Chapter 5- *deflated* interacts genetically with known cell proliferation and cell signalling mutants

5.1 Introduction

As described in the previous chapter, *deflated* is expressed in proliferating and endoreplicating cells and *deflated* mutant phenotypes are consistent with a defect in the regulation of cell proliferation, including the inability to progress normally through the cell cycle. The conserved protein motifs identified in DEFLATED and the mild wing and bristle defects observed in the *deflated* transheterozgous adults suggest that *deflated* may have a role in signalling and that its effects on the cell cycle may be indirect. The aims of the experiments described in this chapter were to further explore *deflated's* role in cell proliferation and cell signalling by examining genetic interactions with other known cell proliferation and cell signalling mutants. A genetic approach was taken for two reasons. One, genetic studies allow for the identification of genetically related genes, particularly those that act in the same biological pathway, and can reveal how biological pathways relate to one and other. Two, the type of role that a gene product plays within these pathways can also be determined, such as whether it acts as an activator or an inhibitor.

This chapter examines the genetic interactions between overexpressed *deflated* and other cell proliferation and signalling genes. Two well characterised developmental systems were studied, the eye and the wing. Both have been extensively used to understand the developmental control of cell proliferation and cell signalling. The eye was used to further explore the role *deflated* plays in regulating S-phase and the wing to identify genes that interact genetically with *deflated*.

The adult eye comprises approximately 800 ommatidia arrayed in an orderly fashion and whose development is well understood (Dickson and Hafen, 1993; Wolff and Ready, 1993). Each ommatidia is made of eight photoreceptor cells and the accessory cone, pigment, and bristle cells. The eye arises from an epithelial sheet of cells known as the eye-antennal imaginal disc. The differentiation of ommatidial cells commences during late third instar larval stage in the wake of the morphogenetic furrow, a constriction of cells that moves from the posterior of the eye disc to the anterior. As cells enter the morphogenetic furrow they become arrested in G1. Upon leaving the furrow some cells have become committed to differentiate. Those that have not enter a synchronous S-phase followed by a synchronous M-phase (known as the second mitotic wave) required to generate enough cells for subsequent differentiation. Perturbations to this strict developmentally controlled cell proliferation and differentiation result in a disruption of the ommatidial array, resulting in a disordered or rough eye phenotype. Therefore, this system allows for easy observation to perturbations of both cell proliferation and differentiation.

The adult wing comprises six longitudinal veins and two cross veins with intervein regions in between. The adult wing arises from the wing imaginal disc, which forms from a small group of 20-40 cells in the embryo that proliferate during the larval stages to form a disc of characteristic size and shape (reviewed in Milan, 1998). Proliferation in the wing disc occurs in an asynchronous pattern under the control of the Decapentaplegic (Dpp) and Wingless (Wg) pathways. Unlike the eye disc, perturbation of cell proliferation in wing discs is generally compensated for via apoptosis and cell proliferation arrest in response to excess cells or increased cell proliferation rates in

response to insufficient cells. Consequently, the resulting adult wing often shows little or no morphological changes (Milan, 1998), with most morphological defects observed in adult wings arising from defects in cell fate specification and differentiation as a consequence of defective cell signalling cascades.

Wing differentiation is under the control of many signalling pathways, including Notch, Epidermal Growth Factor Receptor (EGFR)/Ras, Dpp and Hedgehog/Wingless. Cells receive anterior-posterior information from Dpp signalling cues and dorso-ventral cues from Wingless /Hedgehog signalling (Klein, 2001). The formation of the wing veins occurs through expression of specific transcription factors to specify intervein and provein region and to provide vein identity (de Celis, 2003). This patterning is reinforced by the activation of EGFR and Notch signalling in the proveins. EGFR signalling is thought to activate Notch expression that then signals to cells at the provein-intervein border. Dpp signalling in the proveins results in the differentiation of these cells into veins. Therefore, disruption to signalling pathways often manifests as disruption of wing patterning or wing vein differentiation.

5.2 Overexpression of *deflated* can suppress overexpression of *Cyclin E* but not of *Cyclin A* or *E2f* and *Dp*

5.2.1 Overexpressed *deflated* modifies overexpressed *Cyclin E*

The overexpression of *Cyclin E, Cyclin A*, and the co-expression of *E2f* and *Dp* have been reported to induce ectopic S-phases (Asano et al., 1996; Dienemann and Sprenger, 2004; Du et al., 1996; Duronio et al., 1996; Knoblich et al., 1994; Richardson et al., 1995). When these genes are overexpressed within and posterior to the morphogenetic furrow of the eye imaginal disc (by GMR-Gal4) caused a rough eye phenotype (Figure

5.1A, B, and C). The three rough eye phenotypes varied slightly from each other, although all displayed a reduction in ventral tissue and a disrupted ommatidial array. However, the eyes from *P[GMR-Gal4], P[UAS-CycA]* (Figure 5.1 B) and *P[GMR-Gal4]; P[UAS-E2f1], P[UAS-DP]* (Figure 5.1A) flies had a greater number of bristles than wild type eyes (Figure 5.1 D). *P[GMR-Gal4]; P[UAS-E2f1], P[UAS-DP]* eyes also showed a reproducible furrow running roughly dorso-ventrally, which was not observed in the other two genotypes. *P[GMR-Gal4]; P[UAS-CycE]* eyes, on the other hand, displayed fused ommatidia and were lacking bristles (Figure 5.1 C).

Eye phenotypes resulting from *Cyclin E* or *E2f* and *Dp* overexpression have been previously shown to be modifiable by mutations in other cell cycle regulators (Lane et al., 2000; Staehling-Hampton et al., 1999). To determine whether *deflated* has a role in S-phase, *deflated* overexpression in these three genetic backgrounds was investigated. When one copy of the *deflated* cDNA was overexpressed using GMR-Gal4 (*P[GMR-Gal4]; P[UAS-defl]AV3)* it had no effect on the phenotype of *P[GMR-Gal4]; P[UAS-E2f1], P[UAS-DP]* eyes (Figure 5.1 E). These eyes still lacked cells in the ventral region, the vertical furrow was still present and the eye still contained extra bristles. *deflated* overexpression also failed to suppress the *Cyclin A* overexpression phenotype (Figure 5.1 F). The eye remained disorganised and contained a similar level of extra bristles. *deflated* overexpression, however, did suppress the *Cyclin E* overexpression phenotype. The ommatidia were more orderly arrayed, there were fewer, if any, fused ommatidia and many more ommatidia contained bristles (Figure 5.1 G). The overexpression of *deflated* by GMR-Gal4 in an otherwise wild type background (*P[GMR-Gal4]; P[UAS-defl]AV3)* had no effect on the ommatidial array or bristle number of the adult eye (Figure 5.1 H).

