope was generated by annealing and cloning two *Not*I-compatible, FLAG-containing oligonucleotides (5' GGC CGC TGA TTA CAA GGA TGA CGA TGA CCA GGC 3' and 5' GGC CGC CTT GTC ATC GTC ATC CTT GTA ATC AGC 3') into the unique

*Not*I site of the vector. The correct orientation of the second FLAG oligonucleotide was determined by PCR. The final vector, pCaSpeR-hs(H2xF), was sequenced to confirm the correct frame and orientation of the tags inserted. Different *knirps* fragments were subcloned as *Kpn*I-*Xba*I inserts into pCaSpeR-hs(H2xF), generating hsKni1-429, which contains full-length Knirps, hsKni1-330, which contains the CtBP-independent repression domain of Knirps, hsKni1-105, which contains the Knirps DNA binding domain (aa 1-74) and its nuclear localization signal (aa 75-95; Gerwin et al., 1994), hsKni75-429 and hsKni75-330. All fragments were PCR amplified using as template pBS-N741, which contains a full-length *knirps* cDNA (kindly provided by Michael Levine). To amplify full-length Knirps (1-429), the primers used were DA-502: 5' CGC GCG GTA CCA TGA ACC AGA CAT GCA AAG TG 3' and DA-503: 5' CGG CCT CTA GAG ACA CAC ACG AAT ATT CCC CT 3'. To amplify Knirps75-330 the primers used were DA-504: 5' CGC GCG GTA CCG GAT CCC GCT ACG GAC GTC GC 3' and DA-505: 5' CGG CCT CTA GAT CCT TCT TGA GCG GAA ACG GTG G 3'. To amplify Knirps1-330, DA-502 and DA-505 were used. To amplify Knirps75-429, DA-504 and DA-503 were used. To amplify Knirps1-105 the primers used were DA-502 and DA-773: 5' CGG CCT CTA GAA GGC GCC TTG CCC GCC GCT GC 3'.

Heat-shock experiments. To induce expression of recombinant Knirps proteins, 2-4 hour old embryos collected on apple-juice plates at room temperature (22-23°C) were incubated for 5, 10, 20 or 30 minutes at 38°C in a 10-liter water bath to ensure rapid and even heating. After induction, embryos were allowed to recover in a water bath at room temperature for 30 minutes prior to fixation or sonication. Heat-shock inductions of Knirps1-330 and Knirps1-429 were also performed with no recovery. For the

experiments described in Fig. 3-6, *hairy* expression pattern was monitored after 10 or 30 minutes of heat-shock, *ftz* expression pattern was determined after 5, 10, 15, 20 or 30 minutes of heat-shock, *run* expression pattern was determined after 15 or 30 minutes of heat-shock and *hb* expression pattern was determined after 30 minutes of heat-shock. 30 minues of recovery after heat-shock was applied for all the experiments described in Fig. 3-6.

Crude embryo lysate preparation. Approximately 50 mg of dechorionated embryos were resuspended in 1.2 ml of lysis buffer (25 mM HEPES pH 7.9, 150 mM NaCl, 1 mM DDT, 1 mM PMSF, 1 mM Na-metabisulfite, 1 mM benzamidine, 10 μM pepstatin A) and disrupted by sonication using a Branson Sonifier 250 (2 cycles of 12 pulses each, output 3, duty cycle 60%). After sonication, lysates were centrifuged for 15 minutes at 14,000 rpm using an Eppendorf centrifuge, and the protein concentration of the supernatant was determined using the Bradford assay, with BSA as the standard.

Western blot analysis. Immunoblotting was performed according to standard protocols (Harlow and Lane, 1999) using a tank transfer system (Mini Trans-Blot Cell, Biorad). Sequi-Blot™ PVDF membranes (BioRad) were used and antibody incubation was in TBST (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) supplemented with 5% (w/v) nonfat dry milk as blocking agent. The primary anti FLAG M2 monoclonal antibody (Sigma) was used at 1:10,000 dilution. The secondary ImmunoPure® Goat Anti-Mouse HRP-conjugated antibody (Pierce) was used at 1:20,000 dilution. Western blots were quantitated using a Fluor-S® MultiImager (Biorad) set on high sensitivity and with an exposure time of 50 min. The QuantityOne package (BioRad) was used to

analyze the data. Four independent quantitations of four gels were performed, analyzing lysates from an experiment performed as described for Fig. 3-3 and Table 3-1.

P-element transformation, whole mount *in situ* hybridization. *P*-element transformation vectors were introduced into the *Drosophila* germline by injection of $y\ w^{67}$ embryos and *in situ* hybridizations were performed using digoxigenin-UTP-labeled antisense RNA probes to *eve*, *h*, *kni*, *run*, *ftz* and *lacZ* as described (Small et al., 1992).

***lacZ* reporters**. The *eve* stripe 3/7 and 4/6 *lacZ* reporter genes used in Fig. 3-1 were described in Small et al. (1996) and Fujioka et al. (1999), respectively. Germline mutants of CtBP were generated as previously described (Keller et al., 2000) using $CtBP^{03463}$/TM3, Sb (Bloomington stock no. P1590). The *even-skipped* stripe 2/3 *lacZ* reporter used in Fig. 3-4 contains ~500 bp minimal elements separated by a 340 bp spacer sequence and 2 UAS sites (not utilized in this experiment) fused to the *eve* basal promoter (Keller et al., 2000). The *eve* stripe 3/7 reporter used in Fig. 3-4 (stock E9) was kindly provided by Steve Small and the *eve* stripe 4/6 lacZ reporter (stock B45C52-B) was kindly provided by Jim Jaynes (Fujioka et al. 1999).

Results

Repression by Knirps of *even-skipped* stripe 3/7 enhancer is independent of CtBP, while repression of stripe 4/6 enhancer is CtBP-dependent.

The expression of the endogenous *eve* gene is strongly perturbed by a loss of CtBP, consistent with this corepressor's important role in the activity of gap repressors Giant, Krüppel, and Knirps (Nibu et al. 1998 a, 1998b; Strunk et al., 2001). To study the effectiveness of Knirps repression of individual *eve* regulatory elements, we assayed the expression of *eve-lacZ* reporter genes. Knirps is required for correct regulation of the *eve* stripe 3/7 and 4/6 enhancers, as demonstrated by the expression patterns of *lacZ* reporter genes in *kni* mutant embryos (Fujioka et al., 1999; Small et al., 1996). As previously observed (Keller et al., 2000) the posterior border of *eve* stripe 3 was not derepressed in a *CtBP* mutant, consistent with the CtBP-independent activity of Knirps on this enhancer (Fig. 3-1A, B). In contrast, Knirps repression of *eve* stripe 4/6 is compromised in a *CtBP* mutant background, indicating that the CtBP-independent repression activity of Knirps is insufficient to regulate this enhancer (Fig. 3-1C, D). Therefore, depending on which portion of the *eve* gene is bound by the Knirps protein, its repression activity is either dependent or independent of the CtBP cofactor (Fig. 3-1E).

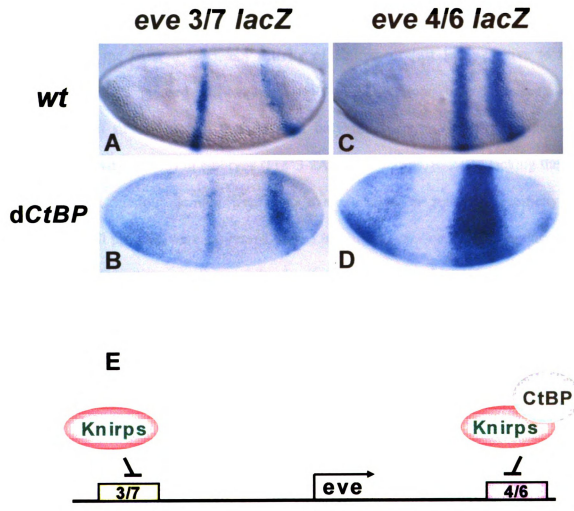

Figure 3-1: CtBP is required for Knirps repression of *even-skipped* (*eve*) stripe 4/6 enhancer, but not stripe 3/7 enhancer.

Expression patterns of *eve* stripe 3/7 (A, B) and *eve* stripe 4/6 *lacZ* reporter genes (C, D) in wild-type and *CtBP* mutant embryos, showing derepression only of the *eve* stripe 4/6 element in the *CtBP* mutant. E. Schematic representation of *eve* regulatory regions, showing cofactor requirements for Knirps repression. Expression patterns were characterized in transgenic embryos by *in situ* hybridization. Embryos are oriented anterior towards the left, dorsal side upwards.

Ectopic expression of Knirps proteins in embryos

To determine whether the *eve* stripe 4/6 enhancer is intrinsically resistant to repression by the CtBP-independent activity of Knirps, or just less sensitive to this activity, we overexpressed full-length (1-429) FLAG epitope tagged Knirps or a truncated form of the protein that contains only the N-terminal, CtBP-independent repression activity (1-330) in embryos (Fig. 3-2A). As controls, proteins lacking the N-terminal DNA binding domain were also overexpressed to test for specificity of repression. All proteins were expressed from a *hsp70* promoter construct introduced by germline transformation into *Drosophila*. In situ analysis showed a uniform distribution of *knirps* mRNA in embryos after heat shock, reaching levels comparable to the endogenous *knirps* gene (Fig. 3-2B). The different forms of the Knirps protein were expressed at similar levels after heat shock induction of the transgenes, with undetectable levels present before heat shock (Fig. 3-2C and data not shown). Heat shock induction of full-length Knirps in the embryo was lethal (data not shown), as is expected for this regulatory factor whose expression usually exhibits tight temporal and spatial regulation.

Differential effects of Knirps protein on the *even-skipped* gene

The effect of misexpression of Knirps proteins was monitored by measuring endogenous *eve* expression by in situ hybridization. Heat shocks of variable duration were performed to test the effects of increasing levels of the Knirps protein (Fig. 3-3). Misexpession of the full-length Knirps protein, 1-429, resulted in repression of stripe 3 expression even after a short (5 minutes) heat shock pulse, with almost as frequent repression of stripe 7 (Fig. 3-3A-B; Table 3-I). Heat shocks of longer duration resulted in

Figure 3-2: Expression of full-length and CtBP-independent portions of the Knirps transcriptional repressor in transgenic *Drosophila*.

A. Structure of proteins expressed from *hsp70* promoter: 1-429, full-length Knirps protein; 75-429, non-DNA binding control protein; 1-330, CtBP-independent Knirps repression domain; 75-330, non-DNA binding control protein. **B.** *In situ* analysis of expression of *knirps* mRNA produced from *hsp70-knirps* transgene before and after heatshock. **C.** Above, proteins expressed from representative lines of the four constructs measured by Western blot. M2 α-FLAG antibody was used to detect recombinant proteins. Lines shown in lanes 2 and 4 were used in subsequent experiments. Lane 8, non heat shock control. Below, Coomassie blue stained gel illustrates equal loading.

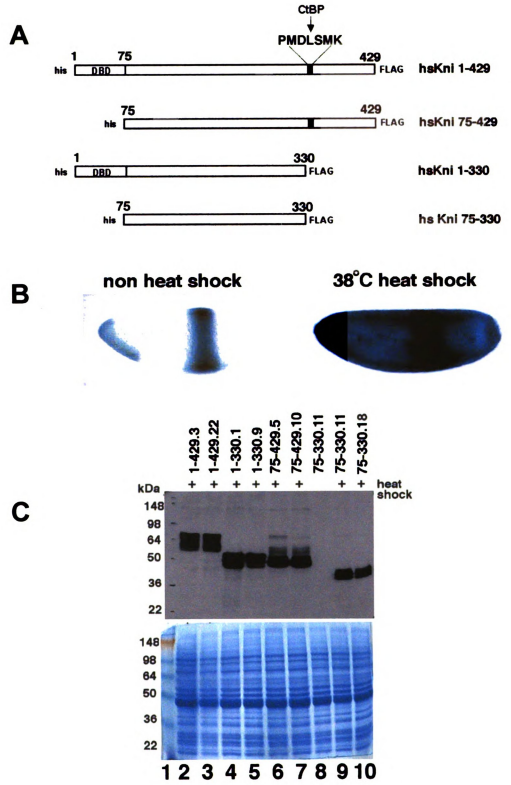

Figure 3-2: Expression of full-length and CtBP-independent portions of the Knirps transcriptional repressor in transgenic *Drosophila*.

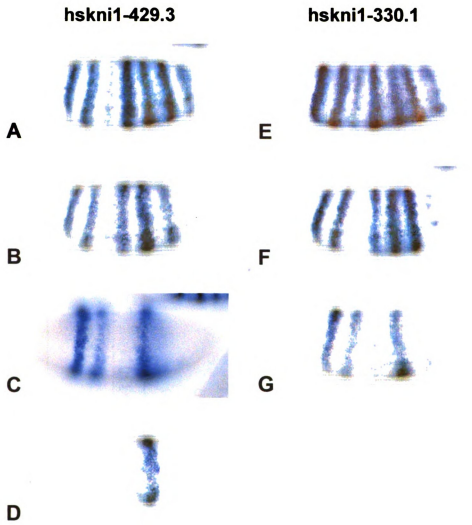

Figure 3-3: Pattern of endogenous *eve* expression in embryos expressing full-length Knirps 1-429 (A-D) and CtBP-independent region of Knirps 1-330 (E-G).

Phenotypes of increasing severity are illustrated. Class I pattern (A, E), repression of stripe 3; Class II (B, F), repression of stripe 3 and 7; Class III (C, G) repression of stripe 3,4,6, and 7; Class IV, all stripes repressed except stripe 5. Endogenous *eve* patterns were visualized by *in situ* hybridization; embryos are oriented with anterior towards the left, dorsal side upwards. More severe phenotypes were produced by expression of full-length Knirps 1-429 than Knirps 1-330, as documented in Table 3-1.

Table 3-1. Percentage of transgenic embryos showing repression of *eve* stripes after heat shock.

heatshock duration	5 min		10 min		20 min	
eve stripe	1-330	1-429	1-330	1-429	1-330	1-429
1	0	0	0	2.5	0	53
2	0	0	0	2.5	0	53
3	10	50	71	72	80	75
4	0	9	11	45	28	66
5	0	3	11	0	10	0
6	0	8	11	39	26	66
7	1.4	40	51	67	71	68
n	718	430	674	446	688	408

significant repression of stripe 4 and 6 (Fig. 3-3C). A 20 minutes heat shock also resulted in repression of stripe 1 and 2, leaving only stripe 5 expression (Fig. 3-3D). The selective repression of a subset of multiple enhancer elements is a striking down a gene entirely. No disruption of the *eve* pattern was noted in lines expressing Knirps proteins lacking amino acids 1-74, demonstrating that an intact DNA binding demonstration of the way short-range repressors can repress individual regulatory elements without shutting domain is required for the effects observed (see Appendix B). The hierarchy of *eve* stripe 3-7 enhancer sensitivity to Knirps is consistent with the relative positions of these stripes within the Knirps protein gradient, whereby stripes 3 and 7 are sensitive to lower concentrations of Knirps than are 4 and 6 (Fujioka et al., 1999; Clyde et al., 2003).

Similar to the case with Knirps 1-429, stripes 3 and 7 were the first to be affected by misexpression of the Knirps 1-330 protein, which bears only the CtBP-independent repression activity. In this latter instance, however, the numbers of embryos showing repression was smaller (Fig. 3-3E, F; Table 3-1). Unexpectedly, overexpression of Knirps 1-330 also led to repression of *eve* stripes 4 and 6, indicating that this regulatory element is sensitive to the CtBP-independent activity of Knirps (Fig. 3-3G). Again, the relative number of affected embryos was smaller, indicative of a quantitative difference in repression between full-length Knirps and the CtBP-independent domain alone (Table 3-1). Unlike the case for Knirps 1-429, no embryos were observed that showed repression of stripes 1 and 2 by overexpression of Knirps 1-330. This result suggests that either these enhancers require still higher levels of Knirps 1-330 to be effectively repressed, or that there are qualitative as well as quantitative differences between the repressors. In a small percentage of cases, stripe 5 expression was also observed to be

repressed in embryos misexpressing Knirps 1-330 and Knirps 1-429 (Table 3-1), however, a small percentage of nontransgenic controls also appear to show loss of stripe 5 expression (not shown), indicating that this phenotype may be a nonspecific heat shock effect.

Heat-shock experiments were also performed with no recovery time after induction to test whether Knirps might be repressing *eve* indirectly. We found that the order of repression of *eve* stripes was identical as in Fig. 3-3, although for each heat-shock regimen repression was not as complete (data not shown), possibly because the *eve* mRNA had less time to turn over. This result is consistent with a direct action of Knirps on *eve* enhancers.

The activity of Knirps 1-330, containing the CtBP-independent domain of Knirps may reflect the previously identified CtBP-independent autonomous activity. Alternatively, some or all of the activity may be due to competition of the DNA binding domain for activator binding sites. Therefore, we overexpressed the DNA binding domain of Knirps (residues 1-105, containing the previously defined DNA binding domain and nuclear localization signal; Gerwin et al., 1994) and determined its effect on *eve* expression pattern. As measured by quantitative Western blotting, this protein was readily induced to levels almost as great as Knirps 1-330. Even at high expression levels, however, Knirps 1-105 was unable to perturb *eve* expression (data not shown), suggesting that Knirps represses *eve* by means other than direct competition for activator binding sites.

Differential effects of Knirps protein on minimal *even-skipped* stipe enhancers

Next, we tested the effects of overexpression of full-length Knirps and the N-terminal, CtBP-independent domain of Knirps on minimal *eve* stripe 2-3, 3/7- and 4/6-*lac Z* reporters. We confirmed that the differential susceptibility to repression of *eve* stripe 3 compared to stripe 2 (Fig. 3-3) was directly associated with the previously defined regulatory regions using a *lacZ* reporter gene coupled to the minimal 500 bp stripe 2 and 3 enhancers. Both full-length Knirps 1-429 and Knirps 1-330 preferentially repressed stripe 3 over stripe 2 (Fig. 3-4B and F). The Knirps 1-429 protein was able to entirely repress stripe 3 in almost all embryos, and stripe 2 in a majority of embryos (Fig. 3-4A-C). In contrast, as noted with the endogenous *eve* gene, the CtBP-independent 1-330 repression domain was less potent than 1-429, resulting in more embryos with only partially repressed *eve* stripe 3, and fewer embryos in which stripe 2 was repressed (Fig. 3-4D-F). Embryos in which both stripes 2 and 3 were repressed were distinguishable from nontransgenic embryos by residual stripe 2 expression in ventral regions and an anterior stripe driven by vector sequences (Fig. 3-4C). The minimal stripe 2 enhancer is probably more sensitive to repression by Knirps 1-330 than the endogenous stripe 2 enhancer because it does not contain all sequences involved in stripe 2 regulation (M. Ludwig and M. Kreitman, personal communication). A previous study found that the minimal stripe 2 element was only slightly affected by Knirps overexpression (Kosman and Small, 1997), but here we are probably achieving higher levels of expression.

To further verify the relative activity of full-length Knirps versus the CtBP-independent activity of Knirps, we tested the effects of overexpression of Knirps proteins in embryos carrying *eve* stripe3/7- and 4/6-*lacZ* reporters (Fig 3-4G-L). As observed on

Figure 3-4: Differental repression of minimal *eve* stripe enhancers by Knirps1-429 and 1-330, demonstrates differential sensitivities to Knirps activity.

Repression of *eve* stripe 2/3-*lacZ* reporter gene demonstrate differential sensitivities of *eve* stripe 2 vs. stripe 3 enhancers to Knirps expression. Patterns of *lacZ* expression in embryos prior to heat shock (A, D) and after heat shock (B, C, E, F). After a 10 minutes heat shock, the majority of embryos expressing Knirps 1-429 showed repression of *eve* stripe 3 (B). After a 30 minutes heat shock, the majority of embryos showed repression of both stripe 2 and 3 (C). A significant percentage of embryos showed only partial repression of stripe 3 upon overexpression of Knirps 1-330 (E). The majority of embryos overexpressiong Knirps 1-330 demonstrated a loss of stripe 3, but not stripe 2 after 30 minutes of heat shock (F).
Effects of overexpression of Knirps1-429 and 1-330 in embryos carrying the *eve* stripe 3/7 *lacZ* reporter (G-I) or *eve* stripe 4/6 *lacZ* reporter (J-L). Full-length Knirps was a more potent repressor, but Knirps 1-330 was capable of repressing the minimal *eve* stripe 4/6 element (see text for details). The pattern shown in H and K is representative of embryos heat-shocked for 15 minutes whereas the pattern shown in I and L is typical of embryos heat-shocked for 30 minutes. Embryos are oriented with anterior towards the left, dorsal side upwards.