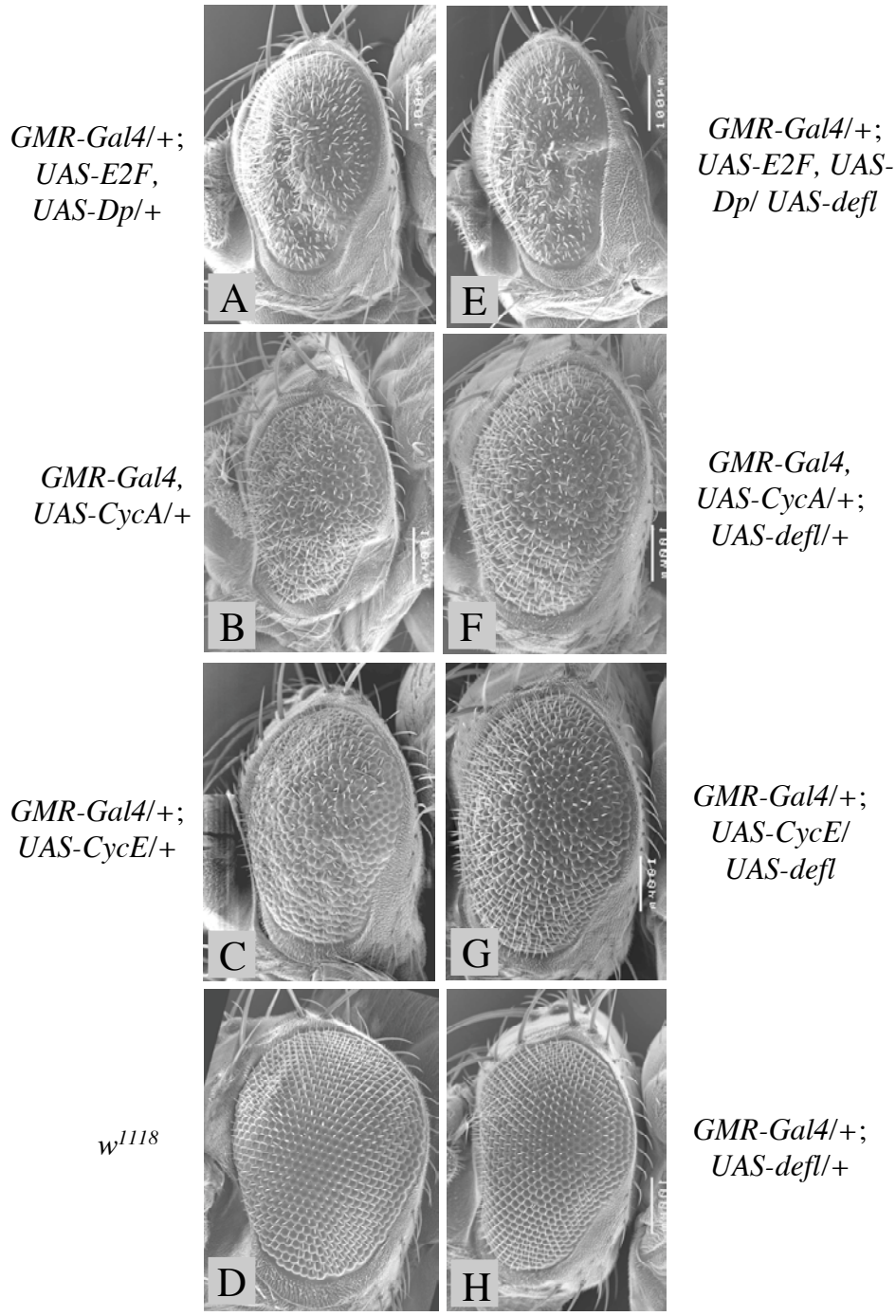


Figure 5.1 Overexpression of *deflated* suppresses overexpression of *CycE*, but not of *CycA*, or *E2f* and *Dp*.

(**A-C**) GMR-Gal4 induced expression of P[UAS-*E2f*] and P[UAS-*Dp*], P[UAS-*CycA*], or P[UAS-*CycE*] results in a rough eye phenotype. (**D and E**) Co-expression of P[UAS-*defl*]AV3 does not modify the eye phenotype of overexpressed P[UAS-*E2f*], P[UAS-*Dp*] or P[UAS-*CycA*]. (**F**) Co-expression of P[UAS-*defl*]AV3 suppresses the eye phenotype of overexpressed P[UAS-*CycE*]. (**G**) Wild type eyes have a regular array of ommatidia, which (**H**) is not disrupted when P[UAS-*defl*]AV3 is overexpressed.

5.2.2 *deflated* overexpression attenuates the ectopic S-phases induced by *Cyclin E* overexpression

In order to understand how *deflated* could suppress *Cyclin E* overexpression but not *Cyclin A* or *E2f* and *Dp,* the effect of *deflated* on S-phase entry in these eye discs was examined. To visualise S-phases, the eye-antennal imaginal discs from third instar larvae were dissected and labelled with BrdU for 30 min (Section 2.24). The synchronous S-phase posterior to the morphogenetic furrow is clearly seen in the wild type eye-antennal imaginal disc (Figure 5.2 A and A′). In the *Cyclin E* overexpressed eye disc the width of the synchronous S-phase band was increased, indicating that ectopic S-phases have occurred (Figure 5.2 B and B′). When *deflated* and *Cyclin E* are co-overexpressed, this band of ectopic S-phases was reduced in both size and intensity. Therefore *deflated* can act to negatively regulate S-phase in these circumstances (Figure 5.2 C and C′). It is interesting to note that the overexpression of *deflated* does not affect the normal band of S-phase cells that are closest to the morphogenetic furrow. When *deflated* is overexpressed in an otherwise wild type background, the pattern of S-phases in the third instar eye imaginal disc is the same as the wild type (Figure 5.2 D and D′). Together these data indicate that *deflated* can act to negatively regulate S-phase, but only when normal cell cycle regulation is perturbed or within cells that should be differentiating rather than proliferating.

To begin to understand why *deflated* overexpression could modify *Cyclin E* but not *Cyclin A* overexpression, third instar eye discs from larvae overexpressing *Cyclin A* were labelled with BrdU. Previous reports of *Cyclin A* overexpression using *sev*-Gal4 showed that ectopic S-phases occurred in cells posterior to the morphogenetic furrow (Dienemann and Sprenger, 2004). However, when GMR-Gal4 was used to overexpress

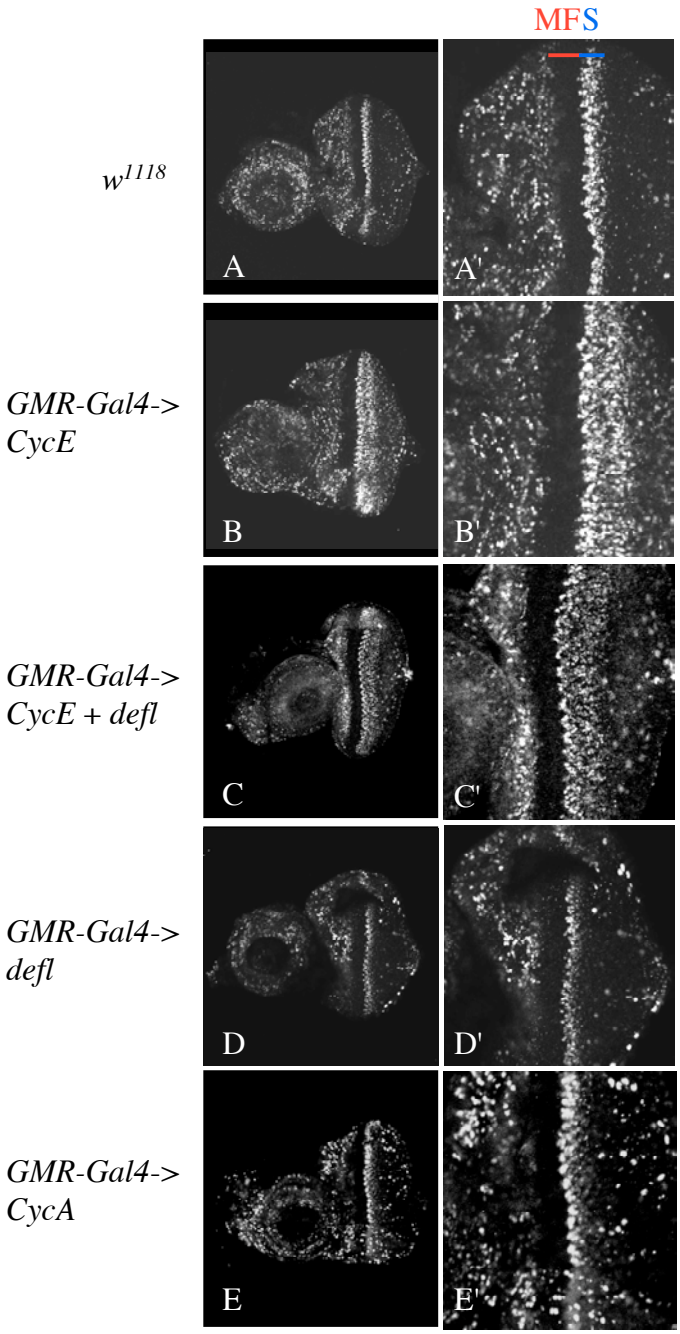


Figure 5.2 *deflated* overexpression suppresses the ectopic S-phases of overexpressed *Cyclin E.*

(**A-E**) Third instar eye-antennal imaginal discs were labelled with BrdU for 30 min to visualise S-phase cells. Discs are shown anterior to the left and posterior to the right. (**A'-E'**)Same imaginal discs shown at higher magnification and with the morphogenetic furrow (MF- red line) and second mitotic wave S-phases (S- blue line) aligned. (**A and A'**) Wild type eye disc showing asynchronous cell divisions in the anterior and the band of synchronous S-phases posterior to the MF. (**B and B'**) GMR-Gal4 induced expression of P[UAS-*CycE*] results in ectopic S-phases posterior to the MF. (**C and C'**) Co-expression of P[UAS-*CycE*] and P[UAS-*defl*]AV3 results in a reduction in the amount and intensity of ectopic S-phases, though the normal S-phases are similar to wild type. (**D and D'**) GMR-induced expression of P[UAS-*defl*]AV3 results in an S-phase pattern indistinguisable from wild type. (**E and E'**) GMR-induced expression of P[UAS-*CycA*] does not result in ectopic S-phases posterior to the MF.

Cyclin A, ectopic S-phases were not observed posterior to the morphogenetic furrow (Figure 5.2 E and E′). Both GMR- and *sev*-Gal4 drive expression in differentiating cells, but as GMR-Gal4 also induces expression in the morphogenetic furrow, it is possible that these cells, or those just posterior, are less sensitive to increased Cyclin A levels. The S-phase pattern in eye imaginal discs overexpressing *E2f* and *Dp* were not examined as it well established that they affect more than just S-phase entry (see discussion) and the cellular effects were not pursued further since *deflated* did not modify the overexpression phenotype.

5.2.3 Halving the wild type dose of *deflated* does not dominantly modify overexpressed *Cyclin E*

To try to understand the genetic relationship between *Cyclin E* and *deflated* further, the effects of heterozygosity for *deflated* on the *Cyclin E* overexpression phenotype was investigated. If the relationship between *deflated* overexpression and reduction of *deflated* is a simple reciprocal relationship then it would be expected that halving the gene dose of *deflated* would enhance the *Cyclin E* eye phenotype. All four *deflated* alleles ($defl^{Z}$, $defl^{L}$, $defl^{P}$, and $defl^{\Delta}$) were introduced into the *P[GMR-Gal4]; P[UAS-CycE]* background and all failed to dominantly modify the eye phenotype (data not shown). It is possible that no modification was observed, despite halving the genetic dose of wild type *deflated,* because there was still sufficient DEFLATED protein to provide wild type function. Alternatively, the overexpression of *Cyclin E* results in an over abundance of protein such that its normal regulation is saturated, masking any effects.

This alternative is given support by the finding that halving the genetic dose of *dpp* (*dpp*^{hr92} or *dpp*^{10638}) does not modify *CycE* overexpression (data not shown) despite previous reports of suppression of the hypomorphic *CycE*^{JP} allele by a number of *dpp* alleles (Horsfield et al., 1998).

5.3 Identification of genes that genetically interact with *deflated*

5.3.1 Generation of an overexpression phenotype

To try to further understand the role of *deflated* in regulating S- and M-phase entry, genetic interactions with other cell proliferation regulators were tested. If *deflated* has a general role in regulating S- and/or M-phase then a number of loci controlling these processes would be expected to modify a *deflated* phenotype. If *deflated* is primarily a positive or negative regulator of cell proliferation then this should be reflected in the nature of the interactions. However, if *deflated* has a complex role, as hinted by the data already obtained, then the interactions may not provide straightforward conclusions.

To test candidate cell proliferation regulators for a genetic interaction with *deflated,* a wing overexpression phenotype was used. The recessive phenotypes (lethality, mild wing and bristle, and chorion phenotypes) described in chapter 3 were pleiotrophic and therefore poorly suited to assessing genetic interactions. Overexpression of the cDNA in a tissue specific manner was chosen since it will affect only one part of the fly and make observed genetic interactions easier to interpret. This type of approach has been successfully employed in genetic screens for modifiers of S-phase regulators such as *Cyclin E* (Lane et al., 2000) and *E2f/Dp* (Staehling-Hampton et al., 1999).

To generate a suitable phenotype, the *deflated* cDNA was overexpressed using a number of Gal4 driver lines and the effects observed. Discernable phenotypes could only be detected when two copies of the *deflated* cDNA (P[UAS-*defl*]AV3; P[UAS-*defl*]Z1) were overexpressed and the flies were raised at 29°C (Gal4 is more active at higher temperatures). This was unexpected since *deflated* is only required at low levels throughout development (Section 4.7.2) and implies that extra *deflated* can be tolerated. Interestingly, even under these conditions only a few of the Gal4 drivers could induce an overexpression phenotype with the wing being the most sensitive to overexpression.

Overexpression of two copies of the *deflated* cDNA by the Gal4 wing drivers, *spalt major*-Gal4 (Figure 5.3 B), MS1096-Gal4 (Figure 5.3 C), and *scalloped*-Gal4 (Figure 5.3 D), resulted in wings that had an extra posterior cross vein (asterisk, Figure 5.3 B) or a reduced and crumpled wing (Figure 5.3 C and D). Low level, ubiquitous Gal4 drivers such as *armadillo*-Gal4 and *escargot*-Gal4 resulted in a mild phenotype in which the machrochaetae on the notum, particularly the scutellum, were frequently duplicated (Figure 5.3 F and G). In addition, an unusual phenotype was observed with the driver T1096-Gal4. The notum contained "scar" like fissures on either side (Figure 5.3 H). The overexpression caused by the Gal4 driver MS1096 was chosen to test genetic interactions, as its effect was moderate so modification (enhancement or suppression) should be easily scored.

5.3.2 *deflated* interacts genetically with *Irregular facets*

In the course of these overexpression experiments, it was noted that overexpressed *deflated* could suppress the *Irregular facets (If)* rough eye phenotype (Figure 5.4 A, B and C). This observation arose because the *scalloped*-Gal4 driver line contains the *If*

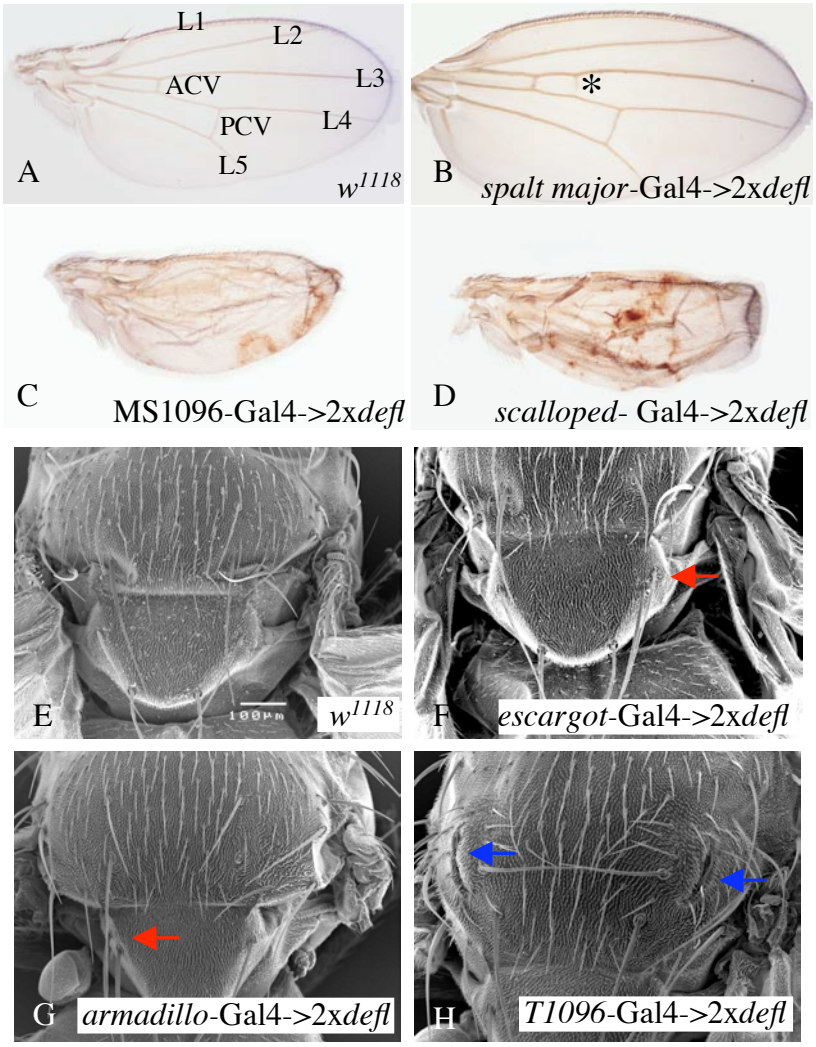


Figure 5.3 Overexpression of two copies of *deflated* results in wing and bristle defects.

(**A**) A wild type wing with the five longitudinal veins (L1-L5) and the two cross veins (anterior- ACV and posterior- PCV) labelled. (**B-D, F-H**) Overexpression of P[UAS-*defl*]AV3, P[UAS-*defl*]Z1 at 29°C resulted in wing and bristle defects. (**B**) Overexpression by *spaltmajor*-Gal4 resulted in an ectopic anterior cross vein (asterisk). (**C and D**) Overexpression by MS1096-Gal4 and *scalloped*-Gal4 resulted in a reduced and crumpled wing. (**E**) A wild type thorax showing the characteristic pattern of macrochatae. The two curled macrochatae are a result of the preparation for scanning electron micrography and do not represent real bristle morphology. (**F and G**) Overexpression by *escargot*-Gal4 and *armadillo*-Gal4 resulted in the duplication of macrochatae (red arrows). (**H**) Overexpression by *T1096*-Gal4 resulted in the formation of 'scar'-like fissures on either side of the thorax (blue arrows).

allele on the second chromosome and, in scoring these flies, a difference between *If* flies with and without the *deflated* cDNA was noted. While *scalloped*-Gal4 is predominantly a wing driver, expression of a UAS-*GFP* construct by this driver showed some fluorescence in third instar eye imaginal discs (data not shown), which implies that *deflated* is overexpressed to a small degree in these eyes when driven by *scalloped*-Gal4. *If* is a gain of function allele of the transcription factor *Krüppel* (Carrera et al., 1998). This allele causes misexpression of *Krüppel* in the eye, which results in a reduced eye with fused ommatidia and necrotic spots (Figure 5.4 A). This phenotype can vary between individuals and is more severe in males. Although the expression of one copy of *deflated* did not modify the *If* phenotype (Figure 5.4 B), the expression of two copies (P[UAS-*defl*]AV3; P[UAS-*defl*]Z1) resulted in strong suppression (Figure 5.4 C).