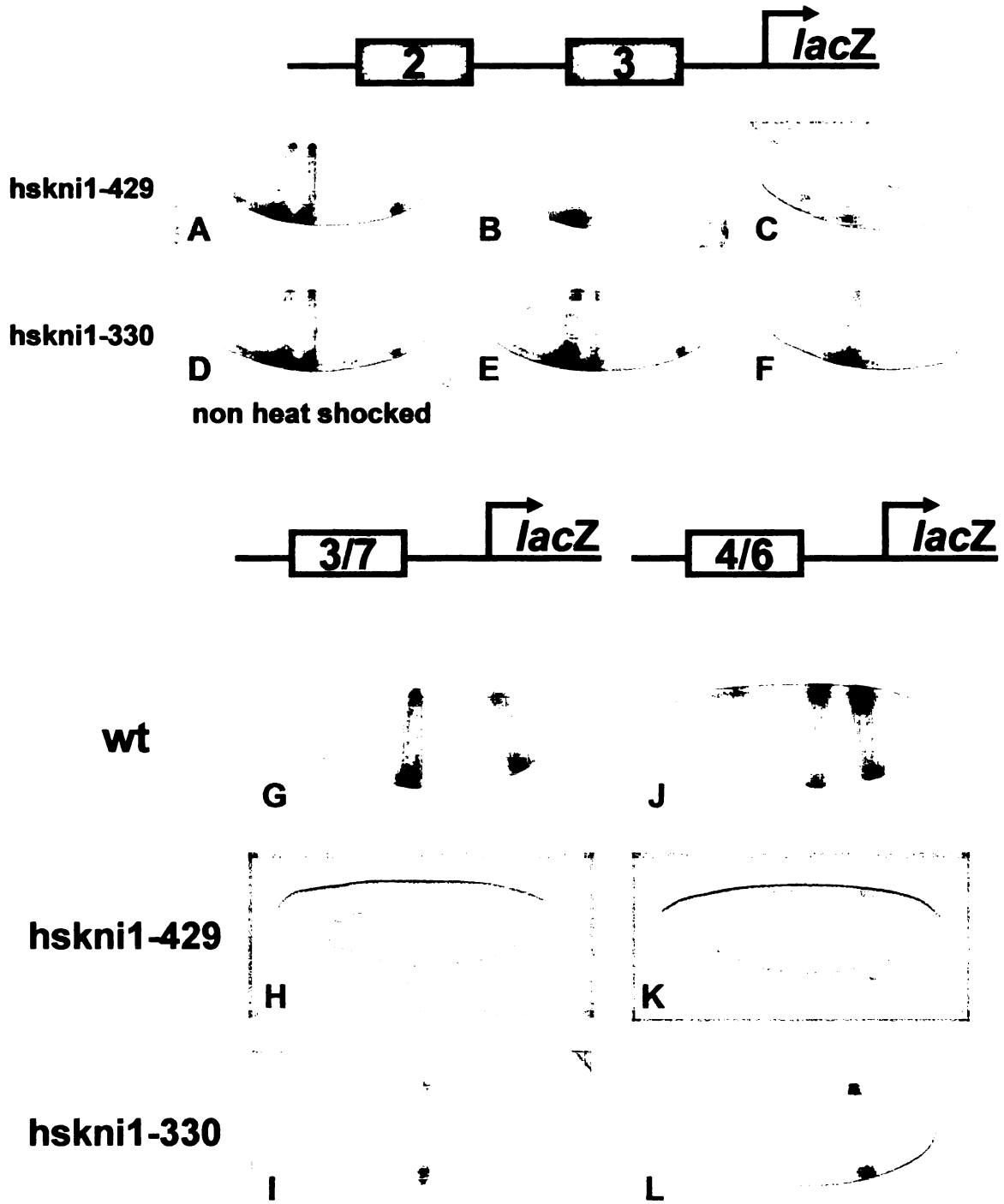

Figure 3-4: Differential repression of minimal *eve* stripe enhancers by Knirps 1-429 and 1-330, demonstrates differential sensitivities to Knirps activity.

the endogenous *eve* gene, full-length Knirps was a more potent repressor than the CtBP-independent domain, causing complete repression of *eve* stripe 4 and 7 and almost complete repression of *eve* stripe 3 and 6 (Fig. 3-4H and K compare with 3-4G and J). A large decrease in the number of stained embryos also indicates that many *lacZ* reporter genes were completely repressed. Knirps 1-330 caused a similar repression pattern, but longer heat shocks were required to achieve comparable repression of the more sensitive *eve* stripe 4 and 7. After 30 min of heat shock, repression of *eve* stripe 3 and 6 was not as complete as that achieved by Knirps 1-429 after 15 min of heat shock (Fig. 3-4I compare with H and L compare with K). Importantly, when expressed at high level, the CtBP-independent repression activity of Knirps was able to completely repress *eve* stripe 4, and partially repress stripe 6, confirming the results observed with the endogenous *eve* gene.

Higher specific activity of Knirps protein containing multiple repression activities.

The lower activity of Knirps 1-330 protein relative to the full-length Knirps protein might be due to a greater potency of the protein containing two distinct repression activities, or it might merely reflect lower protein expression levels. To directly compare levels of ectopically expressed Knirps proteins, lysates from transgenic embryos were subject to Western blot analysis, using the same heat shock regime as that used for the in situ analysis above (Fig. 3-5A). Equivalent amounts of total protein from whole embryo lysates were separated on SDS gels, transferred to membranes and probed with an antibody specific for the C-terminal FLAG epitope. Quantitation of the signals from the blots indicate that the weaker Knirps 1-330 repressor was actually expressed at

Figure 3-5: Quantitation of proteins expressed from *hsp70-knirps* transgenes demonstrates that full-length Knirps 1-429 is less abundant than Knirps 1-330.

A. Western blot analysis of embryos subjected to the same heat-shock regiment (0, 5, 10, 20 min.) used for analysis shown in Table 3-1. Asterisk marks nonspecific cross-reacting protein that was also present in lysates from nontransformant *Drosophila* (presence of nonspecific band appeared to vary with batch of antibody; data not shown). Because this nonspecific band comigrates with the 1-330 protein, the signal from the nonspecific protein (averaged from lanes 5-8) was subtracted from each of the values in lanes 1-4 to determine levels of 1-330 protein. Below, Coomassie stained gel showing equal loading. **B.** Quantitation of Western blots demonstrates an approximately two-fold higher level of Knirps 1-330 protein at each time point than Knirps 1-429, demonstrating that the higher activity of the 1-429 is not due to higher levels of this protein. Standard deviations are shown in B for four separate gels and quantitations of the heatshock experiment shown in panel A.

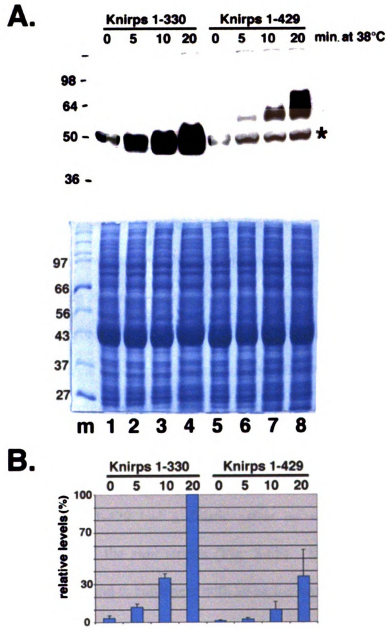

Figure 3-5: Quantitation of proteins expressed from *hsp70-knirps* transgenes demonstrates that full-length Knirps 1-429 is less abundant than Knirps 1-330.

approximately twofold higher levels than Knirps 1-429 at each time point tested (Fig. 3-5B). Therefore, the greater potency of the full-length Knirps is not just a function of greater expression or stability of this protein, but presumably reflects the greater activity of the combined repression domains.

Potency of Knirps 1-429 vs. Knirps 1-330 in regulation of *hunchback, runt, hairy,* and *fushi tarazu.*

To compare the activities of full-length Knirps 1-429 with the Knirps CtBP-independent repression domain on other endogenous target genes, we examined the effects of overexpressing Knirps 1-429 or Knirps 1-330 on *hunchback*, *runt*, *hairy* and *fushi tarazu*. Previous studies demonstrated that the *hunchback* parasegment 4 stripe is very sensitive to low levels of Knirps (Kosman and Small, 1997), and we found that both the full-length Knirps protein as well as the CtBP-independent Knirps repressor strongly downregulated this stripe (Fig. 3-6A-C). Consistent with genetic information about *knirps* regulation of *runt* (Klingler and Gergen, 1993; Kosman and Small, 1997), misexpression of Knirps 1-429 had a drastic effect on *runt* expression, leading to repression of up to six of the *runt* stripes (Fig. 3-6F). Stripe 1 was repressed less frequently than stripes 2-4 and 6, consistent with an earlier report that indicated it was not affected by levels of Knirps sufficient to inhibit stripe 2-3 (Kosman and Small, 1997). Knirps 1-330 was much less effective in perturbing *runt* expression, except for weakened *runt* stripe 3 expression (Fig. 3-6E).

The stripe elements 3, 4, 6 and 7 of *hairy* have been found to be affected by misexpression of Knirps protein or mutations in the *knirps* gene (Pankratz et al., 1990;

Figure 3-6: Effect of expression of Knirps proteins on *hunchback* (*hb*), *runt*, *hairy* and *fushi tarazu* (*ftz*) demonstrates differential activity of Knirps 1-429 versus Knirps 1-330 on all but the most sensitive target genes.

hb, a sensitive target of Knirps (A-C), showed similar repression by both Knirps 1-330 and Knirps 1-429 of the parasegment 4 zygotic expression pattern (arrow) after 30 minutes of heat shock. On other genes, Knirps 1-330 was much less potent than Knirps 1-429. 1-330 repressed only the stripe 3 of *run* (E), while Knirps 1-429 repressed all stripes but *run* stripe 5 (F) after 15 minutes of heat shock (similar patterns were observed at 30 minutes). Similarly, 1-330 had a modest effect only on *hairy* stripes 3/4 (H) and *ftz* stripe 3 (L), while Knirps 1-429 completely repressed *hairy* 3,4 and 7 (I) and extensively disrupted *ftz* expression (K) after 5 and 10 minutes of heat shock respectively (similar patterns were noted at 15 and 30 minutes). Transcripts of endogenous Knirps target genes were visualized by in situ hybridization. All embryos are oriented with anterior towards the left, dorsal side upwards.

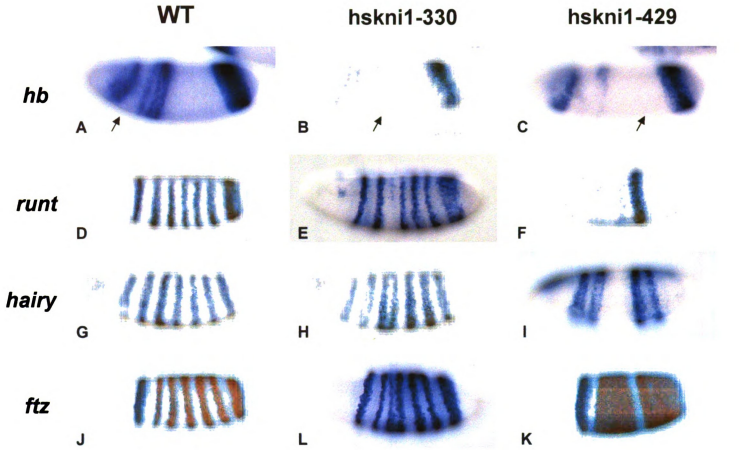

Figure 3-6: Effect of expression of Knirps proteins on *hunchback* (*hb*), *runt*, *hairy*, and *fushi tarazu* (*ftz*) demonstrates differential activity of Knirps 1-429 versus Knirps 1-330 on all but the most sensitive target genes.

Langeland et al., 1994; Kosman and Small, 1997) and binding sites for Knirps protein have been mapped on the *hairy* stripe 6 and 7 enhancer elements (Langeland et al., 1994; Hader et al., 1998). Expression of Knirps 1-429 caused a strong repression of *hairy* stripe 3, 4, and 7 expression, while expression of Knirps 1-330 had no such inhibitory effect (Fig. 3-6G-I). The *ftz* pair-rule gene is also under control of gap gene regulators, as well as primary pair rule genes (Carroll and Scott, 1986; Yu and Pick, 1995). In Knirps 1-429 overexpressing embryos, the central stripes are fused, but overexpression of Knirps 1-330 had a much milder effect, with partial weakening of *ftz* stripes 2 and 3 (Fig. 3-6J-K). As discussed below, the effects of Knirps misexpression on *ftz* might well represent secondary effects mediated through upstream regulators, in particular *eve* and *hairy*. These effects on *eve* and other endogenous pair rule genes support the observation that the CtBP-independent repression domain of Knirps is capable of mediating repression on the most sensitive target genes, but is quantitatively less potent than the full-length protein.

Discussion

Multiple repression activities – quantitative contributions to reaching repression thresholds.

Just as transcriptional activators are known to possess multiple activities to stimulate transcription, a growing number of transcriptional repressors have been found to have multiple activities that are dependent on distinct cofactors. In *Drosophila*, the Brinker repressor can interact with both the CtBP and Groucho corepressors to mediate repression of Dpp regulated genes (Hasson et al., 2001; Zhang et al., 2001). For this repressor, distinct cofactors are required at different promoters. The *tolloid* gene is repressed by Brinker in the blastoderm embryo in a Groucho-dependent manner, while either CtBP or Groucho are sufficient to mediate *brk* autoinhibition. Interestingly, neither cofactor appears to be required for repression of *omb* and *sal*, suggesting a third pathway for repression, possibly direct competition (Hasson et al., 2001; Rushlow et al., 2001). Similarly, the Even-skipped, Runt, and Engrailed proteins repress through Groucho-dependent and –independent pathways, again showing gene-specificity. In none of these cases is it known whether the requirement for specific repression activities at endogenous enhancers reflects qualitatively distinct mechanisms, or alternatively, distinct quantitative requirements for repression levels (Kobayashi et al., 2001; Fujioka et al., 2002; Aronson et al., 1997). Analysis of the Groucho-dependent and –independent activities of Eve protein on *lacZ* reporters, suggest that in combination these two domains do provide quantitatively superior level of repression (Fujioka et al., 2002).

Previous studies of Krüppel, Giant, and Knirps have indicated that CtBP-dependence or –independence of their repression activities varies according to the

specific *cis* regulatory element involved, suggesting that there are particular enhancer architectures that necessitate CtBP activity. The clearest example of enhancer specific requirements for CtBP is shown in the case of *eve* enhancers. In nuclei situated between *eve* stripes 4 and 6, the stripe 4/6 and 3/7 enhancers are both repressed by Knirps in the same nuclei, yet this repression is independent of CtBP on the 3/7 element and dependent on CtBP on the 4/6 element (Fig. 3-1). By expressing increasing levels of the CtBP-independent form of Knirps, the requirement for CtBP is obviated (Fig. 3-3). These results suggest that distinct requirements for the CtBP cofactor at different genes or *cis* regulatory elements can be based on the quantitative levels of repression activity. Indeed, the combination of the CtBP-dependent and CtBP-independent activities make a particularly powerful repressor, as judged by comparison of repression activities of Knirps 1-429 vs. Knirps 1-330 on *eve* (Fig. 3-3 and Table 3-1) and other pair rule genes (Fig. 3-6). These results suggest that both repression domains can be simultaneously engaged on a given cis regulatory element, rather than a particular repression activity being selectively engaged at particular enhancers. Consistent with this picture, when they are assayed separately as Gal4 fusion proteins in embryos, both CtBP-dependent and CtBP-independent repression domains of Knirps have equal, modestly effective repression activities. In contrast, a Gal4 protein containing both domains is much more effective at repressing a strongly activated promoter (Sutrias-Grau and Arnosti, submitted).

A model that explains the quantitative contribution of the CtBP corepressor to Knirps repression activity is shown in Fig. 3-7. At the top, two lines depict the levels of

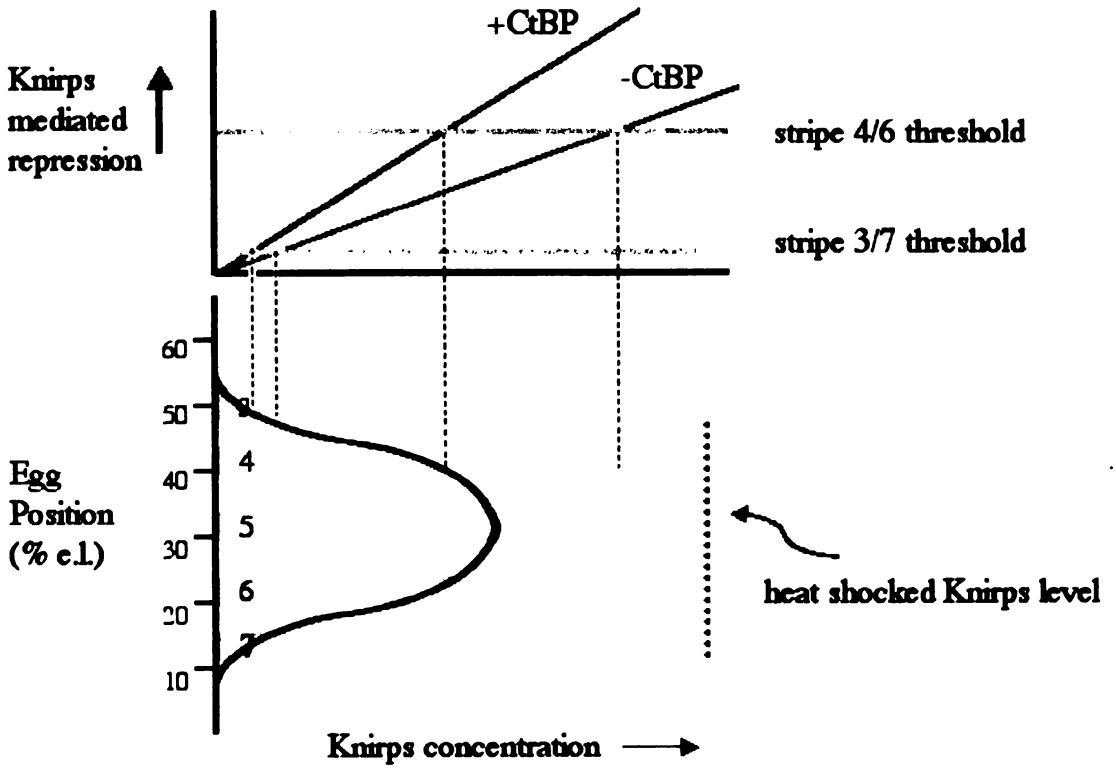

Figure 3-7: Quantitative model for contribution of CtBP activity to repression by Knirps.

Protein levels of Knirps protein (horizontal axis) are read out as differential levels of repressor activity (vertical axis at top). With CtBP, Knirps repression levels increase more sharply with increasing protein levels, allowing the activity to cross critical thresholds at lower protein levels. The position of the Knirps protein levels in the embryo (lower part of figure indicated by % egg length) then dictates where appropriate stripe boundaries will form (vertical broken lines). This model predicts that due to the inherently high threshold of the *eve* stripe 4/6 enhancer, loss of CtBP activity will move the intercept off of the range of physiological Knirps concentrations, while having little effect on the stripe 3/7 position.

repression activity generated by increasing Knirps concentrations, the top line illustrating the levels of repression achieved by the Knirps protein complexed with CtBP.

Thresholds of repression required by the *eve* stripe 3/7 and 4/6 enhancers are depicted by horizontal lines. Below, relative levels of Knirps are shown with respect to position (egg length) in the embryo. At a relatively low level of Knirps protein activity, the *eve* 3/7 enhancer is repressed, and this level of repression activity is achieved at similar levels of Knirps, regardless of whether or not CtBP contributes to repression. Thus, in the absence of CtBP, the positions at which the stripe 3/7 boundaries form shift very little. The much higher level of repression required by the stripe 4/6 element is achieved only near the peak of Knirps protein levels. If CtBP is not complexed with Knirps, the intercept shifts sharply to the right, to a level of Knirps not normally present in the embryo. The sufficient level of repression in the absence of CtBP activity or protein is only achieved under conditions where Knirps is overexpressed, as in Figure 3-3.

Setting Thresholds

The threshold model explains how the contributions of separate repression activities act in a quantitative fashion to meet given thresholds, but what is the basis for distinct repression thresholds? There are at least two variables involved in dictating a threshold, namely, regulatory protein levels, and the nature (number, affinity, and placement) of the relevant binding sites within a regulatory element. Varying intranuclear activator levels can influence repression thresholds, as suggested by regulation of the *Krüppel* gene: Giant requires CtBP for repression of this gene only in nuclei containing peak levels of the Bicoid activator (Strunk et al., 2001). Varying

intranuclear repressor levels will dictate how easily those thresholds are met with or without multiple repression activities. Gap genes, including *knirps*, generate protein gradients that have properties of morphogens, that is, they trigger differential responses at different threshold levels (Kosman and Small, 1997). The stripe 4/6 and 3/7 modular enhancers of the *even-skipped* gene are designed to respond to different levels of Knirps protein, allowing the embryo to establish multiple stripe boundaries with a single protein gradient. The short-range activity of Knirps allows the two enhancers to act independently, so that activators bound to the stripe 4/6 enhancer activate the gene in nuclei where the levels of Knirps are already sufficiently high to inhibit the stripe 3/7 enhancer.

Binding site affinity and number have been clearly established to influence threshold responses in the case of transcriptional activators, such as Bicoid and Dorsal (Jiang and Levine, 1993; Szymanski and Levine, 1995; Struhl et al., 1989). A similar effect is likely to be true for repressors. Sequence analysis of the *eve* gene indicates that there are more high-affinity Knirps binding sites within the *eve* stripe 3/7 element than in the 4/6 enhancer, consistent with relative sensitivities of these elements that we determined experimentally (Fig. 3-3; Papatsenko et al., 2002, Berman et al., 2002). Removal of some of the Knirps binding sites in the *eve* stripe 3/7 enhancer reduces the sensitivity of this element to the Knirps gradient (Clyde et al., in press). However, the number of predicted high affinity binding sites alone is not sufficient information to predict relative sensitivity to Knirps. If it were, one would expect the *eve* stripe 2 enhancer, with 3 predicted Knirps sites, to be more sensitive to Knirps than *eve* stripe 4/6, with only a single site, yet the reverse is true (Berman et al., 2002; Fig. 3-3). This lack of

correlation might be partly attributable to errors in prediction of binding sites, however, additional factors, such as affinity of binding sites and relative placement with respect to other proteins, are likely to make the decisive difference in determining enhancer sensitivity to Knirps. In the case of the Giant repressor, small shifts in the placement of the binding site allows detection of less than two-fold differences in repressor concentrations, a "gene tuning" mechanism that seems to have been invoked during internal evolution of the *eve* stripe 2 enhancer (Ludwig et al., 2000; Hewitt et al., 1999). The stoichiometry of activators to repressors has also been suggested to be a critical factor in determining repression levels, and direct tests indicate that Giant and Knirps respond sensitively to differences in activator binding site number and affinity on defined regulatory elements (Hader et al., 1998; Kulkarni and Arnosti, 2003).

eve stripe 1 lies just posteriorly to the weak anterior domain of *knirps* expression, suggesting a possible role of Knirps in regulating that element, but it is not clear whether the relative sensitivity of other *eve* stripe enhancers normally active outside of the main posterior domain of Knirps expression is of physiological significance. The *eve* stripe 2 pattern lies outside of the normal area of Knirps expression, and is only repressed at highest levels of Knirps (Table 3-1), suggesting that repression might be through cryptic Knirps sites in the element (Berman et al, 2002). The robust activity of the *eve* stripe 5 enhancer even under conditions of high levels of Knirps misexpression underlines that this regulatory element has been designed to function in nuclei containing peak levels of Knirps protein (Fig. 3-3). Similarly, *runt* stripe 5 also resists peak levels of ectopic Knirps (Fig. 3-6). Both of these regulatory elements have few or no predicted Knirps binding sites (Berman et al, 2002). These elements would provide a useful platform to

test the number and placement of novel Knirps binding sites required to bring the element under the control of this repressor.

Knirps regulation of *hb*, *run*, *h*, and *ftz*

The effects of Knirps misexpression on other endogenous pair rule genes reinforce the lessons learned from *eve*, regarding the relative potency of the Knirps repression domains and the sensitivity of different enhancers. Both the CtBP-independent portion of Knirps as well as the intact protein were capable of repressing the *hunchback* parasegment 4 stripe, a highly sensitive target of Knirps (Kosman and Small, 1997). However, *hairy*, *runt*, and *ftz*, previously noted to have a higher threshold to Knirps repression, were noticeably less affected by Knirps 1-330 compared to Knirps 1-429 (Fig. 3-6). Thus, it is likely that CtBP activity contributes quantitatively to repression of other Knirps target genes in addition to *eve*.

Repression of central *run* stripes is consistent with previous findings of direct repression by Knirps and the greater sensitivity of stripes 2-4 relative to stripe 1 (Kosman and Small, 1997). We observed a greater effect of ectopic expression of Knirps on *hairy* than noted in previous experiments, probably on account of higher levels of expression. Knirps expressed under the control of an *eve* stripe 2 enhancer was previously found to have little effect on anterior *hairy* expression, except for a delay in stripe 3/4 separation (Kosman and Small, 1997). Heat shock expression of full length Knirps 1-429, in contrast, resulted in strong repression of *hairy* stripes 3, 4 and 7 (Fig. 3-6I). The *hairy* stripe 3, 4 and 7 enhancers are predicted to contain Knirps binding sites, in contrast to the unrepressed stripe 1 and 5 enhancers (Langeland et al., 1994; La Rosee et al., 1997;

Berman et al., 2002). The weaker Knirps 1-330 protein had an effect similar to that of full-length Knirps expressed from an *eve* stripe 2 expression construct, that is, a delay of stripe 3/4 separation (Fig. 3-6H). Interestingly, *knirps* is important for activation of *hairy* stripe 6, and the protein can bind to the stripe 6 enhancer directly in vitro (Riddihough and Ish-Horowicz, 1991; Langeland et al. 1994). We see no evidence of activation upon overexpression, however, suggesting that such activation might be indirect.

The derepression of *ftz* we observe between stripe 2-4 and 6-7 is likely to be due to indirect effects of repression of *hairy* and *eve* expression; both of these genes are thought to repress *ftz* directly (Jiménez et al, 1996; Manoukian and Krause, 1992). In contrast, previous work involving lower levels of anteriorly expressed Knirps observed only weakened *ftz* stripes 2 and 3, rather than stripe fusion. This lower level of Knirps had a much less profound effect on upstream regulators *hairy* and *eve,* suggesting that Knirps might be a direct gap gene input to this pair rule gene, as suggested by earlier studies (Yu and Pick, 1995; Kosman and Small, 1997).

Repression mechanisms

Our study suggests that the multiple repression activities of Knirps can be simultaneously mobilized to provide quantitatively correct levels of repression activity, and that the design of *cis* regulatory elements can elicit CtBP-dependence. CtBP-independent activity can in some cases be directly attributed to direct competition with activator for DNA binding (Hoch et al., 1992; Nibu et al., 2003), however, the CtBP-independent activity of Knirps can repress activators on elements where sites are not overlapping (Keller et al., 2000; Ryu and Arnosti, 2003), and overexpression of the DNA binding domain of Knirps

(Knirps1-105) is insufficient to mediate repression of endogenous *eve* enhancers (see Appendix B). Cell culture and transgenic embryo assays indicate that both CtBP-dependent and –independent repression activities of Knirps have very similar characteristics with respect to activator specificity, distance dependence, and overall potency, thus the targets and molecular mechanisms might well be similar in each case (Ryu and Arnosti, 2003; Sutrias-Grau and Arnosti, submitted). Key to a deeper understanding of the molecular circuitry controlled by short-range repressors such as Knirps will be biochemical knowledge of the mechanisms of repression employed on these developmentally regulated enhancers.

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Chapter 4

A functional interaction between the histone deacetylase Rpd3 and the *Drosophila* short-range repressor Knirps[6]

Abstract

The *Drosophila* Knirps protein is a short-range transcriptional repressor essential for proper embryonic development. Short-range repressors work over distances of less than 100-150 base pairs to inhibit activators in a local fashion, allowing multiple enhancers to be regulated autonomously. The mechanisms of short-range repression remain poorly understood at the molecular level. Knirps mediates repression in part by recruiting the corepressor CtBP, but it also possesses a CtBP-independent repression activity. To investigate whether Knirps interacts with additional factors, we generated transgenic flies that overexpress inducible, double-tagged versions of Knirps and performed affinity purification experiments. Full-length, recombinant Knirps can be expressed in embryos at the same time as the endogenous factor. The protein is heavily phosphorylated and acts as a functional repressor to control several endogenous Knirps targets. Gel filtration chromatography of embryonic extracts expressing full-length Knirps indicate that the recombinant protein is part of a complex of ~450 kDa, suggesting

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that other factors in addition to CtBP interact with Knirps. In a survey of possible cofactors, we found that Rpd3 (HDAC1) cofractionates with Knirps during gel filtration. To facilitate characterization of novel Knirps-interacting proteins, we developed a tandem affinity chromatography protocol from embryonic extracts overexpressing Knirps and, consistent with the gel filtration results, find that histone deacetylase Rpd3 copurifies with full-length Knirps, but not the CtBP-independent repression domain. To test the functional relevance of this association, we carried out dosage interaction assays, and find that the *rpd3* and *knirps* interact genetically. Altogether these results suggest that histone deacetylation plays a role in short-range repression.

Introduction

Transcriptional repression plays essential roles in establishing cell- and tissue-specific gene expression patterns during *Drosophila* embryogenesis (Pankratz and Jäckle, 1990; Gray et al., 1992; Gray and Levine, 1996). For example, in the blastoderm embryo, the activity of gap-gene repressors restricts the expression pattern of pair-rule genes, generating their characteristic seven-stripe expression pattern, which marks the onset of segmentation (Small et al., 1992; Clyde et al., 2003). Several modes of repression have been proposed, including competition between repressors and activators for overlapping binding sites on the DNA, the formation of inactive heteromeric complexes off the DNA, the local quenching of activators or direct repression of the basal transcription machinery (reviewed in Gray et al., 1995; Gray and Levine, 1996; Nibu et al., 2003). Repressors might also work by modifying, directly or indirectly, the structure of chromatin and

therefore making the DNA template less accessible to activators and/or the basal transcriptional machinery (reviewed in Courey and Jia, 2001).

Transcriptional repressors have been classified according to the range of their activity (Cai et al., 1996; Barolo and Levine, 1997). Short-range repressors work over distances of 100-150 bp to block nearby activators or the core transcription complex. This form of repression allows enhancers to work independently of one another to direct complex, additive patterns of gene expression (Gray et al. 1994). Long-range repressors can function over distances of >1 kb to inhibit multiple enhancers in complex modular promoters, thereby resulting in simple on/off patterns of gene expression (Barolo and Levine, 1997). The molecular mechanisms by which repressors works are poorly understood, although the short-range/long-range distinction may result from the recruitment of different corepressors (Nibu et al. 1998). Short-range repressors interact with the *Drosophila* homolog of mammalian C-terminal binding protein (dCtBP), whereas long-range repressors interact with the Groucho corepressor (Nibu et al., 1998; Courey and Jia, 2001).

The CtBP corepressor is required for full activity of short-range repressors such as Knirps, Krüppel, Giant, and Snail that play important roles in patterning the blastoderm embryo (Nibu et al., 1998a,b; Strunk et al., 2001). This evolutionarily conserved cofactor also interacts with a number of vertebrate transcriptional regulators, including the adenovirus E1A protein, Net, Ikaros, Zeb, and, indirectly, the Retinoblastoma tumor suppressor protein (reviewed in Chinnadurai 2002). Transcription factors typically bind CtBP via a short peptide motif similar to the PLDLS sequence originally identified in E1A (Schaeper et al., 1995). CtBP is homologous to α-hydroxyacid dehydrogenases, and

it contains a conserved NAD binding domain as well as conserved residues in the putative active site (reviewed in Chinnadurai, 2002; Turner and Crossley, 2001). Although not identified in previous studies, recent reports found a weak dehydrogenase activity associated with CtBP (Kumar et al., 2002; Balasubramanian et al., 2003; Shi et al., 2003). CtBP has been found to bind directly to histone deacetylases (HDACs), suggesting that the corepressor may affect transcription by chromatin remodeling (reviewed in Turner and Crossley, 2001; Chinnadurai, 2002). A recent biochemical purification of human CtBP identified additional proteins in a complex, including histone methyltransferases, the CoREST repressor, and a protein homologous to polyamine oxidases (Shi et al., 2003). This additional complexity suggests that CtBP itself may utilize multiple activities to effect transcriptional repression.

CtBP-mediated repression is critical for full activity of short-range repressors. However, several *Drosophila* short-range repressors also possess CtBP-independent repression activities (La Rosee-Borggreve, 1999; Keller et al., 2000; Strunk et al., 2001; Nibu et al., 2003). In the case of Knirps, the CtBP-independent activity has been mapped to an N-terminal repression domain that lacks a CtBP-binding motif, does not bind CtBP in vitro, and is able to repress in the absence of maternal CtBP (Keller et al., 2000). Endogenous targets of Knirps display different requirements for CtBP. For example, the *even-skipped* (*eve*) stripe 3/7 enhancer is regulated by Knirps in a CtBP-independent fashion, whereas the *eve* stripe 4/6 enhancer shows loss of Knirps-mediated repression in the absence of maternal CtBP (Struffi et al. 2004). Moreover, a CtBP-dependent Knirps target, such as the *eve* stripe 4/6, can be repressed by the N-terminus, CtBP-independent repression domain of Knirps when this protein is provided at higher than normal levels

suggesting that CtBP may contribute quantitatively to Knirps repression (Struffi et al. 2004). Cell culture and transgenic embryos assays indicate that both the CtBP-dependent and -independent repression activities of Knirps possess similar functional characteristics with respect to activator specificity, distance dependence and overall potency, suggesting that their activities might be mediated through similar pathways (Ryu and Arnosti, 2003; Sutrias-Grau and Arnosti, 2004).

The ability of Knirps to repress without the contribution of CtBP might suggest either the existence of additional cofactors that mediate short-range repression, or that the Knirps proteins itself has an intrinsic ability to block transcription. Yeast two hybrid analysis has not identified additional Knirps-interacting factors other than CtBP (Keller and Arnosti, unpublished; Giot et al., 2003), therefore we employed biochemical approaches to identify additional cofactors that interact with Knirps. Knirps is found in a complex with apparent molecular size of ~450 kDa, suggesting that other factor/s in addition to CtBP interact with Knirps. The histone deacetylase Rpd3 (HDAC1; De Rubertis et al, 1996) interacts with full-length Knirps during two rounds of affinity purification and the two proteins copurify during gel filtration chromatography, consistent with the hypothesis that Knirps and Rpd3 are present in the same complex. However, Rpd3 does not interact with the N-terminal, CtBP-independent repression domain of Knirps (Knirps1-330), suggesting that the interaction is mediated either through CtBP or via the C-terminus of Knirps. Gene dosage assays suggest that *knirps* and *rpd3* functionally interact *in vivo*. Previous studies designed to test the contribution of Rpd3 in transcriptional repression during early *Drosophila* embryogenesis concluded that this deacetylase was probably not essential for gap gene activity (Mannervick and

Levine, 1999). However, this study was based on a hypomorphic *rpd3* mutant that reduces, but not abolishes, *rpd3* function. Consistent with this hypothesis, we found that only when a null *rpd3* mutant was used in gene dosage assays, a genetic interaction between *knirps* and *rpd3* was detected. Altogether, these results support the hypothesis that histone deacetylation plays a role in short-range repression.

Materials and Methods

Transgenic flies carrying inducible, double-tagged Knirps genes. Details on the generation of transgenic flies expressing either full-length Knirps (1-429) or the N-terminal, CtBP-independent repression domain of Knirps (1-330) were reported elsewhere (Struffi et al., 2004). Each protein is double-tagged, carrying an N-terminal hexahistidine tag and a C-terminal double FLAG tag and is expressed under the control of the *hsp* 70 promoter. Recombinant proteins are functional (Struffi et al., 2004) and can be expressed in embryos older than ~2 hours (see Appendix B).

Heat-shocks. To induce expression of recombinant Knirps proteins, transgenic embryos collected on apple-juice plates at room temperature (22-23°C) were incubated for 30 minutes at 38°C in a 10-liter water bath to ensure rapid and even heating. After induction, embryos were immediately dechorionated and sonicated within 15 minutes from the end of heat-shock.

Western blotting analysis. Immunoblotting was performed according to standard protocols (Harlow and Lane, 1999) using a tank transfer system (Mini Trans-Blot® Cell, Biorad 170-3930). Immun-Blot™ PVDF membranes (BioRad 162-0177) were used and antibody incubation was in TBST (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) supplemented with 5% (w/v) nonfat dry milk as blocking agent. SuperSignal® West Pico Chemiluminescent substrate (Pierce 34080) was used for detecting horseradish peroxidase (HRP) on immunoblots.

Antibodies. FLAG M2 monoclonal antibody (Sigma F3165) was used at 1:10,000 dilution. Rabbit polyclonal antiserum against *Drosophila* Rpd3 (from D. Wassarman,

University of Wisconsin; Pile and Wassarman, 2000) was used at 1:5,000 dilution. Rabbit polyclonal antiserum against *Drosophila* CtBP (dCtBP) was generated against full-length dCtBP and used at 1:20,000 dilution. To generate this antiserum, recombinant full-length, hexahistidine-tagged *Drosophila* CtBP (dCtBP 1-479) from pET15bCtBP$_L$ vector was expressed in *E. coli* BL21-CodonPlus™ RIL competent cells (Stratagene #230240) and purified on Ni-NTA agarose beads (Qiagen 30210) according to the manufacturer's instructions. dCtBP protein (400μg) in 0.2 ml PBS buffer (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.2) was mixed with an equal volume of Titermax Gold adjuvant (Sigma T2684) and injected subcutaneously at multiple sites in a New Zeeland female rabbit. Two secondary boosts were performed similarly after 4 and 12 weeks. Serum was prepared according to Harold and Lane (1999). Monoclonal antibody against *Drosophila* HP1 (C1A9) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa) and used at 1:2,000 dilution. Rabbit polyclonal antiserum against *Drosophila* E(z) (from P. Harte, Case Western Reserve University) was used at 1: 1,000 dilution. Rabbit polyclonal antiserum against *Drosophila* Su(Var)3-9 (from T. Grigliatti, University of British Columbia; Ner et al., 2002) was used at 1:5,000 dilution. Monoclonal antibody against *Drosophila* Sir 2 (from S. Parkhurst, Fred Hutchinson Cancer Research Center) was used at 1:500 dilution. Rabbit polyclonal antiserum against *Drosophila* Brahma (from P. Verrijzer, University of Leiden) was used at 1:1,000. ImmunoPure® Goat Anti-Mouse HRP-conjugated antibody (Pierce 31430) was used at 1:20,000 dilution. Goat Anti-Rabbit HRP-conjugated antibody (BioRad 170-6515) was used at 1:10,000 dilution.

***Drosophila* embryo nuclear extract preparation.** 0-12 hours embryos from hskni1-429.3 were collected on grape juice plates from two population cages. For each extraction, two 0-12 hour collections (the first one stored 12 hours at 13°C) were pooled together. 20-40 grams of embryos were either dechorionated and processed immediately or transferred on a 155-mm Petri dish and floated on a 38°C water bath for 60 min. Heat-shocked embryos were recovered for 30 min at room temperature, prior to dechorionation and homogenization. *Drosophila* standard nuclear extracts (DSNE) were made according to Soeller et al. (1988).

Co-immunoprecipitation (Co-IP) experiments. 200 μl of nuclear extracts (30 μg/μl of total protein) from embryos overexpressing full-length Knirps were incubated overnight at 4°C with 4 μl (4.9μg/μl) of α-FLAG M2 monoclonal antibody or an equivalent amount of α-IgG monoclonal antibody on a rotating wheel. One milliliter of washing buffer (150 mM NaCl, 50mM Hepes pH 7.9, 0.5 mM EDTA, 10% glycerol, 1mM DTT, 1 mM PMSF, 1mM Na-metabisulfite, 1mM benzamidine, 10μM pepstatin A) was added and each sample was supplemented with 10 μl of pre-equilibrated protein G-agarose beads (Cat. #16-266, Upstate) and incubated for 3 hours at 4°C on a rotator. Beads were washed for four times (10 minutes each time) with 1 ml of washing buffer, resuspended in Laemmli buffer, and boiled for 5 min at 95°C.