To further understand the genetic relationships between *deflated* and *Krüppel,* the effect of loss of wild type *deflated* function on *If* was examined. The *If* phenotype in *deflated* heterozygotes looked similar to milder *If* eyes and *deflated* was therefore considered to not be a dominant modifier (Figure 5.4 D). However, the *If* eye phenotype was strongly enhanced in $defl^{P}/defl^{Z}$ (Figure 5.4 E) and $defl^{L}/defl^{Z}$ (data not shown) transheterozygotes. These observations support an antagonistic role for *deflated* in *Krüppel* function as loss of wild type DEFLATED protein caused a worsening of the *If* gain of function phenotype.

5.3.3 Identification of candidate dominant modifiers of the *deflated* overexpression wing phenotype

To test candidate genetic interactors, a recombined second chromosome containing two

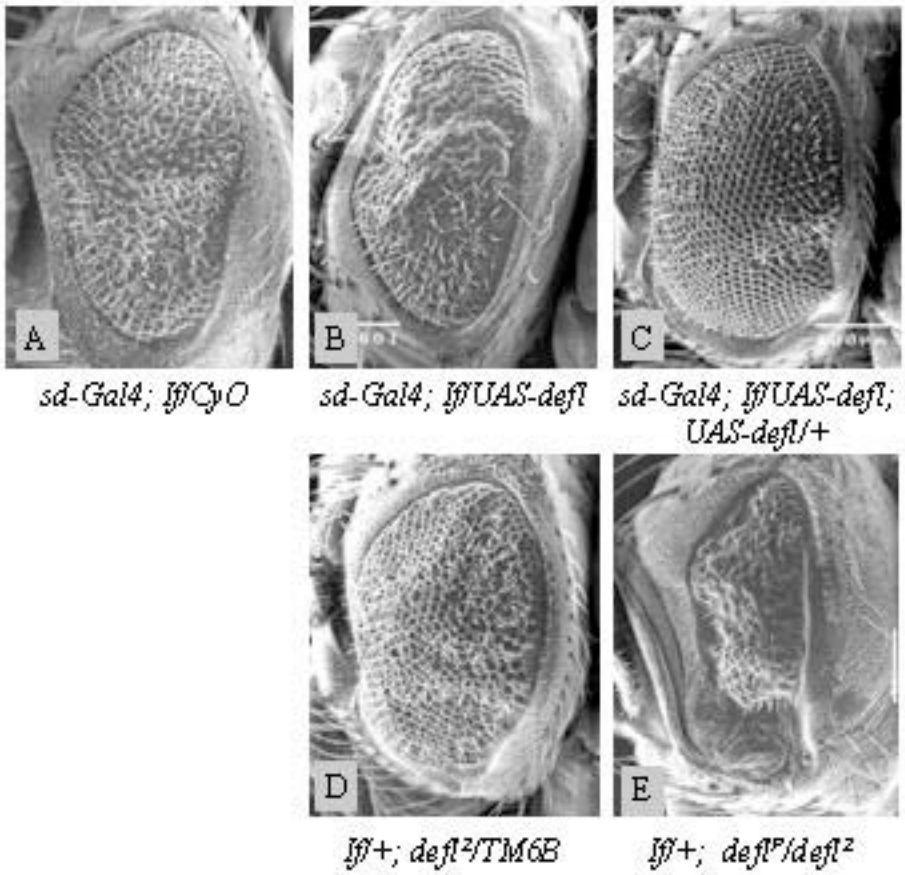


Figure 5.4 *deflated* interacts with *Kr^If*

(**A**) The *Irregular facets* allele of *Krüppel* results in a small, rough eye, which sometimes displays necrotic spots. (**B**) The expression of P[UAS-*defl*]AV3 by *scalloped*-Gal4 at 29°C does not modify the *If* phenotype. (**C**) The expression of two copies of *deflated*, P[UAS-*defl*]AV3, P[UAS-*defl*]Z1, by *scalloped*-Gal4 at 29°C results in a strong suppression of the *If* eye. (**D**) Heterozygosity at the *deflated* locus does not modify the *If* phenotype. (**E**) In a *deflated* transheterozgous background the *If* phenotype is enhanced. A similar result is obtained in a *defl*¹/*defl*² background. All eyes shown are from females.

copies of the *defl* cDNA (P[UAS-*defl*]BB1, P[UAS-*defl*]BA2) was generated and balanced over a *CyO(Act-GFP)* balancer (to aid in genotyping larvae) and driven by the MS1096-Gal4 driver, which is on chromosome X. This combination of *deflated* transgenes was used to make the testing easier due to both transgenes being on the same chromosome. The initial overexpression phenotypes were generated in flies that contained one second chromosome copy and one third chromosome copy (P[UAS-*defl*]AV3, P[UAS-*defl*]Z1). The wing phenotype was less severe in flies containing the two second chromosome transgenes than one on II, one on III combination. This observation indicates that the wing phenotype was dose dependent and likely to be modifiable. The overexpression of P[UAS-*defl*]BB1, P[UAS-*defl*]BA2 resulted in a smaller wing than wild type with ectopic wing vein material anterior to L3 and a slight reduction in the intervein region between L3 and L4 around the anterior cross vein (Figure 5.5 B).

Known cell proliferation genes, cell signalling genes, and other genes that were identified as potential *deflated* interactors were tested for their ability to dominantly modify the *deflated* overexpression wing phenotype. This type of test measures the ability of an allele to modify the *deflated* wing phenotype as a heterozygote, which otherwise shows no phenotype in a wild type background. Modification indicates that the gene tested acts in a similar biological pathway to *deflated*. This is because the wing has become sensitised by the *deflated* overexpression to changes in the levels of proteins that act in similar biological pathways. Many of the alleles tested were loss of function alleles, however some genes were tested for the ability to modify while overexpressed. Many of the candidate genes tested were able to modify the wing phenotype (Table 5.1). The strength of the modification (either enhancement or