Lambda protein phosphatase (λ-PPase) digestion. To test whether recombinant Knirps is a phosphoprotein, crude embryo lysates were prepared from embryos expressing full-length Knirps (hskni1-429.3) and subjected to treatment with increasing amounts of λ-PPase. For each reaction, 20 μl of crude embryo lysate (280 μg of total protein) from 2-4

hour hskni1-429.3 embryos subjected to 30 minutes heat-shock was incubated with 0, 20, 80, 400 or 1600 units of λ-PPase (New England Biolabs, P0753S) in 1X λ-PPase buffer (50 mM Tris-HCl pH 7.5, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01 % Brij 35) supplemented with 2 mM $MnCl_2$, in a final volume of 50 µl. To limit the activity of endogenous phosphatases, reactions were carried out at 4°C for 1 hour. To determine whether the phosphatase activity was the result of λ-PPase rather than due to endogenous activities, 20-mM sodium orthovanadate (Sigma, S-6508), a specific inhibitor of λ-PPase, was added to each reaction and incubated in the same conditions as described. Reactions were stopped by the addition of Laemmli buffer immediately followed by incubation at 95°C for 5 min. Proteins were resolved onto a 8% SDS-PAGE and recombinant Knirps was detected by Western blot using anti-FLAG M2 antibody.

Double affinity purification of recombinant Knirps proteins. Although Knirps is expressed maximally between two and four hours after the embryo has been laid, microarray data from the Berkley *Drosophila* Genome Project (http://www.fruitfly.org) indicate that the gene is expressed at relatively high levels also between 7 and 10 hours. Therefore, for all affinity purification experiments we used 0-12 hour embryos collected at room temperature (22-23°C). Embryos were heat-shocked for 30 min at 38°C as described. 2-4 grams of dechorionated embryos were resuspended in 40 ml lysis buffer (150 mM NaCl, 50 mM Hepes pH 7.9, 10% glycerol, 10 mM imidazole, 20 mM ß-mercaptoethanol, 1 mM PMSF, 1 mM Na-metabisulfite, 1 mM benzamidine, 10 µM pepstatin A) and sonicated using a Branson-250 Sonifier (4 cycles, 20-30 pulses/cycle, output 6, duty cycle 60%, 3 min on ice between cycles, using a medium-tip). Lysates were cleared by centrifugation (20 min at 27,000xg) and 2 ml of washed and pre-

equilibrated Ni-NTA agarose beads (His Select™ HC Nickel, Sigma 6611) were added to the supernatant. After 6 hours at 4°C on a rotating wheel, beads were washed three times with 50 ml of lysis buffer supplemented with 20-mM imidazole and transferred to a 5-ml tube. Proteins were eluted twice using 3 ml of lysis buffer supplemented with 150 mM imidazole. The eluates were pulled together and diluted to 50 ml with 150 mM NaCl, 50 mM Hepes pH 7.9, 10% glycerol, 0.2 mM EDTA, 2 mM DTT, 1 mM Na-metabisulfite, 1 mM benzamidine, 10 µM pepstatin A, 1 mM PMSF. 2-300µl of Protein-G agarose beads (Upstate, 16-266) covalently coupled with anti-FLAG M2 antibody (2 mg of antibody per ml of wet beads) were added to the solution and incubated for 12-18 hours at 4°C on a rotating wheel. Beads were washed three times with 50 ml of the same buffer, transferred to an eppendorf tube and proteins were eluted either with 0.5 ml of buffer supplemented with 0.25 mg/ml of 3X FLAG® peptide (Sigma, F4799), or with 1.2 ml of buffer supplemented with 0.2% sarkosyl (N-lauroyl-Sarkosine, Sigma L-5777). Protein samples were TCA precipitated using 4 mg/ml Na-deoxycholate (deoxycholic acid, Sigma D-6750) as carrier, resuspended in Laemmli buffer and boiled 5 min at 95°C. For each purification experiment, one or two negative controls were used in parallel: either heat-shocked, non-transgenic (*yellow-white*) embryos (*yw*), or transgenic embryos from the same line, that were not heat-shocked.

Chromatographic identification of the Knirps complex. To determine the apparent molecular size of recombinant Knirps, whole-cell extracts from embryos expressing full-length Knirps were subjected to gel filtration chromatography. 0-12 hour embryos from hskni1-429.3 were heat-shocked 30 min and a crude lysate was prepared essentially as described above, using a lysis buffer containing 100 mM NaCl, 50 mM Hepes pH 7.9,

5% glycerol, 0.1 mM EDTA, 1 mM DTT, 1 mM Na-metabisulfite, 1 mM benzamidine, 10 μM pepstatin A, and 1 mM PMSF. Lysates were centrifuged at 27,000xg for 20 min and 300 μl of cleared lysate (6 mg of total protein) was loaded onto a pre-equilibrated Superdex-200 HR 10/30 column (Amersham, 17-1088-01) and eluted with 1.5 volume of lysis buffer at the flow rate of 0.4 ml/min using the ÄKTAexplorer 100 system (Amersham). Fractions (0.5ml) were collected and analyzed by Western blot for the presence of recombinant Knirps, CtBP and Rpd3. The same column was loaded with size markers (MW-GF-1000, Sigma) and run using identical conditions to determine in which fractions the different markers elute.

Genetic interaction between *knirps* and *rpd3*. To test for a genetic interaction between *kni* and *rpd3*, transheterozygous flies for *kni* and *rpd3* were generated and the expression pattern of the Knirps target gene *even-skipped* (*eve*) was monitored by *in situ* hybridization. For each gene, two alleles were used to account for genetic variability. *kni9* (Bloomington stock #3332) carries a null mutation in Knirps and was previously used to test a genetic interaction between *kni* and *CtBP* (Nibu et al. 1998). *kni7G* (Tübingen stock # Z334) is a loss of function mutation caused by a point mutation (C48S) in the DNA binding domain (Gerwin et al. 1994). $rpd3^{04556}$ (Bloomington stock 11633) is a strong hypomorphic mutation caused by a P-element insertion in the 5' untranslated region of *rpd3* and results in a severe reduction in Rpd3 expression (Mannervik and Levine, 1999). $rpd3^{def24}$ (kindly provided by Steward Frankel) is a null allele caused by deletion of ~870 bp from the insertion point of the P-element in $rpd3^{04556}$ into the amino terminal coding region of the gene (Mottus et al., 2000). To distinguish transheterozygous embryos (*rpd3/kni*) from single heterozygous one (*kni*/+ or *rpd3*/+) the

third chromosome was balanced with a *ftz-lacZ* marker (Bloomington stock 2055). A double staining with digoxigenin-UTP-labeled *eve* and *lacZ* antisense RNA indicates the transheterozygous embryos (which are not stained with *lacZ*) from the single heterozygous that are stained with both probes giving a unique staining pattern.

Results

Recombinant Knirps is heavily phosphorylated *in vivo*.

In vivo expression of different domains of Knirps, monitored by Western blotting using α-FLAG M2 antibody which recognizes the C-terminal epitope, leads to the appearance of clusters of bands rather than discrete products (Struffi et al., 2004). In the case of recombinant full-length Knirps (hskni1-429) these products span approximately 25 kDa (from ~55 to ~80 kDa; Fig 4-1, lane 2). Some of the higher molecular size products were no longer detected after an overnight incubation of the extract at 4 °C in the presence of protease inhibitors (data not shown), suggesting that the protein might be post-translationally modified. A search for putative phosphorylation sites using NetPhos2.0 software (http://www.cbs.dtu.dk/services/NetPhos/) revealed 35 predicted phosphorylation sites (29 serine and 6 tyrosine). To directly test whether recombinant, full-length Knirps is phosphorylated *in vivo*, crude lysates from induced hskni1-429 embryos were incubated with increasing amounts of lambda protein phosphatase (λ-PPase), in the absence or presence of sodium orthovanadate, a specific inhibitor of this enzyme. λ-PPase is a Mn^{2+}-dependent protein phosphatase with activity towards phosphorylated serine, threonine and tyrosine residues (Cohen and Cohen, 1989; Zhuo et al, 1993). The presence of λ-PPase caused the conversion of lower mobility products into the fastest migrating species, which has a mass close to the predicted size for recombinant Knirps (~51 kDa). The extent of this conversion depended on the amount of enzyme present and the reaction was complete with a concentration of about 4 U/μg of total protein (Fig. 4-1, lane 7). Importantly, at the highest concentration of enzyme, the

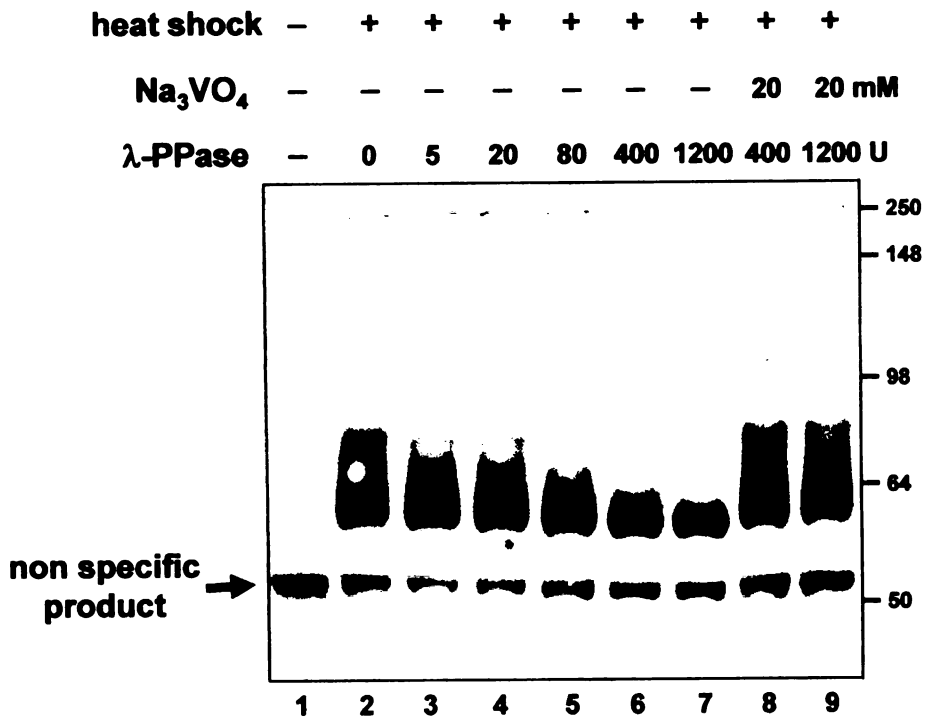

Figure 4-1: Recombinant, full-length Knirps is a phosphoprotein when expressed in the *Drosophila* embryo.

2-4 hours embryos from a line carrying an hsp70-*knirps* transgene (hskni1-429.3) were heat-shocked for 30 minutes at 38°C to express double-tagged, full-length Knirps proteins (lane 2-9) or left at room temperature (lane 1). A cross-reacting product (arrow) serves as convenient marker for equal loading. Crude embryo lysates were incubated with increasing amount of λ-protein phosphatase (λ-PPase) in the absence (lane 1-7) or presence (lane 8-9) of a λ-PPase inhibitor, sodium orthovanadate (Na_3VO_4). Extracts were resolved on an 8% SDS-PAGE and a Western blot was performed using α-FLAG M2 monoclonal antibody, which recognizes Knirps C-teminal epitope tag.

reaction was completely inhibited by the presence of 20mM sodium orthovanadate (Fig. 4-1, lane 9), suggesting that λ-PPase, rather than other endogenous factors, was responsible for this effect. Altogether, these results indicate that recombinant Knirps is heavily phosphorylated *in vivo*. It is possible that the endogenous protein might be as well, but we have not investigated this possibility yet (see discussion).

Knirps is present in a ~450 kDa complex.

To test whether Knirps interacts with additional factor/s other than dCtBP, gel filtration chromatography was performed using whole cell extracts from embryos expressing recombinant full-length Knirps. Although is not known whether endogenous Knirps is able to dimerize, dimerization is not required for DNA binding (Gerwin et al, 1994). *Drosophila* CtBP (dCtBP) is expressed throughout embryogenesis in two major forms ($CtBP_{long}$ and $CtBP_{short}$) with apparent molecular masses of ~42 and ~50 kDa respectively (Appendix A). It is not known whether Knirps interacts with both CtBP forms, but i*n vivo* assays indicate that both forms are able to mediate repression (Sutrias-Grau and Arnosti, 2004). The mammalian homolog of $dCtBP_{long}$ was recently crystallized as a dimer (Kumar et al., 2002). Therefore, if recombinant Knirps interacts exclusively with a CtBP dimer, the complex should have a molecular mass of ~150-180 kDa. Gel filtration chromatography was performed using 0-12 hours embryos expressing full-length, double-tagged Knirps protein (with molecular mass of ~55-80 kDa). Recombinant Knirps eluted predominantly in fraction 17-19 with a peak in fraction 18 (Fig. 4-2A), which corresponds to an apparent molecular size of ~450 kDa, along with both forms of

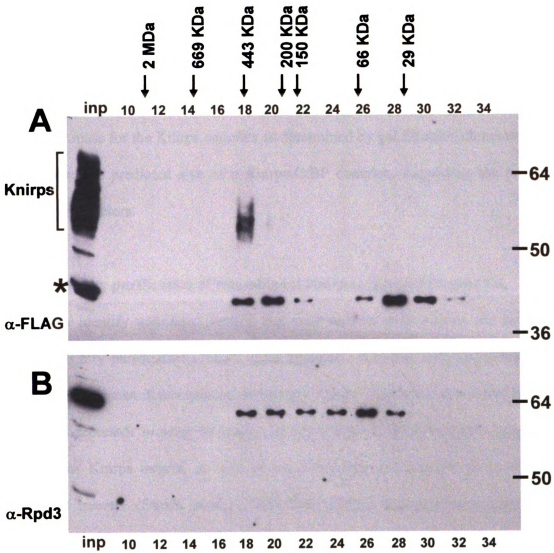

Figure 4-2: Recombinant Knirps is present in a ~450 kDa complex.

A. Recombinant, full-length Knirps elutes during gel filtration chromatography with an apparent molecular mass of ~450 kDa. Gel filtration chromatography was performed using crude embryo lysates from 0-12 hour embryos expressing FLAG-tagged, full-length Knirps. Every other fraction, starting with fraction 10, was resolved on a 10% SDS-PAGE, proteins were transferred onto a PVDF membrane and the blot was probed with monoclonal α-FLAG M2 antibody. Recombinant Knirps (bracket) eluted predominantly in fraction 18, which correspond to an apparent molecular mass of ~450 kDa. The asterisk indicates a non-specific product also present in non-transgenic embryonic extracts (data not shown). **B.** The histone deacetylase Rpd3 (HDAC 1) co-fractionates with Knirps during gel filtration chromatography. Fractions collected from the same experiment as in A were probed using a polyclonal antibody against Rpd3. The peck fraction for Knirps (fraction 18) also contains Rpd3.
Size markers ran in the same conditions eluted as indicated on the top.

dCtBP (data not shown). We could detect a limited amount of recombinant Knirps protein also in fraction 20-29 (data not shown) suggesting that the majority of protein is part of the same complex and very little is present as free monomer. The apparent molecular mass for the Knirps complex as determined by gel filtration chromatography is well above the predicted size of a Knirps-CtBP complex, suggesting the presence of additional cofactors.

Double affinity purification of recombinant Knirps expressed in embryos.

To identify additional factors that may interact with Knirps we developed a tandem affinity purification scheme using transgenic flies that carry inducible, double–tagged *knirps* genes. Recombinant, full-length Knirps was previously found to act as a functional repressor *in vivo*, as judged by the ability of the protein to repress several endogenous Knirps targets, as well as integrated *eve-lacZ* reporter genes, in a dose-dependent manner (Struffi et al., 2004). Recombinant Knirps proteins expressed in embryos did not reach high enough levels to detect differences in the Coomassie-stained protein profile of crude extracts after 30 minutes of heat-shock induction (data not shown). However, recombinant Knirps protein is detected after just 5 minutes of heat-shock induction by immunoblotting using α-FLAG M2 monoclonal antibody (Struffi et al. 2004), due to the enhanced detection achieved with double FLAG-tagged proteins (Hernan et al, 2000). The majority of recombinant, full-length Knirps (~80% of the input) did bind to Ni-NTA agarose in batch purifications as determined by quantitation of Western blots using a Fluor-S® MultiImager (Biorad), although this is not apparent from the fixed-time exposure shown in Fig. 4-3B, where lanes 1 and 2 seem to contain

Figure 4-3: Double affinity purification of recombinant, full-length Knirps from crude embryo lysates.

The starting material for the tandem affinity purification (IN1) was a crude lysate from embryos expressing double-tagged, full-length Knirps (lane 1). Proteins were bound in batch purifications to Ni-NTA beads and eluted in three successive elutions using 150 mM imidazole (E1-3). Imidazole eluates were pooled, diluted ten times and used as starting material for the second affinity purification (IN2). Proteins were bound in batch purifications to α-FLAG M2-Protein G beads and eluted in three successive steps using 0.2% sarkosyl (S1-3). Fractions from each purification step were resolved on 4-12% Nu-PAGE gels (Invitrogen) and transferred to PVDF membrane for Western blotting.
A. Coomassie-stained gel of fractions from the Ni-NTA purification (lane 1-9) shows a clear decrease in protein complexity after a single purification step (compare lane 1 with 6). Silver-stained gel of fractions from the α-FLAG purification (lane 10-18) shows an additional simplification of the protein profile. **B.** Knirps tandem affinity purification. Western blot of fractions as in A, using α-FLAG M2 monoclonal antibody. Recombinant Knirps (arrow) was able to bind to both affinity matrices (lane 6-8 and 15-17). The IgG light chain is indicated (asterisk). **C.** Profile of CtBP in the Knirps double affinity purification. Western blot of fractions as in A, using a rabbit antiserum against $dCtBP_L$, that recognizes both the long- (L) and short-form (S) of CtBP. Both forms of CtBP copurify together with Knirps during two rounds of affinity purifications (lane 15-16).

IN1, input first affinity purification; FTn, flow-through Ni-NTA beads; W1-3, wash 1-3; E1, first imidazole elution; E2, second imidazole elution; E3, third imidazole elution; nB, Ni-NTA beads after three imidazole elutions; IN2, input second affinity purification; FTf, flow-through FLAG beads; W4-6, wash 4-6; S1, first elution with sarkosyl; S2, second elution with sarkosyl; S3, third elution with sarkosyl; fB, FLAG beads after three sarkosyl elutions.
Lane 1 corresponds to 50μg of total protein. Lane 1-5, ~0.007% of the original sample. Lane 6-8, ~0.13% of the original sample. Lane 10-14, ~0.16% of the original sample. Lane 15-18, ~0.08% of the original sample.

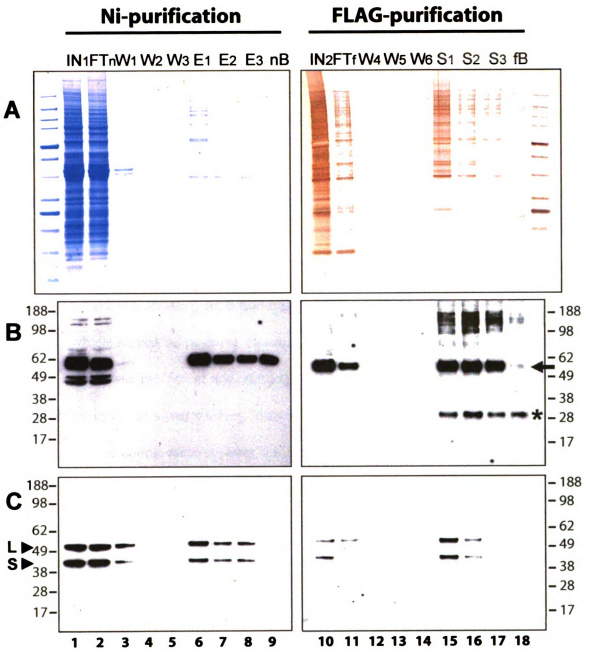

Figure 4-3: Double affinity purification of recombinant, full-length Knirps from embryo lysates.

comparable amounts of Knirps protein. This first purification step was quite efficient in reducing the complexity of the protein profile (Fig. 4-3A, lane 6, compared with 1), but the pattern was still too complex to detect differences between samples before and after heat-shock (data not shown). Approximately 50% of the total Knirps protein (starting material of first affinity purification equal to 100%) was recovered after the Ni-NTA purification. After the second affinity purification step, using αFLAG-ProteinG agarose beads, approximately 10-20% of the total recombinant Knirps could be recovered with two elutions with buffer supplemented with 0.2% sarkosyl. Recombinant Knirps was not detected in fractions obtained from non-induced transgenic embryos or heat-shocked *yw* embryos, which served as negative controls. FLAG peptide elution of double affinity-purified Knirps was inefficient and, starting with 2-4 grams of embryos, we were below the detection limits of silver staining (data not shown). Therefore, we used the detergent sarkosyl to elute bound material after the second affinity purification. Sarkosyl elution was efficient in releasing recombinant Knirps protein bound to FLAG beads (Fig. 4-3B, lane 15-17), but it also elutes other proteins, which probably bind to the beads non-specifically, resulting in a quite complex protein profile (Fig. 4-3A, compare lane 15 with 10).