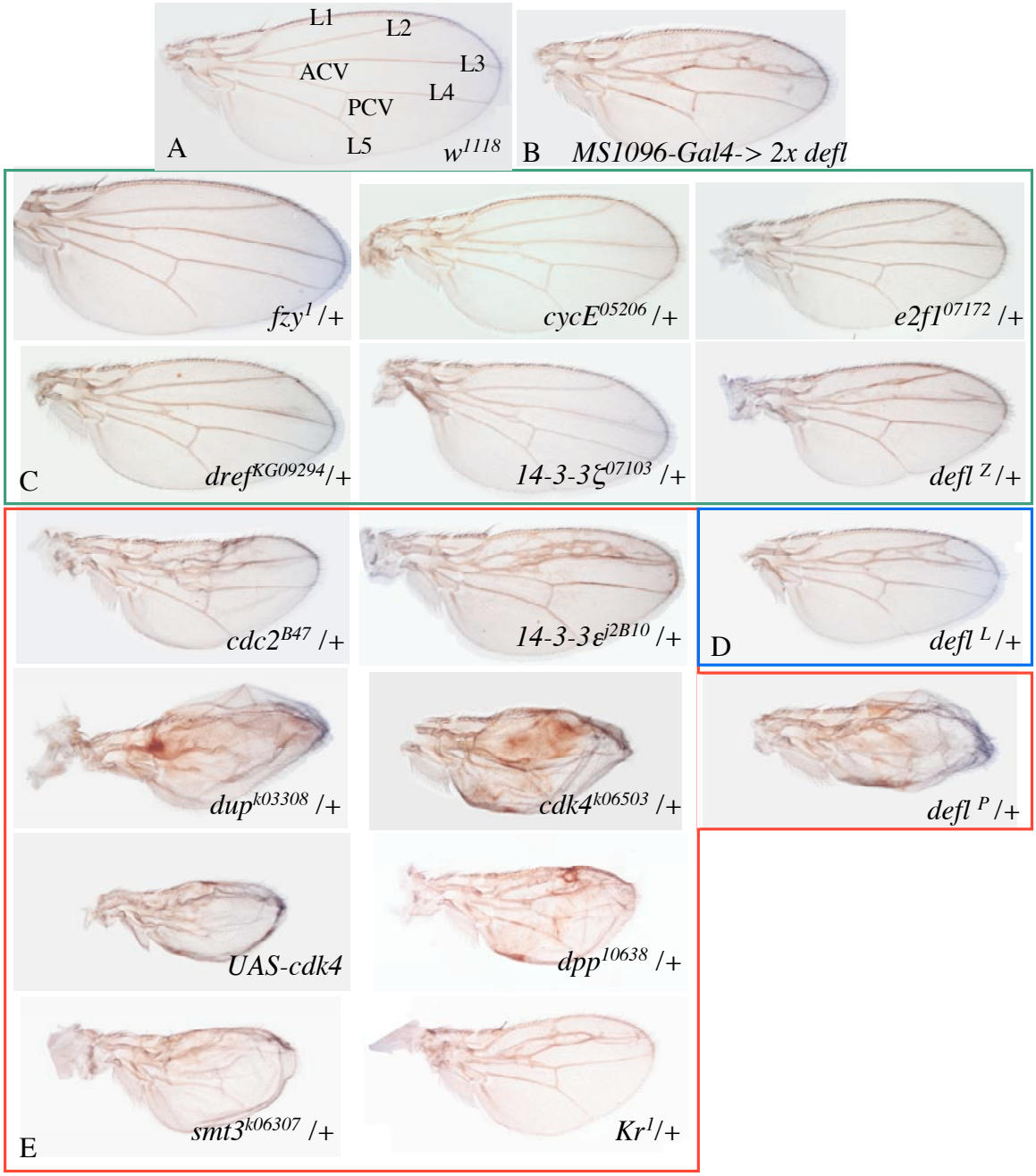


Figure 5.5 Examples of genetic interactors of *deflated* overexpression.

(**A**) A wild type wing is shown with the five longitudinal veins(L1-L5) and cross veins (ACV and PCV) labelled. (**B**) The wings of *MS1096-Gal4; P[UAS-defl]BA2, P[UAS-defl]BB1/+: +/+* individuals (*MS1096-Gal4->2xdefl)* are of reduced size and have extra vein material. **(C-E**) Modifiers of the *deflated* overexpression wing phenotype are shown. The genotypes of the modifying locus are shown with each wing micrograph. (**C**) Suppressors of the wing phenotype are boxed in green. Most suppressors suppress the extra wing veins, though some also have an increased wing size. (**D**) $defl^{L}$ does not modify the *deflated* overexpression wing phenotype. (**E**) Enhancers of the wing phenotypes are boxed in red. They show increased amount of ectopic wing veins, a reduction in wing size, or a disruption in wing structure.

suppression) was assigned on a scale of 1 to 5, with 1 being a slight modification and 5 being a strong modification and 0 being no modification. The main character that was scored was the reduction or enhancement of the ectopic veins anterior to L3, but the size and shape of the wing was also taken into account.

5.3.4 Genetic interactions with cell proliferation alleles

A number of cell proliferation regulators were chosen to be tested for their ability to dominantly modify the *deflated* overexpression wing phenotype. These included alleles of the E2f-Dp S-phase regulatory network, both S-phase and M-phase cyclins, DNA replication factors, positive regulators of M-phase entry and M-phase progression, and negative regulators of M-phase. The strongest suppressors were $E2f1^{07172}$, $Cyclin\ E^{05206}$ and $fizzy^{1}$ (Table 5.1). All three alleles resulted in loss of the ectopic vein material and with both $Cyclin\ E^{05206}$ and $fizzy^{1}$, the total wing area also increased in size (Figure 5.5 C). Moderate suppressors included $Cyclin\ A^{03946}$, $three\ rows^{1}$, $aurora^{87Ac\text{-}3}$, and UAS-*string* (Table 5.1).Weaker suppressors included $Dp^{49Fk\text{-}1}$, $E2f2^{76Q1}$, $string^{01235}$ and UAS-*cdk1* (Table 5.1).

Cell proliferation alleles that enhanced the *deflated* overexpression phenotype included a series of alleles of *double parked* (*dup*) (Table 5.1). dup^{k03308} was a strong enhancer, causing a further reduction in wing size, blistering and the absence of veins (Figure 5.5 E). Unexpectedly, *cdk4* enhanced the *deflated* overexpression wing phenotype strongly as both an heterozygote ($cdk4^{k06503}$) and also when overexpressed (UAS-*cdk4*) (Table 5.1). In both cases the resulting wings were small and blistered (Figure 5.5 E). $cdc2^{B47}$ was found to act as a moderate enhancer of the wing phenotype (Table 5.1), producing

Table 5.1 Genetic modifiers of MS1096-Gal4->2x *defl* cDNA

Allele	Function	Strength of effect on MS1096-> 2x *defl* †
	Suppressors§	
Cell Cycle§		
E2F^{07172} (LOF)	Positive regulator of S-phase	4
Dp^{49FK-1} (hypomorph)	Positive regulator of S-phase	1
E2F2^{76Q1} (LOF)	Negative regulator of S-phase	1
CycE^{05206} (hypomorph)	S-phase cyclin	4
CycA^{03946} (P-element insertion)	M-phase cyclin	3
stg^{01235} (P-element insertion)	Positive regulator of M-phase	2
thr^{1} (amorph)	Metaphase-Anaphase transition	3
fzy^{1} (amorph)	APC activator	4
aur^{87Ac-3} (amorph)	Mitotic kinase	3
UAS-cdk1-myc	S-phase cyclin dependent kinase	2
UAS-stg	M-phase phosphatase	3
Cell Cycle and Cell Signalling		
14-3-3ζ^{07103} (hypomorph)	M-phase regulator and Ras signalling	4
Dref^{KG09294} (hypomorph)	Positive regulator cell proliferation and Ras signalling	4
Cell signalling		
cact^{4} (?)	Toll signalling cascade	4
Unknown		
defl^{Z}	Unknown	3
	Enhancers	
Cell Cycle		
CycE^{k05007} (P-element insertion)	S-phase cyclin	1
cdc2^{B47} (amorph)	Positive regulator of M-phase	3
stg^{4} (amorph)	Postive regulator of M-phase	2
cdk4^{K06503} (P-element insertion)	Cell growth- Cyclin D dependent kinase	4
UAS-cdk4	Cell growth- Cyclin D dependent kinase	5
dup^{a1} (hypomorph)	S- and M-phase regulator	2
dup^{a3} (hypomorph)	S- and M-phase regulator	3
dup^{K03308} (P-element insertion)	S- and M-phase regulator	5

Enhancers cont.		
Cell Cycle and Cell Signalling		
14-3-3ε^{j2B10} (LOF)	M-phase regulator and Ras signalling	2
nkd (?)	Wg signalling cascade	5
dpp^{10638} (P-element insertion)	Decapentaplegic signalling cascade	5
dpp^{hr92} (LOF)	Decapentaplegic signalling cascade	2
Transcription factors		
Kr^{1}	Gap rule	3
Protein modification		
smt3^{k06307} (P-element insertion)	Sumoylation	4
Unknown		
defl^{P}	Unknown	3
Non Modifiers		
Cell Cycle		
Orc5^{2} (?)	DNA replication	0
lat^{1} (?)	DNA replication	0
CycA^{C8LR1} (null)	M-phase cyclin	0
wee^{DS1} (hypomorph)	M-phase checkpoint	0
wee^{ES1} (amorph)	M-phase checkpoint	0
UAS-E2f and Dp	Positive regulator of S-phase	0
Unknown		
defl^{L}	Unknown	0

† Candidate alleles were tested for their ability to modify the wing phenotype of two copies of *deflated* (P[UAS-*defl*]BB1, P[UAS-*defl*]BA2) overexpressed by MS1096-Gal4. The strength of the interaction is on a scale of 0-5, where 0 is no modification and five is the strongest suppression or enhancement scored.

§ They are grouped according to their ability to suppress, enhance or not modify, and subgrouped by the cellular process in which they are involved. For each allele the type of allele is shown in brackets, if known. LOF- loss of function.

smaller wings that contained small blisters (Figure5.5 E). Weak enhancers of *deflated* overexpression included *string*^{4} and *Cyclin E*^{k05007} (Table 5.1).

Alleles of genes that were found to not modify the *deflated* overexpression wing phenotype included those involved in DNA replication (*Orc5*^{2} and *lat*^{1}), the M-phase checkpoint (*wee*^{DS1} and *wee*^{ES1}), *Cyclin A*^{C8LR1}, and co-overexpression of *E2f* and *Dp* (Table 5.1). This last result is consistent with the findings observed in the eye, where overexpressed *deflated* could not modify overexpressed *E2f* and *Dp*.