To determine whether the affinity purification protocol used was suitable to retain Knirps-interacting factors, we tested whether the corepressor CtBP was co-purifying together with recombinant Knirps protein during both purification steps. Indeed, both forms of CtBP co-purified with recombinant Knirps (Fig. 4-3C) and were not detected in fractions from heat-shocked, non-transgenic *yw* embryos (data not shown), suggesting

that this purification protocol is suitable to detect protein-protein interactions that occur under physiological conditions.

Histone deacetylase Rpd3 co-purifies with recombinant, full-length Knirps.

Limiting amount of embryos expressing recombinant Knirps forced us to take a candidate approach for the identification of Knirps-interacting factors. Proteins implicated in transcriptional repression and gene silencing in *Drosophila* include methyltransferases such as Su(Var)3-9 (Ner et al. 2002; Shotta et al., 2002) and E(z) (Carrington and Jones, 1996; Laible et al. 1997), histone deacetylases including Rpd3 (Chen et al. 1999) and Sir2 (Rosenberg et al. 2002), and ATP-dependent nucleosome remodeling factors such as Brahma (Elfring et al. 1998; Kal et al., 2000). To test whether any of these proteins interact *in vivo* with Knirps, we performed immunoprecipitation experiments using α-FLAG M2-beads and nuclear extracts prepared from embryos expressing FLAG-tagged, full-length Knirps. FLAG-bound proteins were resolved on 10% SDS PAGE gels, transfer to PVDF membranes and subjected to Western blotting analysis using antibodies against each of the above factors (Fig. 4-4A, and data not shown). Rpd3 was consistently immunoprecipitated using α-FLAG M2-beads, but not α-IgG-beads (Fig. 4-4A, lane 4 versus 5). To confirm this interaction we performed tandem affinity purification experiments using transgenic embryos expressing double-tagged, full-length Knirps (*hsp70-knirps1-429.3* embryos after heat shock; Fig. 4-4B). As negative controls, double-affinity purifications were performed in parallel using the same amount of material from heat-shocked, non-transgenic embryos (*yellow white* [*yw*]), as well as from non-induced, transgenic embryos (*hsp70-kni 1-429.3* non heat-shocked;

Figure 4-4: Histone deacetylase Rpd3 (HDAC 1) copurifies with full-length Knirps.

A. Rpd3 co-immunopurifies with α-FLAG antibody (lane 4), but not α-IgG antibody (lane 5) from embryonic nuclear extracts overexpressing recombinant full-length Knirps. Nuclear extracts from transgenic embryos expressing FLAG-tagged, full-length Knirps were immunoprecipitated using α-FLAG M2-Protein G-beads. FLAG-bound material was resolved on a 10% SDS-PAGE gel, proteins were transferred to a PVDF membrane and Western blotting was performed using a polyclonal antibody against *Drosophila* Rpd3.

B. Rpd3 copurifies with recombinant, full-length Knirps during tandem affinity purification. Crude extracts from embryos expressing double-tagged, full-length Knirps were subjected to two successive affinity purification steps (as described in Fig. 4-3). Fractions from each purification step were subjected to Western blotting using α-Rpd3 (fractions are as in Fig. 4-3). After two affinity purifications, fractions that contained recombinant Knirps (S1-S2; see Fig. 4-3B) also contained Rpd3 (Fig. 4-4B, lane 15-16).

C. After double-affinity purification, Rpd3 is present exclusively in fractions that express recombinant, full-length Knirps. Tandem affinity purification experiments were performed in parallel using equivalent amounts of embryos expressing double-tagged, full-length Knirps (kni 1-429 after heat shock; lane 3 and 6) or not expressing Knirps (*yellow white* (*yw*) after heat shock [lane 1 and 4]; non heat-shocked kni1-429 [lane 2 and 5]). After the second affinity purification, FLAG-bound materials were eluted using 0.2% sarkosyl and resolved on 10% SDS-PAGE gels, proteins were transferred to a PVDF membranes and Western blotting was performed using a monoclonal antibody against FLAG (lane 1-3) or a polyclonal antibody against *Drosophila* Rpd3 (lane 4-6). Rpd3 is detected only in the sarkosyl eluates from embryos that express recombinant, full-length Knirps (lane 6), but not in the eluates from the two negative controls (lane 4-5).

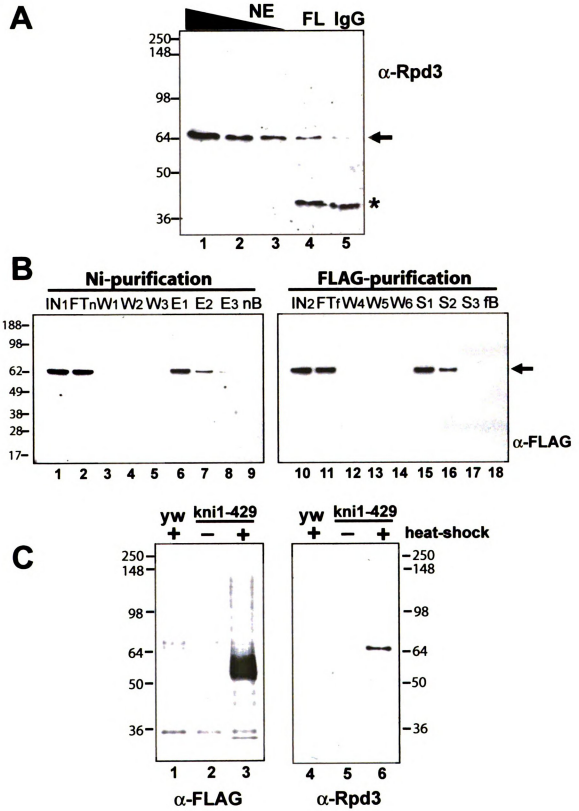

Figure 4-4: Histone deacetylase Rpd3 (HDAC 1) copurifies with full-length Knirps.

Fig. 4-4C). After two successive purification steps, Rpd3 was detected only in samples expressing recombinant Knirps, but not in the two negative controls (Fig. 4-4C, lane 3 versus 1-2). Altogether, these results suggest that Rpd3 physically interacts, directly or indirectly, with full-length Knirps. If the two proteins are present in the same complex, they should also co-fractionate during gel filtration chromatography. Consistent with this hypothesis, fraction 18 where the majority of recombinant Knirps eluted, also contained Rpd3 (Fig. 4-2B). The majority of Rpd3 eluted in fraction 26, which correspond to the monomeric form, but significant amounts were also present in higher molecular size fractions, consistent with the possibility that the protein is part of many different complexes.

Rpd3 does not interact with the N-terminal, CtBP-independent repression domain of Knirps.

Mammalian CtBP has been shown to interact with the histone deacetylase HDAC 1 (Shi et al. 2003). Therefore, it is possible that the Knirps-Rpd3 interaction we detected during our tandem affinity purification experiments is an indirect interaction mediated through dCtBP. To test this hypothesis, we performed double affinity purification experiments using transgenic embryos expressing the N-terminal, CtBP-independent repression domain of Knirps (Fig. 4-5). Neither forms of dCtBP bound to double-tagged Knirps 1-330 and both were quantitatively lost in the flow-through from the Ni-NTA column (Fig. 4-5B). Some Rpd3 was able to bind to Ni-NTA agarose beads, however, and this material was efficiently eluted with imidazole and was present in the input of the second affinity purification (Fig. 4-5C, lane 5). The affinity of Rpd3 for Ni-NTA

Figure 4-5: Rpd3 does not copurify with the N-terminal, CtBP-independent repression domain of Knirps during double-affinity purification.

Tandem affinity purification experiments were performed using crude extracts from embryos expressing double-tagged, Knirps 1-330. Fractions were resolved on a 10% SDS-PAGE, transferred to a PVDF membrane and Western blotting was performed using antibodies against FLAG (A), CtBP (B), and Rpd3 (C). Fractions are as in Fig. 4-3.

A. Double affinity purification of Knirps 1-330. The N-terminal, CtBP-independent repression domain of Knirps was expressed by heat shocking embryos carrying an *hsp70-knirps 1-330* transgene for 30 minutes. Crude extracts (lane 1) were first bind to Ni-NTA-agarose beads and eluted with imidazole (lane 3). Imidazole eluates were pooled (lane 5) and bound to αFLAG M2-Protein G agarose beads. FLAG-bound material was eluted with sarkosyl (lane 7-8). **B.** CtBP does not interact with Knirps 1-330. Both forms of CtBP were quantitatively lost in the flow-through of the first affinity column (lane 2, compare with 1). **C.** Rpd3 does not interact with Knirps 1-330. Some Rpd3 is able to bind to the Ni-NTA column and is eluted with imidazole (lane 3). However, this material is unable to efficiently bind to α-FLAG beads and is present in the flow-through of the second affinity column (lane 6).

Lane 1-2, ~0.16% of the original sample. Lane 3, ~0.3% of the original sample. Lane 4, ~0.2% of the original sample. Lane 5-9, ~20% of the original sample.

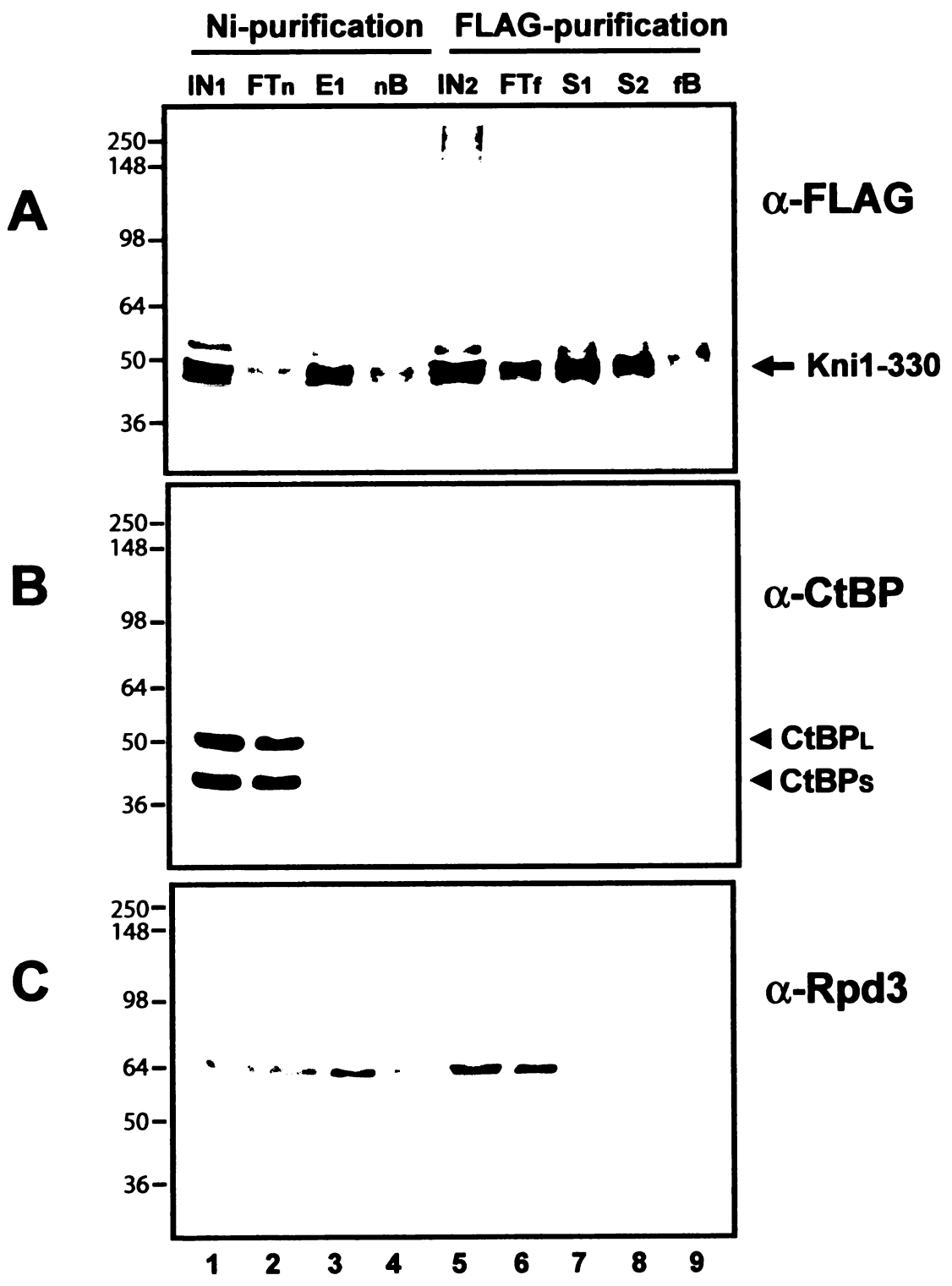

Figure 4-5: Rpd3 does not copurify with the N-terminal, CtBP-independent repression domain of Knirps during double-affinity purification.

agarose beads was also observed using extract from non heat-shocked transgenic embryos carrying an *hsp70-knirps1-429.3* transgene (data not shown), suggesting it is not dependent by the presence of Knirps 1-330. However, Rpd3 was unable to bind to α-FLAG beads when extracts expressing double-tagged, Knirps 1-330 were used (Fig. 4-5C, lane 7-8). These results are consistent with the hypothesis that Rpd3 directly interacts with CtBP or with the C-terminus of Knirps.

rpd3 genetically interacts with *knirps*

To test whether *rpd3* genetically interacts with *kni*, we generated transheterozygous embryos for both genes (*rpd3/kni*) by crossing two heterozygous flies (*kni*/+ and *rpd3*/+) and determining the expression pattern of *eve* in blastoderm embryos from the singly heterozygous parents as well as from the doubly heterozygous F_1 embryos. To determine whether the results obtained were allelic-specific, we chose two alleles for each gene and checked all four combinations. In order to distinguish transheterozygous embryos from singly heterozygous embryos, the third chromosome was balanced with a *ftz-lacZ* reporter and the F_1 embryos obtained by crossing the two singly heterozygous parents were hybridized with digoxigenin-labeled antisense mRNA for *eve* and *lacZ*. Embryos heterozygous for each *rpd3* allele ($rpd3^{04556}$/+ and $rpd3^{def24}$/+) showed a normal *eve* expression pattern (Fig. 4-6A and data not shown). Control embryos obtained from the mating of *kni9*/+ or *kni7G*/+ males with normal (*yw*) females showed a reduction or complete repression of *eve* stripe 5 expression in approximately 10% of the embryos (Fig. 4-6B and data not shown). This effect is

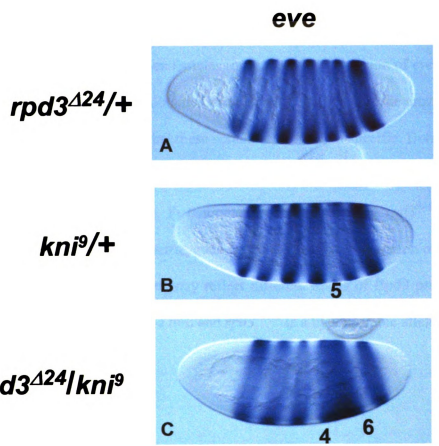

Figure 4-6: Genetic interactions between *rpd3* and *knirps*.

Embryos that are heterozygous for the $rpd3^{def24}$ null mutation ($rpd3^{\Delta 24}$/+) exhibited a normal *even-skipped* (*eve*) expression pattern (A). Embryos that are heterozygous for the kni^{9} null mutation (kni^{9}/+) exhibited reduced or complete repression of *eve* stripe 5 expression (B) in approximately 10% of the embryos. F_1 embryos obtained by mating *kni* heterozygous males with $rpd3^{\Delta 24}$ heterozygous females showed derepression of *eve* stripe 4/6 expression (C).

Embryos were hybridized with digoxigenin-labeled *eve* antisense RNA (A-C) and digoxigenin-labeled *lacZ* antisense RNA (C), and visualized by histochemical staining.

probably indirect, since *eve* stripe 5 does not have predicted binding sites for Knirps (Clyde et al, 2003). F_1 embryos obtained by mating $rpd3^{def24}$ heterozygous males (or females) with *kni9* heterozygous females (or males) showed fusion of *eve* stripe 4-6 (Fig. 4-6C). Similar results were obtained with $rpd3^{def24}$/*kni7G* transheterozygous embryos (data not shown), suggesting that these results are not allele-specific. Derepression of *eve* stripe 4/6 did not allow determining whether *eve* stripe 5 expression is also affected in the doubly heterozygous embryos. F_1 embryos obtained by mating $rpd3^{05446}$ heterozygous females with *kni9* or *kni7G* heterozygous males did not show alteration of the *eve* pattern (data not shown). These results may reflect different levels of Rpd3 protein in the two alleles tested, since $rpd3^{def24}$ is a null and $rpd3^{05446}$ is a hypomorphic allele.

Discussion

In order to identify functionally-relevant Knirps cofactors, we took a biochemical approach and employed recombinant Knirps proteins expressed in the embryo as affinity matrixes. To this goal, we created transgenic flies that carry double-tagged versions of Knirps (the full-length protein, Knirps1-429, and the N-terminal, CtBP-independent repression domain, Knirsp1-330) under the control of the heat-shock inducible, *hsp70* promoter. Upon heat shock of such transgenic embryos, Knirps is expressed ubiquitously throughout the embryo and induction can be achieved at the blastoderm stage, when endogenous Knirps is maximally expressed (Struffi et al., 2004). Recombinant Knirps proteins are functional as judged by their ability to repress several endogenous Knirps targets including *eve*, *hairy*, *runt* and *hunchback* (Struffi et al., 2004). The recombinant proteins are double-tagged (hexahistidine-tagged at the N-terminus and double FLAG-tagged at the C-terminus) allowing the use of affinity purification methods to purify Knirps-interacting factors.

First, we found that recombinant, full-length Knirps expressed in the embryo is heavily phosphorylated (Fig. 4-1). All recombinant Knirps proteins tested, which include Knirps 1-330, Knirps 75-330, Knirps 75-429 and Knirps 1-429, appeared to be phosphorylated when expressed *in vivo* (Fig. 4-1 and data not shown). Knirps is not known to be post-translationally modified, but it has 35 predicted phosphorylation sites. Unfortunately, the poor quality of available Knirps antibodies for Western blotting analysis precluded us from testing whether the endogenous Knirps protein is phosphorylated as well. The short-range repressor Giant, when overexpressed in

Drosophila embryos, was also found to be phosphorylated, but the functional relevance of this modification in Giant-mediated repression was not investigated (Capovilla et al., 1992). A recent study of the mammalian homolog of the *Drosophila* short-range transcriptional repressor Snail showed that phosphorylation regulates the subcellular localization and activity of this repressor (Dominguez et al., 2003). Testing whether endogenous Knirps is phosphorylated and whether this post-translational modification plays a role in Knirps repression was beyond the scope of this work and was not pursued further.

To test whether Knirps interacts with other co-factors in addition to CtBP, we determined the apparent molecular mass of the Knirps complex by gel filtration chromatography using crude embryonic extracts expressing recombinant, full-length Knirps. The apparent size of the complex is ~450 kDa, well above the expected size of a Knirps-CtBP complex (Fig. 4-2A). This result is the first indication that the short-range repressor Knirps may interact with additional factors other than CtBP. The majority of recombinant Knirps was found in the high molecular weight fraction (fraction 18), and very low amounts eluted as a free monomeric form (expected to elute in fraction 26-28). This unexpected result could be explained assuming that the recombinant protein is expressed at relatively low levels compared to its associating factors. Gel filtration chromatography separates proteins not only according to their mass, but also according to their shape, therefore, we should take these results as an indication, rather than a proof, that the size of the Knirps complex is around 450 kDa.

The double affinity purification protocol we developed is suitable for detecting physiologically relevant interactions, since we were able to co-purify CtBP together with

Knirps along each step of the tandem affinity purification (Fig. 4-3B, C). We found that both forms of CtBP have the ability to bind to full-length Knirps (Fig. 4-3C) and are present together with Knirps in the same fractions during gel filtration chromatography (data not shown). It is not known whether endogenous Knirps interacts with both CtBP forms, but *in vivo* repression assays using Gal4-$CtBP_{long}$ and Gal4-$CtBP_{short}$, indicate that both forms are able to mediate similar levels of repression (Sutrias-Grau and Arnosti, 2004). The unbiased identification of Knirps-interacting factors will involve mass spectrometry analysis of peptide-eluted material from double affinity purification of embryo expressing full-length Knirps versus embryos that are not expressing the recombinant protein (either because non transgenic or because Knirps was not induced). These experiments are in progress, but in the meantime we took a candidate approach and tested whether several proteins that have been involved in transcriptional repression were present in fractions enriched for Knirps. Embryonic nuclear extracts overexpressing recombinant, full-length Knirps were immunoprecipitated using α-FLAG and probed using a panel of antibodies (see material and methods for the antibodies tested; data not shown). The histone deacetylase Rpd3 (HDAC 1; De Rubertis et al., 1996) was consistently found in the FLAG-bound fraction (Fig 4-4A). Only a minority of Rpd3 did bind to Knirps, which was in part expected, since Rpd3 is an abundant protein and it is probably present in several different complexes. We could unambiguously detect an Rpd3-Knirps interaction only when the immunoprecipitation experiments were performed using nuclear extracts prepared from embryos overexpressing recombinant full-length Knirps (Fig. 4-4A), but not when crude embryo lysates overexpressing Knirps were used (data not shown). This is probably due to the low levels of expression of

recombinant Knirps. We confirmed this interaction by testing whether Rpd3 was copurifying together with Knirps during double affinity purification experiments. Rpd3 was detected in the bound fraction after two rounds of affinity purification only when the starting material contained recombinant full-length Knirps, but not from two different negative controls (Fig. 4-C). Gel filtration chromatography supports the hypothesis that Knirps and Rpd3 interact, since both proteins eluted in the same chromatographic fraction (Fig. 4-2B).

Mammalian CtBP 1 was recently found to interact with HDAC 1 (Shi et al., 2003), therefore we tested whether Rpd3 was interacting with Knirps indirectly, through CtBP. Double affinity purification experiments were carried out using embryos overexpressing the N-terminal, CtBP-independent domain of Knirps. Although able to bind (non-specifically) to Ni-NTA agarose beads, Rpd3 was quantitatively lost during the second affinity purification, suggesting that the Rpd3-Knirps interaction is mediated either through the C-terminal domain of Knirps or via CtBP (Fig. 4-5).

Previous analysis of gene expression in *Drosophila* embryos lacking maternal *rpd3* concluded that this deacetylase was not required for short-range repression (Mannervik and Levine, 1999). However, we found that *knirps* genetically interacts with *rpd3* (Fig. 4-6). This interaction is detectable only when a null mutant for each gene is used, and also in this case the effects observed are not as severe as a complete loss of *knirps* function, suggesting that Rpd3 contributes only partially to Knirps activity. In their analysis, Mannervik and Levine used a hypomorphic allele for *rpd3* ($rpd3^{04556}$) which also in our hands did not show appreciable interaction with two different *kni* null alleles. Therefore, it is likely that low Rpd3 levels in the $rpd3^{0455}$ mutant are still sufficient to

maintain Knirps function and only when the Rpd3 level is further reduced (as in the *rpd3*^{del24} mutant), it is possible to detect a genetic interaction with *knirps*. These results suggest that Rpd3 contributes to Knirps-mediated repression. Although additional experiments are required to test the molecular basis behind the genetic interaction we observed between *knirps* and *rpd3*, it is plausible to assume that histone deacetylation is involved in short-range transcriptional repression mediated by Knirps. Whether Rpd3 plays a role in the repression activity of other *Drosophila* short repressors remain to be determined. However, if deacetylation contributes to short-range repression, this chromatin modification must be localized to a single nucleosome, since the range of activity for short range repressors is about 100-150 bp. Yeast Rpd3 is capable of targeting deacetylation to a single nucleosome (Deckert and Struhl, 2002). Therefore, it would be important to determine the acetylation state of the chromatin surrounding Knirps target sites in the presence and absence of the repressor.

Our data supports the hypothesis that Rpd3 is recruited to Knirps indirectly through the corepressor CtBP (Fig. 4-5) and this interaction is important for Knirps-mediated repression (Fig 4-6). However, Rpd3 was previously found to functionally interact with the Groucho corepressor (Chen et al, 1999), which mediates long-range transcriptional repression (Paroush et al., 1994; Fisher and Caudy, 1998). Since both short- and long-range transcriptional repressors interact with Rpd3, what dictates the range of repression activity? Groucho is able to form high-order oligomers (Chen et al., 1998) and oligomerization was recently shown to be essential for Groucho-mediated repression (Song et al., 2004). The ability to polymerize may perhaps allow the corepressors to spread along the chromatin template recruiting histone deacetylases

and/or other chromatin modifying activities to a large domain, whereas short-range repressors may lack the capacity to spread. Alternatively, short- and long-range repressors may recruit specific factor/s which in turn dictates the range of activity over which the repressor is able to function.

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Chapter 5

Conclusions and future directions

Conclusions

The main conclusions of my work are the following:

1) Knirps is able to repress certain targets without the contribution of CtBP. For instance, the *eve* stripe 3/7 enhancer is regulated by Knirps also in a maternal mutant for *CtBP* (Fig. 3-1).

2) CtBP may contribute quantitatively rather than qualitatively to Knirps repression. My data (presented in Chapter 3) support the hypothesis that targets that require low level of Knirps activity can exhibit CtBP-independent repression activity, whereas targets that require higher levels of Knirps activity will need the combined activities of Knirps and CtBP. However, CtBP does not seem to contribute a unique repression activity because a CtBP-dependent target, such as the *eve* stripe 4/6 enhancer, was able to be repressed by the N-terminal, CtBP-independent repression activity of Knirps when provided at higher than normal levels.

3) Knirps does not regulate *even-skipped* by competition for DNA binding. Repression by simple competition has been suggested for many enhancers in *Drosophila* where DNA binding sites for activators and repressors often overlap. However, overexpression of the Knirps DNA binding domain was unable to affect *eve* expression pattern (Appendix C).

4) Recombinant Knirps is present in a complex of approximately 450 kDa (Fig. 4-1), which strongly suggests the existence of additional Knirps cofactors other than the known corepressor CtBP.

5) The histone deacetylase Rpd3 (HDAC 1) interacts biochemically and genetically with Knirps. This interaction is most likely mediated through CtBP, since Rpd3 is lost when the N-terminal, CtBP-independent repression domain of Knirps was used during tandem affinity purification experiments (Fig. 4-5).

My results suggest that histone modifications are likely to play a role in short-range repression. Therefore, the difference between short-and long-range repressors may result in the ability to localize or spread certain patterns of histone modifications. Chromatin immunoprecipitation (ChIP) experiments will help to determine whether histone deacetylation is indeed involved in short-range repression, as my results are suggesting, and whether the deacetylation is localized in the case of short-range repressors and spread over a large chromatin domain in the case of long-range repressors.

I also developed an *in vivo* biochemical purification strategy which is suitable to identify Knirps-interacting factors in an unbiased way. For this purpose, I generated transgenic flies that carry inducible, double-tagged *knirps* transgenes. Upon heat shock, I was able to express recombinant Knirps proteins in the embryo and at the same time the endogenous gene is expressed. Double-tagged Knirps proteins (full-length and N-terminal repression domain) were functional since they were able to repress a number of endogenous Knirps targets in a dose dependent manner (results in Chapter 3). Using these transgenic embryos, I was able to affinity purify recombinant Knirps protein on a small scale and showed that CtBP copurifies together with full-length Knirps at each step of the purification. Using the same method I also confirmed that Rpd3 copurifies with Knirps, and these interactions are strong enough to be retained through two rounds of affinity

purification. The discovery that Rpd3 functionally interacts with Knirps is novel and was previously unnoticed (Mannervik and Levine, 1999).

The importance of multiple repression domains within the same protein is not well understood, but it might be an effective way to tune the repression activity required at different targets along a gradient of repression activity. In regions of the embryo where low levels of repressor are present, effective repression may be achieved by the combined contribution of both repression activities, or by a single one, provided that a sufficient amount of repressor binds to the target.

Future directions

The following are possible future directions that will increase our knowledge of the mechanisms of transcriptional repression mediated by Knirps and possibly other short-range repressors.

1) The double-affinity purification protocol I developed is suitable to identify Knirps-associating factors in an unbiased way. To this aim, I suggest using nuclear extracts from embryos overexpressing recombinant Knirps proteins as the starting material for the tandem affininity purification. I also suggest that the bound complexes at the end of the purification be eluted with triple-FLAG peptide. Concentrating the starting material and specifically eluting Knirps complexes should be sufficient to detect differences in protein profiles between positive and negative controls. These bands would then be excised and submitted to mass spectrometric analysis.

2) The ability to overexpress a transcriptional repressor such as Knirps ubiquitously and

uniformly throughout the embryos will allow us to create genetic switches which could be investigated using chromatin immunoprecipitation (ChIP) assays (Orlando, 2000). Endogenous targets of gap-gene regulators including Knirps are expressed in discrete domains, so that the population of nuclei at the blastoderm stage is a mix of on and off states. Therefore, ChIP performed on wild type embryos will always give a high noise/signal ratio, making the results hard to interpret. A better way to obtain meaningful mechanistic information about short-range repression *in vivo* would be to create embryos that express activator/s and repressor/s in all (or a vast majority) of nuclei. Ubiquitous activation could be achieved by crossing a UAS-*lacZ* reporter line with an *actin5C*-Gal4-activator line, whereas ubiquitous repression could be achieved by heat shock of a transgenic line expressing a heat-shock inducible repressor, such as the lines I generated during my research.

3) ChIP assays could also be used to determine the protein complexes and chromatin modifications associated with short-range repression mediated by Knirps. For instance we could directly test whether Rpd3 is present at the *eve* stripe 4/6 enhancer, where I expected to find this protein based on the results presented in chapter 4 (Fig. 4-6), versus an *eve* enhancer not regulated by Knirps, such as *eve* stripe 5. For these experiments, full-length, double-tagged Knirps is expressed ubiquitously in 2-4 hour embryos and ChIP is performed using α-FLAG (or α-Knirps) and an irrelevant antibody. A sequential IP is performed using α-Rpd3 followed by PCRs using primers specific for *eve* stripe 4/6 and *eve* stripe 5. In this way we can determined whether both proteins are simultaneously present on a Knirps target. ChIP assays using embryos expressing or non-expressing Knirps ubiquitously together with antibodies against deacetylated histones will allow to

determine the state of the chromatin upon Knirps expression. All these experiments could also be carried out overexpressing the N-terminal, CtBP-independent domain of Knirps to determine whether this portion of Knirps mediates repression through similar mechanisms.

4) Production of a highly specific Knirps antisera that recognize endogenous Knirps, will allow to determine the apparent molecular size of endogenous Knirps complexes as well as to perform direct affinity purification experiments from wild-type embryos. The Knirps antiserum I raised against Knirps 75-330 is able to recognize the endogenous protein, but it also cross-reacts with additional factors (Fig. B-1). Purification of the antiserum could be achieved by affinity chromatography using a GST-Knirps75-330 column and eluting the bound antibody with glicine pH 1.9 according to published protocols (Harlow and Lane, 1999).

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Appendix A

Multiple forms of the CtBP corepressor are present during *Drosophila* embryogenesis

Introduction

At the onset of my research project, our laboratory did not have antibodies against CtBP and Knirps. These reagents could be particularly useful for a number of projects including mine; therefore, I decided to generate polyclonal antibodies against both proteins. *Drosophila CtBP* was shown to be maternally-contributed and three mRNAs were dynamically expressed during all stages of development (Poortinga et al. 1998). However, no data were available about the dCtBP protein. To understand the functions of this corepressor it will be important to determine whether the different mRNAs for CtBP give rise to functionally different proteins. To start addressing this question we should determine whether these proteins are present at similar steady-state levels during development and whether they all are nuclear proteins. The original yeast two hybrid screen that identified CtBP as the corepressor for several short-range repressors found that the short isoform of dCtBP (383 aa) interacted with Knirps (Nibu et al., 1998). Whether Knirps was able to interact with other CtBP isoforms was not known. An antibody against CtBP would have been useful for addressing these and other questions.

Therefore, a rabbit polyclonal antiserum was raised against bacterially-expressed, full-length dCtBP protein (aa 1-479). The antiserum was able to recognize both bacterially-expressed and endogenous dCtBP proteins. Three different cross-reacting

bands were detected using *Drosophila* whole-cell extracts and nuclear fractions. A developmental Western using embryonic extracts of different stages showed that all forms are present at similar levels throughout all stages of embryogenesis.

Material and methods

Generation of a polyclonal antibody against dCtBP. Rabbit polyclonal antiserum against *Drosophila* CtBP (dCtBP) was generated against full-length dCtBP and used at 1:20,000 dilution. To generate this antiserum, recombinant full-length, hexahistidine- and FLAG-tagged *Drosophila* CtBP (dCtBP 1-479) from pET15bCtBP$_L$ vector (generated by David Arnosti) was expressed in *E. coli* BL21-CodonPlus™ RIL competent cells (Stratagene #230240) and purified on Ni-NTA agarose beads (Qiagen 30210) according to the manufacture's instructions. dCtBP protein (400 μg) in 0.2 ml PBS buffer (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.2) was mixed with an equal volume of Titermax Gold adjuvant (Sigma T2684) and injected subcutaneously at multiple sites in a New Zealand White female rabbit. Two secondary boosts were performed similarly after 4 and 12 weeks. Serum was prepared according to Harold and Lane (1999).

Western Blotting analysis. Immunoblotting was performed according to standard protocols (Harlow and Lane, 1999) using a tank transfer system (Mini Trans-Blot® Cell, Biorad 170-3930). Immun-Blot™ PVDF membranes (BioRad 162-0177) were used and antibody incubation was in TBST (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) supplemented with 5% (w/v) nonfat dry milk as blocking agent. SuperSignal® West Pico Chemiluminescent substrate (Pierce 34080) was used for detecting horseradish peroxidase (HRP) on immunoblots. Rabbit antiserum against CtBP

(αCtBP) was used at 1:20,000 dilution (any batch of serum collected between 01-05-02 and 04-25-02). Goat anti-Rabbit HRP-conjugated antibody (BioRad 170-0615) was used at 1:10,000 dilution.

Embryo collection. Wild-type *Drosophila* embryos from Canton S flies were collected on apple-juice plates from well-fed, one week old flies using standard techniques. Embryos were collected and aged at 25°C. After a prelaying period of four hours (two changes) to avoid stored embryos, apple-juice plates were changed every hour and embryos were aged for the appropriate amount of time (i.e. 6-7 hour embryos were a 0-1 hour collection aged 6 hours; 16-18 hour embryos were 0-2 hour aged 16 hours).

Embryonic extracts. All extracts were prepared from Canton S embryos. Whole cell extract (WCE) from staged embryos was prepared as described in chapter 4. *Drosophila* standard nuclear extract (DSNE) from 0-12 hour embryos was prepared according to Soeller et al. (1988). *Drosophila* embryonic nuclear extract (DENE) was prepared from 0-12 hour embryos according to Heierman and Pongs (1985), except that KCl was used in place of NaCl. Soluble nuclear fraction (SNF) from 0-12 hour embryos was prepared according to Kamakaka and Kadonaga (1994).

Results

The rabbit polyclonal antiserum raised against $dCtBP_{long}$ was able to recognize bacterially expressed $CtBP_{short}$ (aa 1-386) and $CtBP_{long}$ (aa1-479) proteins (Fig. A-1A, lane 2 and 4, and data not shown). Recombinant $CtBP_{short}$ and $CtBP_{long}$, bearing a hexahistidine repeat and FLAG epitope tag, migrated slightly slower than the endogenous proteins (Fig. A-1A). Two prominent bands were detected using whole-cell *Drosophila* embryonic extracts (WCE) which migrated with the expected mobilities for $CtBP_{short}$ and $CtBP_{long}$ (Fig. A-1A, lane 6). Using *Drosophila* embryonic nuclear extracts prepared according to high-salt extraction protocols (DSNE and DENE), we could detect both forms of dCtBP as well (Fig. A-1A, lane 8-9). However, using nuclear extracts prepared according to a low-salt extraction protocol (SNF), we detected only one form of CtBP ($CtBP_{short}$) and additional high molecular products (around 90 kDa) of unknown identity (Fig. A-1A, lane7). Altogether these results demonstrate that both forms of dCtBP are expressed in embryos and the proteins are present in nuclear fractions. Western blotting analysis of cytosolic and nuclear embryonic fractions using α-CtBP indicate that both forms of CtBP are present at similar levels in the nucleus and cytosol of *Drosophila* embryos (data not shown).

On a high-resolution, 8% SDS-PAGE gel the fast mobility cross-reacting band detected using α-CtBP appears to be a doublet (Fig. A-1C). These two bands could correspond to the two shorter isoforms of dCtBP, which differs by three amino acids (383 and 366 aa). Alternatively, one of these products could correspond to a post-translational modification of $dCtBP_{short}$, possibly a phosphorylation product. Mammalian CtBP was originally identified as a phosphoprotein (Boby et al. 1993) and both forms of *Drosophila*

Figure A-1: Characterization of a polyclonal antiserum raised against $dCtBP_{long}$

A. Polyclonal antiserum raised against $dCtBP_{long}$ recognizes both forms of CtBP in bacterial extracts as well as in *Drosophila* embryonic extracts. Western blot analysis was performed using 1:20,000 dilution of serum from rabbit 208, which was immunized against bacterially expressed $dCtBP_{long}$ protein (01-05-02 bleed). Proteins were resolved on a 10% SDS-PAGE and transferred to a PVDF membrane for Western blotting.
The antiserum recognizes bacterially expressed $CtBP_{long}$ (lane 4) and $CtBP_{short}$ (lane 2 [arrow]; in this case a very faint band is detected due to lower loading). *Drosophila* whole-cell embryonic extract (lane 6) or embryonic nuclear extracts prepared using high-salt extraction protocols (lane 8 and 9), showed bands that correspond to both forms of dCtBP. A low-salt nuclear extract (lane 7) was enriched of $dCtBP_{short}$. The two dCtBP forms have an apparent mass of ~50 and ~40 kDa.
B. Silver-stained 10% SDS-PAGE gel of fractions as in A, showing relative amounts of total proteins.
C. High-resolution gel electrophoresis of crude embryonic *Drosophila* extracts was followed by Western blotting using α-CtBP. The higher mobility band (~40 kDa) is resolved in a doublet (lane 2 represent a lower exposure of lane 1).

A. Lane 1 and 2, ~1ng and ~5 ng of bacterially expressed $dCtBP_{short}$. Lane 3 and 4, ~1 ng and ~5 ng of bacterially expressed $dCtBP_{long}$. Lane 5, 10 µl of extract from Schneider cell line 2. Lane 6-9, ~40 µg of total protein from 0-12 hour embryonic *Drosophila* nuclear extracts prepared according to different protocols. Lane 6, soluble nuclear fraction (Kamakaka et al. 1991). Lane 7, *Drosophila* standard nuclear extract (Soeller et al., 1988). Lane 9, *Drosophila* embryonic nuclear extract (Heiermann and Pongs, 1985).

B. Lane 1 and 2, ~5 ng and ~50 ng of bacterially expressed $dCtBP_{short}$. Lane 3 and 4, ~5 ng and ~50 ng of bacterially expressed $dCtBP_{long}$. Lane 5, 20 µl of extract from Schneider cell line 2. Lane 6-9, ~2 µg of total protein from 0-12 hour embryonic *Drosophila* nuclear extracts prepared according to different protocols.

C. Lane 1, 50 µg of total protein from whole-cell embryo extracts. Lane 2 is a light exposure of the higher mobility product. Proteins were separated on a 15-cm long, 8% SDS-PAGE gel.