Three of the genes tested for genetic interaction with *deflated* are involved in regulating the cell cycle as well as having roles in regulating the Ras signalling pathway. These are the 14-3-3 isoforms ε and ζ and the transcription factor DREF. 14-3-3 proteins have established roles in the regulation of M-phase entry (Sections 1.1.3 and 1.1.4) as well as regulating Raf, MEK and MAPK activation (Section 1.2.1). The two different 14-3-3 isoforms differed in their ability to modify the *deflated* overexpression wing phenotype. *14-3-3ε*^{J2B10} was a weak enhancer with the resulting wings containing an increase in ectopic vein material (Figure 5.5 E). On the other hand, *14-3-3ζ*^{07103} was a strong suppressor and the resulting wings displayed no ectopic vein material (Figure 5.5 C). The transcription factor DREF was originally identified as required for the transcription of DNA replication and S-phase genes such as *Cyclin A* and *E2f1* (Ohno et al., 1996; Sawado et al., 1998), but it also transcriptionally activates members of the Ras signalling pathway (Ryu et al., 1997; Yoshida et al., 2004). The *Dref*^{KG09294} allele was a strong suppressor of the *deflated* wing overexpression phenotype, resulting in wings lacking the ectopic vein material (Figure 5.5 C).

5.3.5 *deflated* interacts genetically with various cell signalling pathway components

The L5 wing vein defect observed in transheterozygous adult *deflated* escapees suggested that *deflated* may affect signalling cascades involved in wing vein differentiation (Section 4.7.5). The *deflated* overexpression wing phenotype (Figure 5.5 B) supports this conclusion. The formation of ectopic vein material anterior to L3 indicates that, when overexpressed, *deflated* interferes with normal differentiation, possibly through perturbation of cell signalling pathways. To explore this possibility, a number of alleles of genes encoding components of various signalling cascades were tested for their ability to dominantly modify the *deflated* wing overexpression phenotype.

Only one allele tested was able to suppress the wing phenotype. This was the Toll pathway component, $cactus^{4}$ (Table 5.1). The two *dpp* alleles tested, dpp^{hr92} and dpp^{10638}, enhanced the phenotype (Table 5.1), with dpp^{hr92} was weakly enhancing and dpp^{10638} strongly enhancing. The resulting wings from the dpp^{10638} cross were reduced in size and blistered, with some extra wing vein material (Figure 5.5 E). *Naked cuticle* (*nkd*), a member of the Wingless signalling cascade, was also a strong enhancer (Table 5.1). Taken together, these data show that *deflated* can interact genetically with a number of cell signalling components.

5.3.6 *deflated* also interacts genetically with *Krüppel* in the wing

Since *deflated* antagonises the effects of *Krüppel* misexpression in the eye (Figure 5.4), it was assessed whether *Krüppel* could also dominantly modify the *deflated* overexpression wing phenotype. Kr^{1} was able to moderately enhance the *deflated* wing phenotype (Table 5.1). The resulting wings were reduced in size and the proximal-distal

axis was shortened. There was a further decrease in the intervein region between L2 and L3 and the presence of some ectopic vein material (Figure 5.5 E). This result further supports an antagonistic role between *deflated* and *Krüppel*, as reducing *Krüppel* activity enhances the *deflated* overexpression phenotype.

5.3.7 *deflated* interacts with *smt3*, which encodes SUMO

An allele of *smt3*, which encodes the ubiquitin-like protein SUMO, was tested to assess whether the conserved putative SUMO-modification site (a.a. 872-875; Figure 4. 2) in DEFLATED may be functional. Unlike ubiquitin, the conjugation of SUMO to proteins does not target them for degradation by the proteosome, but is thought to protect them from degradation by preventing ubiquitination (Shih et al., 2001). Halving the dose of wild type SUMO ($smt3^{k06307}$) resulted in enhancement of the *deflated* overexpression phenotype (Table 5.1). The resulting wing was reduced in size and contained blisters (Figure 5.5 E). This finding indicates that DEFLATED may be directly modified by SUMO conjugation with the modification serving to negatively regulate DEFLATED function. Alternatively, SUMO may regulate another protein that interacts with DEFLATED.

5.3.8 Genetic interaction between the *deflated* alleles

The *deflated* alleles generated in Chapter 3 were tested for their ability to suppress the *deflated* overexpression wing phenotype. If the phenotype was due to excess but normal function of *deflated,* then it would be expected that reducing the dose of wild type *deflated* by mutation would suppress the wing phenotype. Unexpectedly, only $defl^{Z}$ could suppress the wing overexpression phenotype. Wings from $defl^{Z}$ heterozygotes resulted in a near absence of the ectopic wing vein material (Figure 5.5 C). $defl^{L}$ failed

to modify the phenotype and *defl*^{P} enhanced the *deflated* wing overexpression phenotype (Table 5.1). In *defl*^{L} heterozygote wings, the amount and positioning of the ectopic veins remained unchanged (Figure 5.5 D). However, wings from *defl*^{P} heterozygotes were smaller and blistered and the veins were not easily observed (Figure 5.5 E). Taken together, these data indicate that the genetic relationships between the *deflated* alleles and its overexpression are not straightforward, indicating perhaps that not all of the *deflated* alleles are hypomorphic in nature.

5.4 Discussion

In an attempt to further define the cellular role(s) of *deflated*, a series of genetic studies were performed. It should be noted that in interpreting these data it was assumed that the interacting loci were indeed the candidates tested even though there is the possibility that other unknown loci on the chromosome may be mediating the interaction. The genetic interactions observed with overexpressed *deflated* support a role for *deflated* in regulating the cell cycle. In particular, interactions with *Cyclin E* suggested that *deflated* has a negative role in regulating S-phase in the differentiating part of the eye imaginal disc. These genetic interaction data also support a possible role for *deflated* in cell signalling, a finding given further weight by the nature of the phenotypes observed when *deflated* is overexpressed. Duplication of macrochaetae and disruption of normal wing vein specification and formation are very suggestive of perturbation of signalling pathways. Surprisingly, these interactions show that *deflated* is a likely antagonist of *Krüppel,* a finding that could not be predicted by the putative protein domains or expression patterns.

5.4.1 *deflated's* role in regulating S-phase

Overexpression of *deflated* was not able to suppress the rough eye phenotype of *Cyclin A* (Figure 5.1 F). Unlike previous reports (Dienemann and Sprenger, 2004), *Cyclin A* was found to not induce ectopic S-phases posterior to the morphogenetic furrow (Figure 5.2 E and E′), which indicates that the rough eye phenotype in Figure 5.1 B is not due to an increase in cells entering S-phase. Whatever the cause of the rough eye phenotype, it is unlikely that *deflated* is involved as *deflated* failed to modify the *Cyclin A* overexpression rough eye phenotype. This study overexpressed *Cyclin A* using GMR-Gal4 (which drives expression in the morphogenetic furrow and all the posterior cells), whereas the previous study used *sev*-Gal4 (which is expressed in a subset of the photoreceptor cells). It is possible that cells in the morphogenetic furrow or just posterior are not sensitive to increases in Cyclin A levels and are therefore not induced into an ectopic S-phase. Cells further away from the furrow, such as those in which *sev*-Gal4 is expressed may be more sensitive and able therefore to undergo an ectopic S–phase. A likely explanation for the proposed increased resistance to Cyclin A levels in the morphogenetic furrow is the expression of *roughex* in the furrow, which is a known inhibitor of Cyclin A and is one of the gene products required for the G1 arrest (Section 1.3.3 ; Avedisov et al., 2000; Thomas et al., 1994).

Overexpressed *deflated* was also not able to modify the effects of co-expression of *E2f1* and *Dp* in the eye. Since the co-overexpression of *E2f* and *Dp* is known to induce cellular processes such as apoptosis as well as ectopic S-phases (Asano et al., 1996; Du et al., 1996), the rough eye phenotype is likely to be caused by the additive or synergistic effects of these different cellular processes. As *deflated* overexpression failed to modify this eye phenotype, it is likely that *deflated* does not play a role in all

of the same cellular processes as *E2f* and *Dp*. Alternatively, if *deflated* is a transcriptional target of E2F (Section 4.7.2), then it would already be upregulated when *E2f* and *Dp* are overexpressed and further overexpression may not have an affect. The finding that overexpression of *E2f* and *Dp* in the wing did not modify the *deflated* wing overexpression phenotype (Table 5.1) argues against the second alternative because if *deflated* was a transcriptional target then increasing its expression through overexpression of *E2f* and *Dp* would result in enhancement of the phenotype. In conclusion, *deflated* overexpression and *E2f* and *Dp* overexpression do not interact genetically, indicating that even though *deflated* mutants phenocopy *E2f* and *Dp* mutants (4.7.3), the proteins are likely to act in separate cellular pathways.