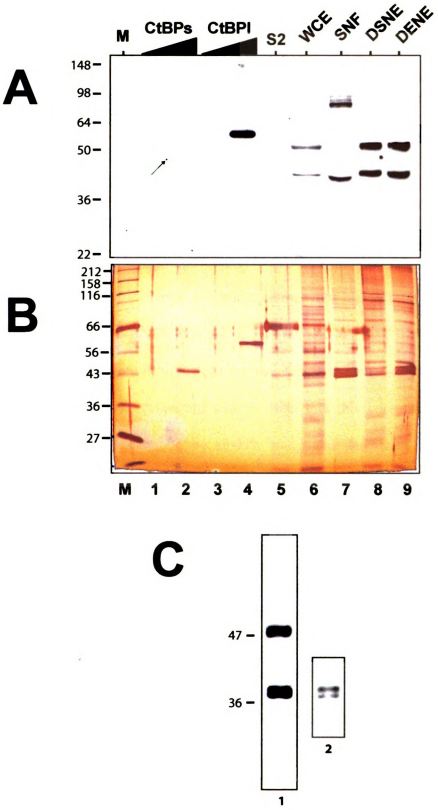

Figure A-1: Characterization of a polyclonal antiserum raised against dCtBP$_{long}$.

CtBP have a number of predicted phosphorylation sites. A search for putative phosphorylation sites using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) of the long form of dCtBP (479 aa) revealed 17 predicted phosphorylation sites (11 serine, 5 threonine and 1 tyrosine).

Next, I performed Western blotting analysis using α-dCtBP and wild-type (Canton S) crude embryonic extracts prepared from staged embryos (Fig. A-2). Each sample represented a 60-minutes collection aged for different amount of time to cover the entire duration of embryogenesis. Two forms of dCtBP of approximately equal intensities were detected throughout all stages of embryogenesis. The higher mobility doublet is not resolved on a 10% SDS-PAGE minigel and I referred to these isoforms as $CtBP_{short}$ throughout this dissertation. Both forms are present from the onset of embryogenesis (0-2 hours; Fig. A-2, lane 1-2) consistent with the fact that this corepressor is maternally contributed. $CtBP_{long}$ appeared to be slightly more abundant than the shorter forms ($CtBP_{short}$) between 2 and 5-6 hours (lane 3-6), whereas it was less abundant than $CtBP_{short}$ towards the end of embryogenesis (lane 9-14).

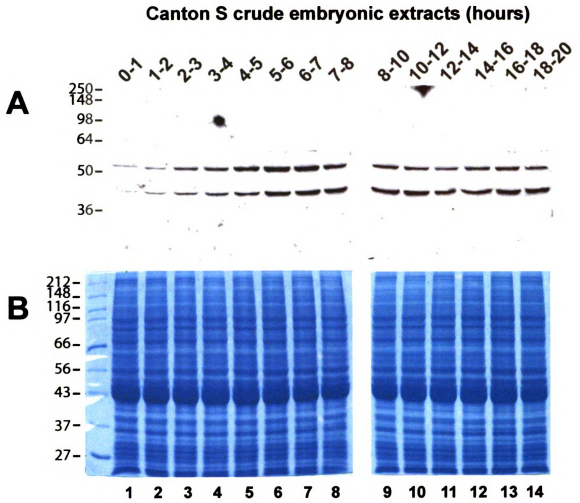

Figure A-2: Developmental Western of *Drosophila* embryonic extracts using α-CtBP antiserum.

A. Both forms of CtBP are expressed at similar levels throughout all stages of *Drosophila* embryogenesis. Embryonic whole-cell extracts were prepared from staged Canton S embryos of the indicated age (expressed in hours after egg deposition). 40 μg of total protein were resolved on a 10% SDS-PAGE gel, transferred to PVDF membranes and Western blotting was performed using polyclonal α-CtBP antiserum (1:20,000 dilution).
B. Coomassie-stained gels with the same amount of material used in A showing equal loading.

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Appendix B

Characterization of *Drosophila* transgenic lines expressing full-length, double-tagged Knirps

Introduction

As a first step in the long-term goal of understanding the mechanisms of transcriptional repression mediated by the *Drosophila* Knirps protein, I proposed to biochemically identify Knirps-interacting proteins. Initial attempts to identify proteins from embryonic nuclear extracts that interact with GST-Knirps chimeric fusions were not successful. Fusion proteins containing the entire repression domain of Knirps were insoluble and fusions to the N-terminal repression domain did not specifically interact with any proteins visible on silver stained gels (data not shown). Possible targets of Knirps may not recognize bacterially expressed GST-Knirps, or might not readily exchange from endogenous complexes. Therefore, I developed a protocol for the affinity purification of Knirps complexes from embryos overexpressing double-tagged versions of Knirps. To this aim, I generated transgenic flies carrying stably integrated *hsp70-knirps* transgenes that express recombinant full-length Knirps in the embryo at the same time as endogenous Knirps expression (see Fig. 3-2). This appendix reports the initial characterization of *hsp70-knirps1-429* transgenic lines, which are able to express double-tagged, full-length Knirps protein upon heat-shock. Five minutes of heat shock were found to be sufficient for detecting the recombinant protein. Induction could be achieved starting from 2-3 hours old embryos and, upon full induction, recombinant Knirps protein persisted in the embryo for approximately 2 hours.

In parallel, I generated a polyclonal antiserum against Knirps. This reagent would allow us to determine the size of endogenous Knirps complexes, the relative abundance of this transcription factor during different developmental stages, and to directly purify Knirps complexes from wild-type embryos.

Material and Methods

Generation of transgenic flies carrying inducible, double-tagged Knirps genes. See Chapter 3 for a detailed description.

Heat-shock experiments. Transgenic embryos from *hsp70-knirps1-429* of the indicated age were collected on apple-juice plates at room temperature (22-23°C). To induce expression of full-length Knirps proteins, transgenic embryos were incubated for the indicated time at 38°C in a 10-liter water bath to ensure rapid and even heating. After induction, embryos were immediately dechorionated and lysed.

Crude embryo lysate preparation. See Chapter 3 for a detailed description.

Dose-dependent induction of full-length Knirps. 1-5 hour old embryos from *hsp70-knirps1-429.3* line were heat-shocked for increasing amount of time (5-120 minutes) at 38°C. After heat shock, embryos were immediately dechorionated and lysed within 15 minutes from the end of induction.

Induction of full-length Knirps in staged embryos. Embryos from *hsp70-knirps1-429.3* line were collected at room temperature (22-23°C) for one hour and allowed to age for 0, 1, 2, 3, 4, 5, or 6 hours at 25°C (final age from 0-1 to 6-7 hour). To ensure the proper and uniform age of the embryos, apple-juice plates were changed three times over a period of 4 hours before starting the embryo collection. Each collection of embryos was

heat-shocked for 30 minutes at 38°C, immediately dechorionated and lysed within 15 minutes from the end of the heat-shock.

Turnover of recombinant Knirps protein. To determine how long full-length, double-tagged Knirps protein persists in the embryo after induction, 1-5 hours embryos from *hsp70-knirps1-429* (line 3) were heat-shocked for 30 minutes at 38°C and recovered at room temperature for 10, 30, 45, 60, 90, 120, or 180 minutes before sonication. After the heat shock embryos were transferred to a water bath at room temperature to ensure rapid return to normal temperature.

***In situ* antibody staining.** Embryos were fixed and stained according to previously published protocols (Ashburner, 1989), using the Vectastain® Universal Elite ABC kit (Vector Laboratories, Burlingame, CA). Polyclonal antibody against Knirps (#566) was kindly provided by John Reinitz and used at 1:25 dilution. FLAG M2 monoclonal antibody (4.9 mg/ml; F3165, Sigma) was used at 1:4,000 dilution.

Generation of a polyclonal antiserum against Knirps. Rabbit polyclonal antiserum was generated against the N-terminal, CtBP-independent repression domain of Knirps (Knirps 75-330). Hexahistidine- and FLAG-tagged Knirps 75-330 from pET15bKni75-330F was expressed in *E. coli* BL21 CodonPlus™ RIL competent cells (Stratagene #230240) and purified on Ni-NTA agarose beads (Qiagen 30210) according to the manufacturer's instruction. . Knirps 75-330 protein (400 μg) in 0.2 ml PBS buffer (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.2) was mixed with an equal volume of Titermax Gold adjuvant (Sigma T2684) and injected subcutaneously at multiple sites in a New Zealand White female rabbit. Two secondary boosts were

performed similarly after 7 and 16 weeks. Serum was prepared according to Harold and Lane (1999).

Results

Polyclonal antiserum against Knirps recognizes the endogenous protein.

The rabbit antiserum raised against Knirps 75-330 was able to recognize bacterially expressed Knirps75-330 and Knirps1-429 proteins (Fig. B-1A, lane 1-2). It was also able to recognize recombinant Knirps proteins expressed in the embryo (Fig. B-1A, lane 7-9) and endogenous Knirps from embryonic nuclear extracts (Fig. B-1A, lane 10). However, the antiserum was unable to recognize endogenous Knirps from crude lysates prepared from 2-4 hour embryos, probably because of the low level of the endogenous repressor (Fig. B-1A, lane 4-6). These results were compared with those obtained with a Knirps antibody (#566) obtained from John Reinitz and successfully used in *in situ* antibody staining (Fig. B-4A and B). The two antibodies gave similar results with bacterially expressed Knirps, but the antiserum I raised appeared to be able to detect all three recombinant Knirps protein tested, whereas #566 detected only recombinant full-length Knirps. However, additional cross-reacting bands were present using both antibodies, which probably correspond to non-specific products. After purification (possibly using GST-Knirps beads), the antiserum I raised will likely be a useful reagent for immonoaffinity purification of endogenous Knirps complexes.

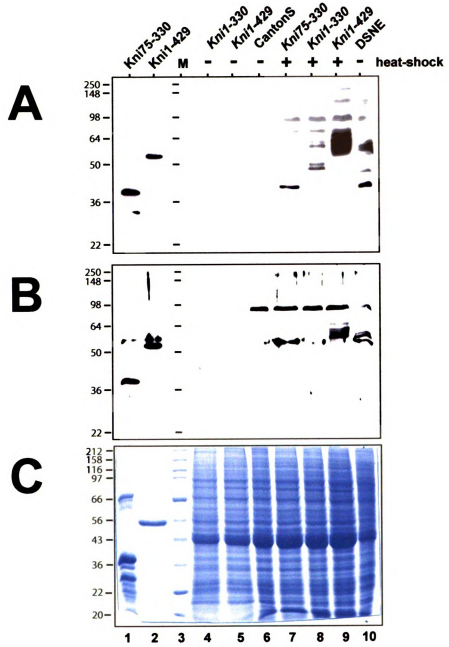

Figure B-1: Characterization of a polyclonal antiserum raised against Knirps.

A. Western blotting using of a rabbit polyclonal antiserum raised against Knirps 75-330 (1:20,000 dilution of serum 04-26-02 from rabbit 209). **B.** Western blotting using rat αKnirps (1:5,000 dilution). **C.** Coomassie stained gel of an identical gel as in A-B.
Lane 1-2, 10 µl of bacterially expressed and affinity purified Knirps 75-330 (lane 1) and Knirps 1-429 (lane 2). Lane 4-10, 50 µg of total proteins from crude lysates of 2-4 hour embryos (lane 4-9) and nuclear extract (lane 10). Lane 6, Canton S embryos. Lane 4 and 7, *hsp70-knirps1-330.1* embryos. Lane 5 and 9, *hsp70-knirps1-429.3* embryos. Lane 7, *hsp70-knirps75-330. 10* embryos. (-) before and (+) after 30 minutes of heat shock.

Generation and characterization of *Drosophila* transgenic lines expressing full-length, double-tagged Knirps.

Ectopic expression of a transcription factor may result in lethality. Therefore, I decided to place the double-tagged *knirps* transgene under the inducible *hsp70* promoter using the injection vector pCaSpeR-hs. In some cases, transgenes based on this vector did show leaky expression at 25°C (Steller and Pirrotta, 1985). Therefore, to reduce the likelihood of basal Knirps expression flies were always kept at temperatures of 22-23°C or lower. For *hsp70-knirps1-429*, 32 transgenic lines from male survivors were obtained by injection of approximately 1000 embryos, suggesting that the transgene does not have a deleterious effect on the ability to generate transgenic animals. Nine lines were further analyzed for their ability to induce recombinant Knirps upon heat-shock. The results for seven lines are reported in Fig. B-2A. All lines did express a cluster of bands of approximately the expected molecular mass for full-length, double-tagged Knirps (~55 kDa). Expression was completely heat-shock-dependent with no cross-reacting bands detected from embryos that were not heat-shocked (Fig. B-2A, line 8 and data not shown). The majority of lines expressed similar steady-state levels of Knirps and one line (*hsp70-knirps1-429.3*) was chosen for all further experiments.

Heat-shock induction in *Drosophila* is achievable during most of the life cycle except during late oogenesis and early embryogenesis (Parsell and Lindquist, 1994). Early *Drosophila* embryos are refractory to heat shock as a result of nuclear exclusion of the heat shock transcription factor dHSF. From cycle 13 onward (~2 hours after egg deposition) the transport factor dKap-α3 is present and dHSF is localized within the nucleus thus allowing the embryo to respond to heat-shock (Fang et al., 1991). In the

Figure B-2: Characterization of *Drosophila* transgenic lines expressing full-length, double-tagged Knirps proteins.

A. Heat-shock induction of recombinant, full-length Knirps in different transgenic lines. (Top panel) Western blotting analysis using α-FLAG M2 monoclonal antibody of whole-cell extracts from 0-12 hour embryos carrying *hsp70-knirps1-429* transgene (line 1-7) after 30 minutes of heat-shock at 38°C (+ hs). Lane 9, ~5 ng of bacterially expressed and affinity purified $dCtBP_{long}$ (used for size comparison). (Bottom panel) Coomassie blue stained gel with identical amounts of embryonic extract used in the Western blot shown on the top.

B. Heat-shock induction of recombinant, full-length Knirps in transgenic embryos of different stages. (Top panel) Western blotting analysis using α-FLAG M2 monoclonal antibody of whole-cell extracts from embryos carrying *hsp70-knirps1-429* transgene (line 3) of the indicated age (in hours after egg deposition). See text for details.
(Bottom panel) Coomassie blue stained gel with identical amounts of embryonic extract used in the Western blot shown on the top.

C. Dose-dependent induction of recombinant, full-length Knirps.
1-5 hour old embryos from *hsp70-knirps1-429* (line 3) were heat-shocked for increasing amount of time (5-120 minutes) at 38°C. (Top panel) Western blotting analysis using α-FLAG M2 monoclonal antibody of whole-cell extracts from transgenic embryos after heat shocks of variable length. (Bottom panel) Coomassie blue stained gel with identical amounts of embryonic extract used in the Western blot shown on the top.

All Western blotting were done using α-FLAG M2 monoclonal antibody at 1:20,000 dilution.
Lane 1-27 (except 9), whole-cell *Drosophila* embryonic extract from transgenic lines carrying *hsp70-kni1-429* transgenes before (-) and after (+) heat shock. ~50 μg of total protein/lane.

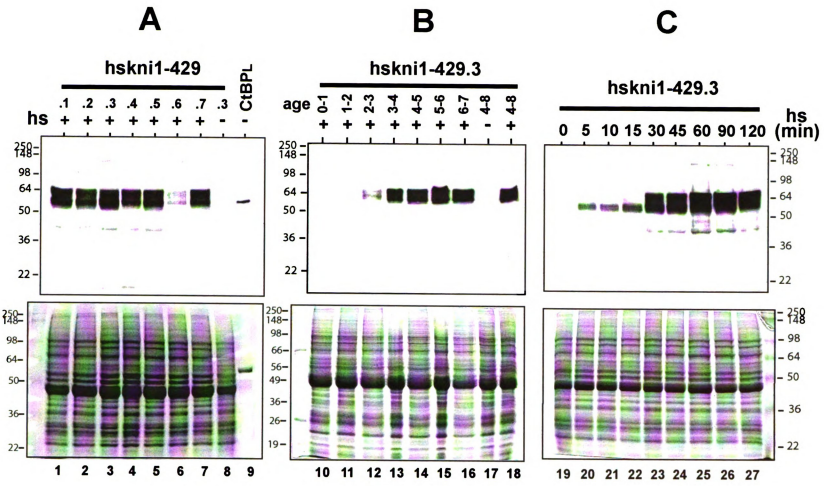

Figure B-2: Characterization of *Drosophila* transgenic lines expressing full-length, double-tagged Knirps proteins.

early embryo, Knirps is maximally expressed between 2 and 4 hours after egg deposition. For the identification of physiologically relevant Knirps-interacting factors using full-length, double-tagged Knirps as affinity matrix, it would be important to achieve expression at the same or overlapping time as the endogenous *knirps* expression. Therefore, I tested how early the induction of recombinant Knirps proteins could be achieved, by heat-shocking embryos of different ages and performing Western blotting using α-FLAG antibody. Induction of full-length Knirps was possible as early as 2-3 hours post-fertilization (Fig. B-2B). *In situ* hybridization experiments using digoxigenin-UTP-labeled antisense mRNA for *knirps* also indicate that the induction of *hsp70-knirps* transgenes can be achieved at the blastoderm stage, when the endogenous gene is maximally expressed (Fig. 3-2B). A dose-dependent induction indicated that 5 minutes of heat shock were sufficient for detecting the recombinant protein, and 30 minutes of heat-shock were sufficient to reach the maximum level of recombinant Knirps expression (Fig. B-2C).

To determine the *in vivo* stability of recombinant, full-length Knirps after a pulse of induction, transgenic embryos carrying *hsp70-knirps1-429* were subjected to 30 minutes of heat-shock at 38°C, followed by a recovery period at room temperature of variable duration before the embryos were lysed. Recombinant Knirps proteins were detected using α-FLAG M2 monoclonal antibody. Following a pulse of induction that expresses near maximum levels of Knirps, the recombinant protein was detectable in embryos up to ~2 hours after the end of the heat shock (Fig. B-3).

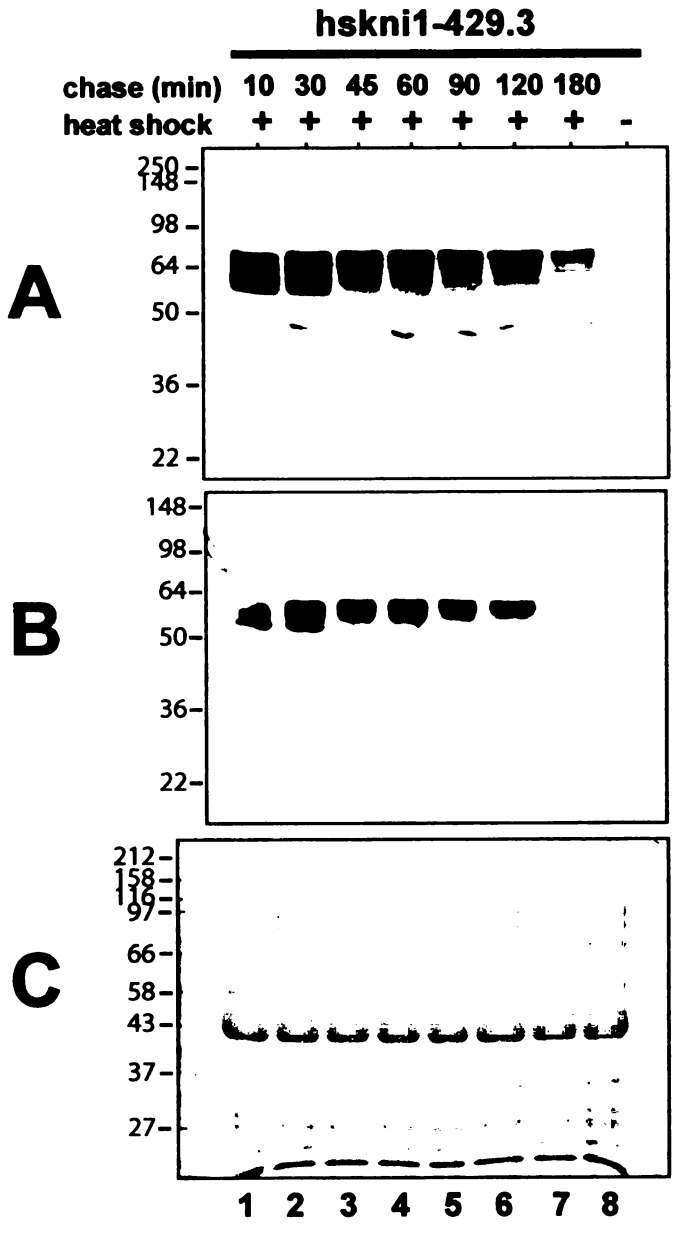

Figure B-3: Recombinant full-length Knirps persist in the embryo for ~2 hours after a pulse of induction.