The finding that *deflated* overexpression suppressed the ectopic S-phases induced by *Cyclin E* overexpression suggests that *deflated* may act a negative regulator of S-phase. This finding seems to contradict the observations of Chapter 4, in which cells from $defl^{P}$ homozygous brains were less likely to be in S-phase at any given time, which indicates that *deflated* may be required positively for S-phase. However, brains from $defl^{L}$ homozygous individuals showed a slight increase of cells in S-phase, therefore supporting a conclusion that *deflated* is a negative regulator of S-phase. Since *deflated* overexpression only had an effect on the ectopic S-phases that occurred in cells that should be differentiating in the eye (Figure 5.2 C and C′), it is possible that *deflated* may play different roles depending on the requirements of the cell. In cells that should be proliferating, such as the brain, *deflated* is required for S-phase, but in cells that should be differenting, *deflated* in required to inhibit S-phase. In the wing the genetic relationships between *deflated* and *Cyclin E* further obscure *deflated's* role in regulating S-phase. Two alleles of *Cyclin E* were tested, $Cyclin\ E^{05206}$ strongly suppressed the

deflated wing overexpression phenotype, whereas *Cyclin E*^{k05007} weakly enhanced the phenotype. These allelic differences indicate that even within one tissue *deflated* seems to have opposing roles in regulating S-phase.

In order to understand *deflated's* interaction with *Cyclin E* further, it was assessed whether decreasing the genetic dose of wild type *deflated* could modify the rough eye phenotype. It was found that none of the four *deflated* alleles modified the rough eye phenotype. Alleles of *dpp*, which is a known genetic interactor of *Cyclin E*, also failed to modify. It is possible that overexpressed *Cyclin E* may result in too much Cyclin E protein for modifications to be observed. This notion is supported by the finding that many more interactors were identified when a *Cyclin E* hypomorphic allele, *Cyclin E*^{JP}, was used (Brumby et al., 2004) rather than *Cyclin E* overexpression (Lane et al., 2000). The *dpp* interaction was observed using the *Cyclin E*^{JP} allele (Horsfield et al., 1998). Since this allele is sensitive to modifiers, it would be interesting to see if the *deflated* alleles could dominantly modify this allele. Brumby et al. (2004) did find that a deficiency (*Df(3L)AC1*) covering the *deflated* region (67A2-67D7-13) enhanced the *Cyclin E*^{JP} phenotype. If *deflated* is a negative regulator of S-phase, as the overexpression interactions suggest (Figure 5.2), then it is unlikely that *deflated* is the causative locus in the deficiency. However, if *deflated* is a positive regulator of S-phase, as the analysis of brains from *defl*^{P}/*defl*^{P} second instar larvae seems to suggest (Section 4.7.6), then it is possible that *deflated* is the causative locus identified by Brumby et al. (2004).

5.4.2 *deflated* may be involved in linking DNA synthesis with M-phase entry

What is the role of *deflated* in regulating S-phase? At this stage the data is contradictory. Many cell cycle regulators interact genetically with *deflated* when it is overexpressed in the wing (Table 5.1) but interpreting these interactions has proved difficult. As already discussed, the interactions with *Cyclin E* and *E2f* and *Dp* in the developing eye and the requirements for *deflated* in second instar larval brains suggest opposing roles for *deflated* in S-phase regulation. However, from the genetic interactions observed between *deflated* overexpression and various cell cycle regulators, it appears that *deflated* may be involved in linking DNA synthesis and its completion with M-phase entry. Five distinct sets of data are consistent with this interpretation.

Firstly, a number of positive regulators of cell cycle progression, *E2f1*, *fzy*, *thr,* and *aur* suppressed the *deflated* overexpression wing phenotype. This indicates that the wing phenotype is in part due to an increase in cell proliferation. Therefore, from these data, it appears that overexpressed *deflated* causes an increase in cell cycle progression, which can be reduced by decreasing the levels of these positive regulators.

Secondly, two genes for which the observed interactions were consistent were *cdc2* (cdk1) and *dup*, which both have roles in ensuring that DNA synthesis must occur before mitosis can begin. Halving the dose of *cdc2* resulted in suppression of the wing phenotype and overexpression of cdk1 resulted in enhancement. Taken together these data suggest that the overexpression of *deflated* either results in the upregulation of cdk1 or acts in a pathway that can be affected by cdk1 activity. All three alleles of *dup* that were tested enhanced the *deflated* overexpression wing phenotype. *dup* encodes the

cdt1 homologue, which is a pre-replication complex component (Thomer et al., 2004). Homozygous *dup* mutants are unable to replicate their DNA in postblastoderm cycles yet enter mitosis with unreplicated DNA (Whittaker et al., 2000). As mitosis and DNA synthesis are uncoupled in *dup* mutants, and *dup* can enhance the *deflated* overexpression phenotype, it is possible that overexpressed *deflated* may also cause an uncoupling of mitosis and DNA synthesis. If this is the case then an explanation can be provided for the finding that halving the dose of *cdc2* suppresses the *deflated* overexpression phenotype, while increasing the cdc2 enhances the *deflated* overexpression phenotype. Prevention of M-phase entry by decreasing *cdc2* results in fewer cells overexpressing *deflated* entering M-phase with unreplicated or partially replicated DNA and the severity of the phenotype is thereby reduced.

Thirdly, the notion that overexpressed *deflated* uncouples DNA synthesis with M-phase entry fits with the finding that *wee* alleles fail to modify the overexpression phenotype. The M-phase checkpoint is thought to be triggered by the presence of unreplicated DNA. The checkpoint is not triggered if DNA synthesis is not initated, as in *dup* mutants, and therefore M-phase occurs (Whittaker et al., 2000). Therefore, changes in *wee* gene dosage would have no effect. If DNA replication does not occur when *deflated* is overexpressed then the observation that *lat* and *Orc5* also fail to modify the overexpression phenotype also makes sense.

Fourthly, the genetic interactions with the two 14-3-3 isoforms could be due to the roles that these proteins play in regulating M-phase, their roles in regulating Ras signalling (see Chapter 6), or to some other cellular process. In *Drosophila* 14-3-3ε is required to time mitosis and effects a mitotic delay following irradiation in post-blastoderm

embryos by keeping cdk1 in check, whereas 14-3-3ζ is required for correct chromosome segregation in syncytial embryos (Su et al., 2001). Halving the dose of *14–3-3ε* enhanced the *deflated* overexpression phenotype, which indicates that if 14-3-3 and *deflated* interact in regulating M-phase then overexpression of *deflated* is likely to result in an increase in M-phase entry. This interpretation is consistent with the interpretations of the *dup* and *cdc2* interactions (see above). Halving the 14-3-3ζ dose resulted in the suppression of the *deflated* overexpression phenotype and this finding is also consistent with the interpretation that *deflated* overexpression may induce inappropriate M-phase entry. Reducing the amount of 14-3-3ζ may prevent or slow down APC/C activation, affecting the timing of chromosome segregation and mitotic exit, thereby counteracting the effects of premature M-phase entry.

Fifthly, the finding that a *Dref* allele can dominantly suppress the *deflated* overexpression phenotype also supports an interpretation that *deflated* overexpression causes premature M-phase. DREF is required for the transcription of many cell cycle genes including those required for M-phase entry (Hyun et al., 2005). When DREF activity is reduced the transcription of these M-phase regulators is also reduced and therefore M-phase entry would take longer. However, since DREF induces the transcription of many cell cycle genes the interaction observed may reflect a different role for *deflated*.

In contrast the evidence discussed above, the finding that heterozygosity and overexpression of the M-phase regulator *string* (Section 1.3.3) do not consistently modify the *deflated* overexpression phenotype clouds the argument that *deflated* overexpression causing premature M-phase entry. stg^{01235} was observed to be a weak

suppressor, whereas *stg*^{4} caused weak enhancement, and overexpression of *string* resulted in moderate suppression. At this point is is difficult to reconcile these findings with the postulated role of *deflated* in regulating M-phase entry once S-phase is completed.

The genetic interactions observed between *deflated* and *cdk4* suggest that *deflated* may affect the regulation of cellular growth and M-phase entry. However, as *cdk4* was found to enhance the *deflated* overexpression phenotype when the former was both heterozygotic or overexpressed, it is likely that *deflated* has an indirect effect on cellular growth and the role *cdk4* plays in M-phase. It is possible that when *cdk4* is overexpressed alone it could result in a small and crumpled wing and therefore not really be enhancing the *deflated* overexpression phenotype. However, previous studies have shown that the overexpression of *cdk4* without co-overexpression of *Cyclin D* in the wing does not result in a phenotype (Datar et al., 2000). Therefore the severe phenotype observed must be due to either enhancement of *deflated* overexpression or an additive effect of overexpression of the two genes. Since CyclinD/Cdk4 can also promote G2-M progression (Datar et al., 2000; Neufeld et al., 1998), it is possible that the genetic interactions observed may reflect a potential role for *deflated* in the regulation of M-phase entry. Thus, the finding that *cdk4* enhances when either heterozygous or when overexpressed may reflect both of these roles.