Transgenic embryos from *hsp70-knirps1-429* (line 3) were heat-shocked for 30 minutes at 38°C and recovered at room temperature (22-23C) for variable time before lysis. Whole-cell extracts were prepared after the indicated recovery time (from 10 to 180 minutes).

A, B. Western blotting analysis using α-FLAG M2 antibody (1:20,000 dilution) of whole-cell extracts from transgenic embryos expressing full-length Knirps after 30 minutes of heat-shock and 10-180 minutes of recovery. The picture in panel B is a lighter exposure of the same blot used in A.

C. Coomassie blue stained gel loaded with identical amount of extracts as in A.

To determine whether recombinant, full-length Knirps is a nuclear protein, *in situ* antibody staining were performed using embryos from *hsp70-knirps1-429.3*, before and after induction. After heat shock induction, recombinant Knirps was expressed ubiquitously throughout the embryo (Fig. B-4E compare with C). Higher magnification photographs of the surface of the embryo shows that, upon Knirps expression, the staining is present in a punctuate pattern similar to the endogenous Knirps protein pattern (Fig. B-4F compare with B). Uninduced embryos showed background levels of staining and the pattern was completely uniform (Fig. B-4C and D). Embryonic nuclear extracts were prepared from embryos over expressing recombinant, full-length Knirps and the protein was found predominantly in the nuclear fraction (data not shown). Finally, the repression activities of recombinant Knirps proteins (full-length Knirps and the N-terminal, CtBP-independent repression domain: Knirps 1-330) on endogenous Knirps targets including *eve*, *hb*, *run*, *h*, and *ftz* clearly prove that at least some of the recombinant protein is imported into the nucleus and is able to repress endogenous Knirps targets (Figs. 3-3 and 3-6).

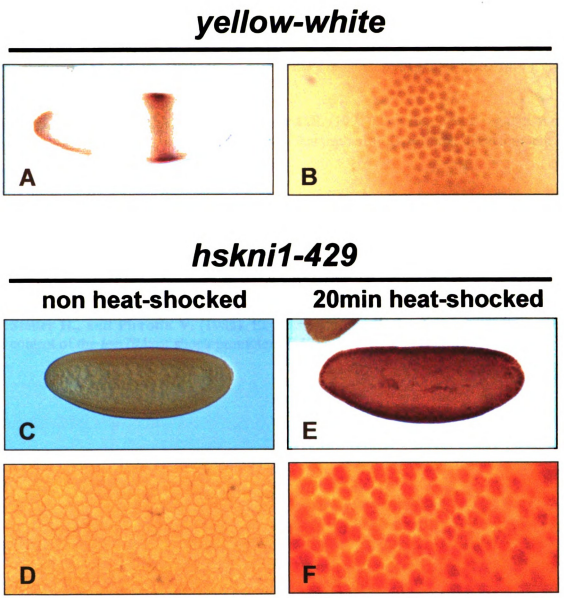

Figure B-4: Recombinant full-length Knirps is expressed ubiquitously and in a punctuate pattern in blastoderm embryos.

A-B. *In situ* antibody staining of a blastoderm *yellow-white* embryo using α-Knirps antiserum (antibody #566 from John Reinitz). A, 20X. B, 100X.
C-F. *In situ* antibody staining using α-FLAG M2 monoclonal antibody of transgenic embryos from *hsp70-knirps1-429* line 3 that were either heat shocked for 20 minutes (E and F) or kept at room temperature (C and D). C and E, 20X. D and F, 100X.

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Appendix C

Additional experiments not included in chapter 3

Introduction

This appendix documents several experiments that were suggested by three anonymous reviewers and whose results were referenced in chapter 3 as "data not shown".

The first question raised by the reviewers of my original manuscript[7] regarded whether the effects observed on *even-skipped* (*eve*) and other Knirps targets upon overexpression of recombinant Knirps proteins represented direct or indirect effects. Following a heat-shock treatment of increasing durations (from 5 to 30 minutes at 38C), the embryos were allowed to recovered for 30 minutes at room temperature prior to fixation, allowing in principle sufficient time for indirect effects to occur, through changes in the expression of other genes. Therefore, it was suggested to repeat the heat-shock experiments without recovery time and determine whether the expression pattern of *eve* looked similar to the one shown in Fig. 3-3. The heat-shock experiments were conducted without recovery time and the results obtained (Fig. C-1) was essentially the same as those obtained with a 30 minutes recovery period, suggesting that we are probably looking at direct effects.

[7] Now published as: **Struffi P., Corado M., Kulkarni M., and Arnosti D.N.** (2004). Quantitative contributions of CtBP-dependent and –independent activities of Knirps. Development **131**: 2419-2429.

An additional question concerned whether some of the repression activities observed upon overexpression of recombinant Knirps proteins were mediated by direct competition of the DNA binding domain (DBD) of Knirps with activator/s binding sites. Overexpression of full-length Knirps or the N-terminal, CtBP-independent repression domain of Knirps resulted in repression of a subset of *even-skipped* (*eve*) regulatory elements which was attributed to the different potency of the two repression domains of Knirps. However, both proteins have an intact DNA binding domain (DBD). Therefore, some (or all) of the repression activities observed could in principle result from direct competition of the Knirps DBD with overlapping DNA binding sites for activator/s. To test this hypothesis, I generated transgenic lines expressing the DNA binding domain and nuclear localization signal (NLS) of Knirps (Knirps1-105). As well as for the other recombinant Knirps proteins expressed in embryos, Knirps 1-105 is double-tagged and expressed under the *hsp70* promoter. I measured heat-shock induction of this protein by Western blotting analysis, and quantitated the level of protein expression with respect to the other forms of Knirps. A transgenic line expressing the DBD of Knirps at comparable levels as for the full-length (Knirps 1-429) and the N-terminus domain of Knirps (Knirps 1-330) used in chapter 3 was selected. Knirps 1-105 was induced at increasing levels and its effects on *eve* expression pattern were determined. The DBD of Knirps was unable to mediate repression of *eve*, including the most sensitive Knirps target (*eve* stripe 3/7), even under high level of protein expression (Fig. C-2). These results demonstrate that Knirps represses *eve* by means other than direct competition for activator binding sites. These results also suggest that the repression activity measured with the N-terminal

region of Knirps (Knirps 1-330) is contributed by the previously identified CtBP-independent repression domain, and not through the Knirps DBD.

To assess the contribution of the CtBP-dependent repression domain of Knirps alone to the overall Knirps repression activity, I generated transgenic flies overexpressing the previously identified minimal CtBP-dependent repression domain (Knirps 202-358; Keller et al., 2000) fused in frame with the Knirps DBD and NLS (Knirps 1-105). The expression pattern of *eve* was unaffected upon overexpression of the CtBP-dependent domain of Knirps. However, Western blotting analysis of transgenic embryos overexperissing Knirps1-105/202-358 showed an apparent proteolysis between the two domains and therefore the lack of repression is not informative (Fig. C-3).

Materials and Methods.

Heat-shock experiments. See Chapter 3 for details. Heat-shock inductions of *hsp70-knirps1-429* (line 3) and *hsp70-knirps1-330* (line 1) embryos were performed without recovery. 10-12 minutes were required to process the embryos from the end of the heat shock induction to the start of fixation/sonication.

Plasmid construction. See Chapter 3 for details regarding contruction of Knirps-1-105. To test the contribution of the CtBP-dependent repression domain alone to the overall repression activity of Knirps, I generated transgenic flies carrying the minimal CtBP-dependent repression domain of Knirps (residues 202-358; Keller et al., 2000) fused in frame with the Knirps DNA binding domain (residues 1-105). Knirps 202-358 was amplified using primers DA776 (5'-CCG CGC TCT AGA GCT GCC GCT GCA GCG GCT TCT GCT GCC-3') and DA775 (5'-CGG CCT CTA GAC ACC TCC ACT

TCT TGA TCC TCG GAG CC-3') and pBS-N741 as DNA template. The PCR product was restricted with *Xba*I and cloned into Knirps1-105 restricted with *Xba*I and dephosphorylated. The correct orientation of the insert was determined by PCR. This cloning strategy introduces two additional codons (Ser and Arg) between residues 105 and 202 of Knirps. The final vector consist of pCaSpeR-hs(H2xF) (Struffi et al., 2004) containing a *KpnI-XbaI* insert corresponding to Knirps1-105/202-358. The insert was sequenced to confirm the correct sequence and frames.

Results

Repression of *even-skipped* enhancer elements by ectopic Knirps is a direct effect.

Ectopic expression of recombinant Knirps proteins (full-length and N-terminus, CtBP-independent repression domain) in blastoder embryos led to differential repression of *even-skipped* (*eve*) and other Knirps targets (Fig. 3-3 and 3-6). In the initial experiments, after heat-shock inductions of variable duration, the embryos were allowed to recover for 30 minutes prior to fixation. This window could allow sufficient time for indirect effect to occur, through changes in the expression of other genes. As suggested by an anonymous reviewer, all heat-shock experiments were also conducted without recovery time to determine whether the patterns of repression on *eve* would differ.

For recombinant full-length Knirps (1-429), after 10 minutes of heat shock with no recovery time, *eve* stripe 3 and 7 were partially repressed (Fig. C-1A), similar to the results reported in Fig. 3-3. Lengthening the heat shock to 15 minutes led to complete loss of *eve* stripe 3 and 7 expression with weakening of the next most susceptible target, *eve* stripe 4 and 6 (Fig. C-1B). After 20 minutes of heat shock with no recovery, all *eve*

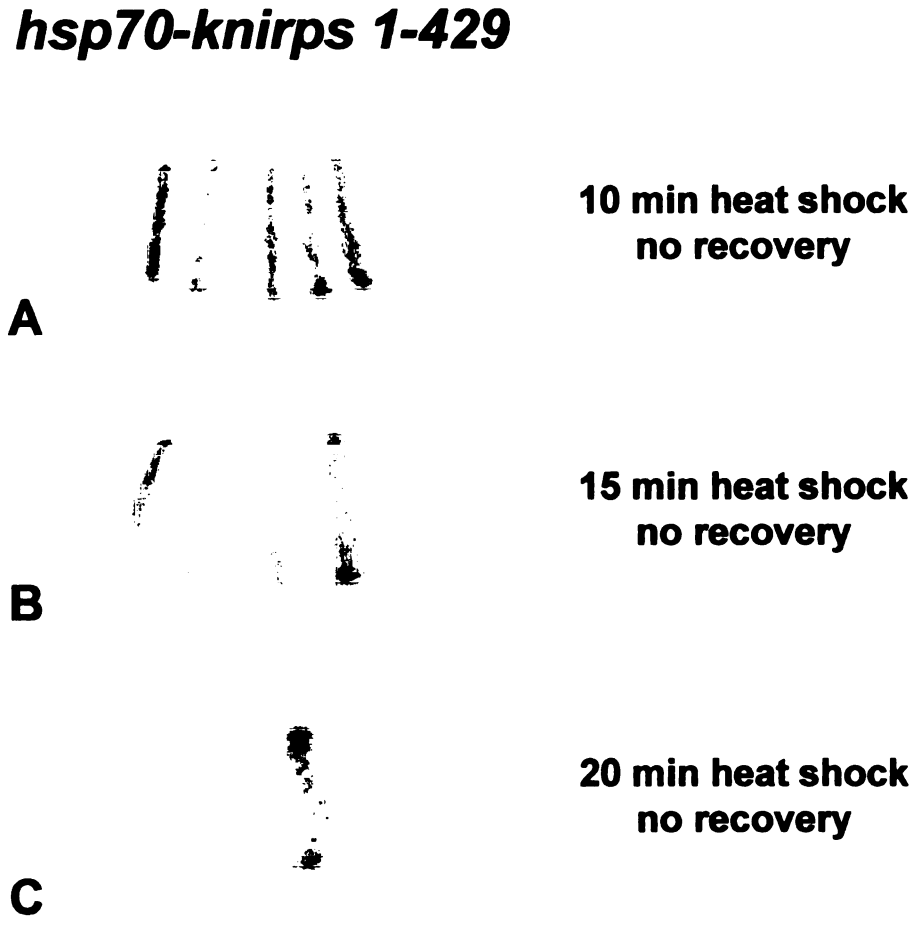

Figure C-1: Direct repression of *even-skipped* (*eve*) by ectopic full-length Knirps.

Embryos carrying an *hsp70-knirps1-429* transgene were heat-shocked for 10 (A), 15 (B), or 20 minutes (C) at 38°C and immediately fixed for *in situ* hybridization (no recovery), to reduce indirect effects.

In situ hybridization was performed using digoxigenin-UTP-labeled antisense mRNA probe to *eve*. Surface views of blastoderm embryos are shown. Embryos are oriented anterior towards the left, dorsal side upwards.

stripes except *eve* stripe 5 were repressed, similar to the finding shown in Fig. 3-3, except that residual staining for the other stripes remains, presumably because the *eve* mRNA has had less time to turn over (Fig. C-1C). The order of disappearance of the *eve* stripes is in all cases identical, indicating that the differential sensitivity of *eve* enhancers to misexpressed Knirps identified previously is similar.

The pattern of repression of *eve* stripe enhancers obtained by misexpression of Knirps 1-330 was also similar to that reported in Fig. 3-3, but residual *eve* mRNA was detectable. With no recovery after heat shock, *eve* stripe 4 and 6 appeared to be less effectively repressed that in Fig. 3-3 (data not shown). However, the minimal *eve* stripe 4/6-*lacZ* reporter was repressed by Knirps 1-330 in experiments performed with no recovery (Fig. 3-4K and L). In conclusion, it appears that the results shown in Fig. 3-3 are essentially the same as those obtained using a protocol without the recovery period. Therefore, the differential repression of *eve* stripe elements observed upon misexpression of recombinant Knirps proteins is likely to be a direct effect.

Competition for DNA binding does not play a role in Knirps-mediated repression of *eve* stripe elements.

Some of the repression activities observed upon misexpression of recombinant Knirps proteins on *even-skipped (eve)* stripe elements could be mediated by the DNA binding domain of Knirps directly competing for activator binding sites. To test the relevance of direct competition in *eve* regulation by Knirps, I created transgenic embryos that express double-tagged Knirps 1-105 upon heat shock. Knirps 1-105 contains the previously defined DNA binding domain (DBD) of Knirps (aa 1-74) as well as the

Figure C-2: Competition for DNA binding does not play a role in Knirps-mediated repression of *even-skipped* (*eve*) stripe elements.

A. Quantitation of proteins expressed from *hsp70-knirps1-105*.
Transgenic embryos (from line 11a) were heat-shocked at 38°C for increasing amounts of time and immediately lysed. Crude embryonic extracts (50 μg of total protein/lane) were resolved on a 4-20% SDS-PAGE gel (Gradipore, NG21-420) and recombinant Knirps proteins were detected by Western blotting analysis using α-FLAG M2 monoclonal antibody (1:10,000 dilution). Protein levels were quantitated relative to the level of Knirps 1-330 and are reported on the bottom in percentage (100% being the level of Knirps 1-330 after 20 minutes of heat shock). Asterisk marks a nonspecific cross-reacting protein that was also present in lysates from non-transgenic embryos (data not shown).

B. *eve* expression pattern in transgenic embryo misexpressing Knirps 1-105.
Transgenic embryos (from *hsp70-knirps1-105.11a*) were heat-shocked for 30 minutes at 38°C and immediately fixed. *In situ* hybridization was performed using digoxigenin-UTP-labeled antisense mRNA probe to *eve*. Surface view of a blastoderm embryo oriented anterior towards the left, dorsal side upwards.

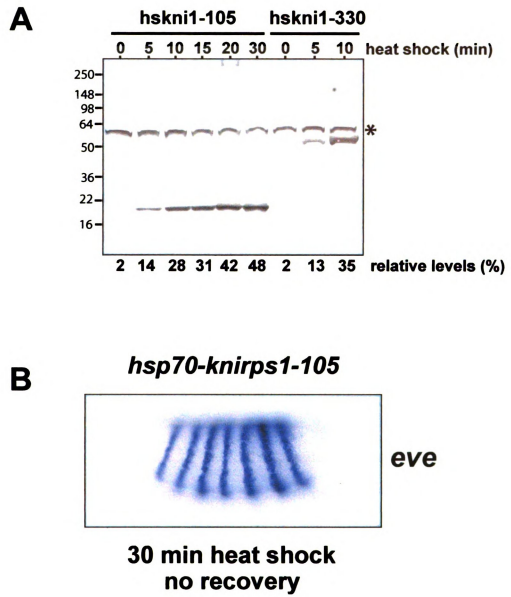

Figure C-2: Competition for DNA binding does not play a role in Knirps-mediated repression of *even-skipped* (*eve*) stripe elements.

putative nuclear localization signal (aa 75-93; Gerwin et al., 1994). Dose-dependent induction of Knirps 1-105 protein was monitored by Western blotting and quantitated with respect to the other forms of Knirps as described in chapter 3. Quantitation of Western blots indicated that transgenic embryos from *hsp70-knirps 1-105* (line 11a) expressed the DBD of Knirps at levels higher than the full-length protein, and somehow lower than that of Knirps 1-330 (Fig. C-2A). Double-tagged Knirps 1-105 was unable to mediate repression of *even-skipped*, even of the most sensitive *eve* stripe 3/7 enhancer when embryos were heat-shocked up to 30 minutes (Fig. C-2B). This result suggests that the CtBP-independent repression activity of Knirps is not simply a measure of residual direct competition activity. The results are consistent with the model that Knirps represses *eve* by means other than competition for activator binding sites.

Transgenic embryos misexpressing the CtBP-dependent repression domain of Knirps do not affect *eve* expression pattern.

To test the contribution of the CtBP-dependent repression activity of Knirps relative to the repression activity of the full-length protein, I created lines carrying a transgene that express under heat shock conditions a fusion protein between the Knirps DBD and the minimal CtBP-dependent repression domain of Knirps (Knirps1-105/202-358). Misexpression of the CtBP-dependent domain of Knirps did not alter *eve* expression pattern (Fig. C-3B) although a similar Gal4 fusion protein (Gal4-Knirps 202-358) was able to repress an *eve* stripe2/3-lacZ reporter using an *in vivo* repression assay (Keller et al., 2000). Unlike other recombinant Knirps proteins tested, Knirps 1-105/202-358 was subjected to considerable proteolysis *in vivo* (Fig.C-3A).

Figure C-3: Misexpression of the CtBP-dependent repression domain of Knirps does not affect *even-skipped* (*eve*) expression pattern.

A. Quantitation of proteins expressed from *hsp70-knirps1-105/202-358*.
Transgenic embryos (from line 1a) were heat-shocked at 38°C for increasing amounts of time and immediately lysed. Crude embryonic extracts (50 μg of total protein/lane) were resolved on a 4-20% SDS-PAGE gel (Biorad 161-1105) and recombinant Knirps proteins were detected by Western blotting analysis using α-FLAG M2 monoclonal antibody (1:10,000 dilution). Asterisk marks a nonspecific cross-reacting protein that was also present in lysates from non-transgenic embryos (data not shown).

B. *eve* expression pattern in transgenic embryo misexpressing Knirps 1-105/202-358.
Transgenic embryos (from *hsp70-knirps1-105.1a*) were heat-shocked for 30 minutes at 38°C and immediately fixed. *In situ* hybridization was performed using digoxigenin-UTP-labeled antisense mRNA probe to *eve*. Surface view of a blastoderm embryo oriented anterior towards the left, dorsal side upwards.

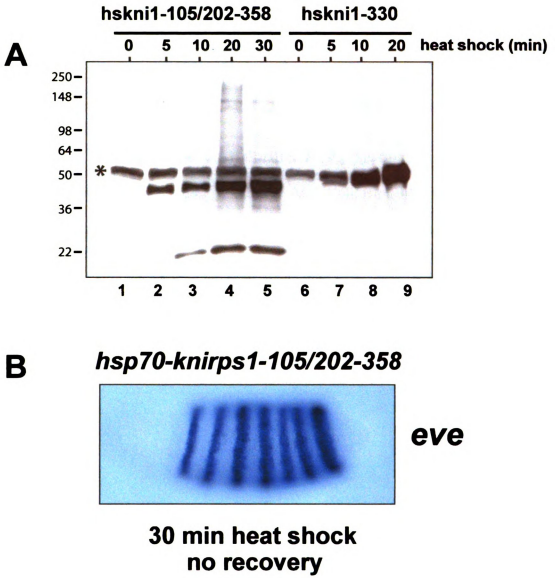

Figure C-3: Misexpression of the CtBP-dependent repression domain of Knirps does not affect *even-skipped* (*eve*) expression pattern.

Therefore, protein stability might be a factor that explains the lack of repression activity displayed by the CtBP-dependent domain of Knirps.

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