Taken as a whole, these genetic interaction data do support a case for *deflated* being involved in linking DNA replication with M-phase entry. This model for *deflated* function is also consistent with the findings of cell cycle progression in second instar larval brains. In brains from homozygous *defl*^{L} and *defl*^{P} individuals, cells were less

likely to be in M-phase, indicating that *deflated* is required for M-phase entry (Section 4.7.6). To test this hypothesis, further analyses of the genetic interactions that focus on DNA replication and M-phase entry would be required.

5.4.3 The *deflated* overexpression phenotype suggests a role for *deflated* in cell signalling

Many cells and tissues are able to tolerate *deflated* overexpression with no obvious developmental perturbations. Two copies of the cDNA transgene were required before phenotypes were observed and even then only the wing and the notum showed abnormalities (Figure 5.3). The wing phenotypes observed included extra veins, a reduction in the wing size, or a reduction in the intervein region. These phenotypes may well be due to perturbation of cell proliferation, however they are equally reminiscent of cell signalling phenotypes, particularly of member of Ras and Dpp pathways (Ralston and Blair, 2005).

The conclusion that *deflated* overexpression may perturb signalling pathways is further supported by the duplication of mainly scutellar macrochaetae when *deflated* is overexpressed. Macrochaetae are sensory organs and their specification and formation are under the control of a number of signalling pathways, including Notch, EGFR/Ras, Dpp, and Gbb (Culi et al., 2001; Tomoyasu et al., 1998; Wharton et al., 1999). The mild macrochaetae phenotype observed when *deflated* is overexpressed is most like that seen in *glass bottom boat* (*gbb*) mutants or when there is a slight reduction in EGFR signalling (Clifford and Schubach, 1989; Diaz-Benjumea and Garcia-Bellido, 1990; Wharton et al., 1999). Simarily to the *deflated* overexpression phenotype, *extramacrochaetae* mutants also display extra macrochaetae over the notum, due to its

role in regulating responsiveness to the proneural signals of *achaete* and *scute* (Cubas and Modolell, 1992). Its activity is regulated by both EGFR and Notch signalling. *emc* mutants also show ectopic wing veins and *emc* acts antagonistically with Ras during wing vein differentiation (Baonza and Garcia-Bellido, 1999). Since *deflated* and *emc* have similar phenotypes it is possible that they provide similar function or act in similar pathways. Both ectopic wing vein formation and macrochaetae duplication observed when *deflated* is overexpressed strongly supports a role for *deflated* in regulating cell signalling.

To test the role of *deflated* in cell signalling, *cactus* from the Toll signalling pathway, *dpp* from the Decapentapelgic (TGFβ) pathway and *nkd* from the Wingless pathway were tested for their ability to modify the *deflated* overexpression phenotype.

cactus was found to dominantly suppress the *deflated* wing phenotype (Table 5.1). Toll signalling is involved in dorsal/ventral patterning of the egg and embryo and in innate immunity (Hoffmann, 2003). CACTUS (an IκB homologue) binds DORSAL (an NF-κB homologue) and prevents it from entering the nucleus in the absence of signal (Kidd, 1992). Upon signalling, CACTUS becomes phosphorylated, ubiquitinated, and degraded by the proteosome. DORSAL is also phosphorylated and is able to enter the nucleus and transcriptionally activate ventral genes and suppress dorsal genes (Drier et al., 1999; Whalen and Steward, 1993). There is no evidence to suggest that the Toll pathway plays a role in wing development. However, CACTUS and DORSAL play Toll-independent roles, and seemingly cooperative roles, in muscle and neuromuscular junction development and function in larva and adults (Bolatto et al., 2003; Cantera et

al., 1999), so it is possible that the suppression of with *deflated* overexpression in the wing reflects an unknown function of *cactus*.

In mammals, NF-κB is activated in response to apoptotic signals. It acts in a protective capacity in response to tumour necrosis factor receptor, but can induce a pro-apoptotic response to p53 (Ryan et al., 2000). Other pathways can also activate NF-κB, including a Raf/MAPK-dependent but Ras-independent pathway (Ghoda et al., 1997; Schouten et al., 1997). While it is not known if DORSAL is involved in apoptotic pathways in *Drosophila,* it is formally possible that it may and the genetic interaction observed between *cactus* and *deflated* reflects this role. While it is difficult to know how *deflated* and *cactus* interact at this point, it can be concluded that *deflated* overexpression may result in increased CACTUS levels or may affect some other process that is ameliorated by reduced CACTUS levels.

Dpp signalling is required for proper wing vein formation, with the cross veins being most sensitive to Dpp loss. The *deflated* wing overexpression phenotype is similar to increased Dpp signalling (Conley et al., 2000) but it more closely phenocopies ectopic *gbb* driven by *ptc*-Gal4 (Khalsa et al., 1998). The protein encoded by *gbb*, like Dpp, is a BMP ligand and the relative abundance of the two is important for proper wing development, as demonstrated by the fact that ectopic *dpp* can counteract ectopic *gbb* (Khalsa et al., 1998). Therefore, the finding that *dpp* alleles dominantly enhance the *deflated* phenotype (Table 5.1 and Figure 5.5 E) is difficult to interpret if Dpp signalling is considered on its own. However, if the defect is an increase in Gbb signalling, then loss of *dpp* would exacerbate the phenotype as the balance between Dpp and Gbb activities would be further disrupted.

This postulated disruption of relative Dpp and Gbb levels could also explain the interaction with *naked cuticle* (Table 5.1). *naked cuticle* is an antagonist of the Wingless signalling pathway (Zeng et al., 2001) that can function to repress Dpp signalling in establishing dorso-ventral patterning (Johnston and Shubiger, 1996). Therefore halving the dose of *naked cuticle* will increase Wingless signalling, which would cause a reduction in Dpp signalling. Together these data support a conclusion that overexpressing *deflated* may perturb Dpp signalling either directly or indirectly.

5.4.4 *deflated* is an antagonist *of Krüppel*

The finding that *deflated* interacts antagonistically with *Krüppel* was unexpected. In *Drosophila, Krüppel* has been most studied in its role as a gap gene during embryogenesis (Pankratz and Jackle, 1993). It is required to specify segments during embryogenesis by repressing the expression of other gap and pair-rule genes. In mammals there are 16 Krüppel-like factors (Suske et al., 2005). Interestingly, some of them have opposing roles in regulating cell proliferation (Ghaleb et al., 2005). KLF4 acts as a negative regulator of cell proliferation, in part through the p53 pathway (Yoon et al., 2003), whereas KLF5 is a positive regulator of cell proliferation and mediates the oncogenic potential of Ras (Nandan et al., 2004). KLF2 represses the transcription of WEE1 in response to DNA damage, thereby preventing M-phase entry (Wang et al., 2005) and it also upregulates the cdk inhibitor p21 (Wu and Lingrel, 2004). KLF6 can also prevent cell proliferation by inhibiting Cyclin D1 (Benzeno et al., 2004). Unlike the mammalian proteins, there is no experimental evidence to suggest that *Drosophila* genes *Krüppel*, *Krüppel-homologue 1* or *Krüppel-homologue 2* have a role in the regulation of cell proliferation but shuch a role cannot be excluded.

The ability of overexpressed *deflated* to suppress the eye phenotype of *If*, the loss of wild type *deflated* to enhance the phenotype, and the ability of *Krüppel* to dominantly enhance overexpressed *deflated,* when taken together, implies an antagonistic relationship between the two gene products. It is possible that *Krüppel* antagonises *deflated* but, as the loss of *deflated* in the transheterozygotes does not on its own result in a rough eye phenotype, the *If* phenotype could not be due to reduced *deflated* function. Therefore, it is more likely that *deflated* antagonises *Krüppel*. This antagonism could be upstream of Krüppel, directed at Krüppel itself, or by activating Krüppel targets (Figure 5.6). It is unlikely that it is directed at Krüppel targets for two reasons. Firstly, it is expected that there are many Krüppel targets. Secondly, the targets are likely to differ between the eye and the wing. Since *deflated* has the same effects on Krüppel acitivity in two different tissues, it is unlikely to regulate all Krüppel targets. Alternatively, *deflated* could be upstream of *Krüppel*, for example affecting its transcription or translation, or it could directly affect *Krüppel* as an inhibitor by affecting its cellular localisation or its turnover. Further studies would be required to determine how *deflated* antagonises *Krüppel* and how this relates to the functional capacity of *deflated*. While it is possible that there is an unknown function for *Krüppel* in cell proliferation or cell signalling regulation, it is more likely that *deflated* affects Krüppel's known roles.

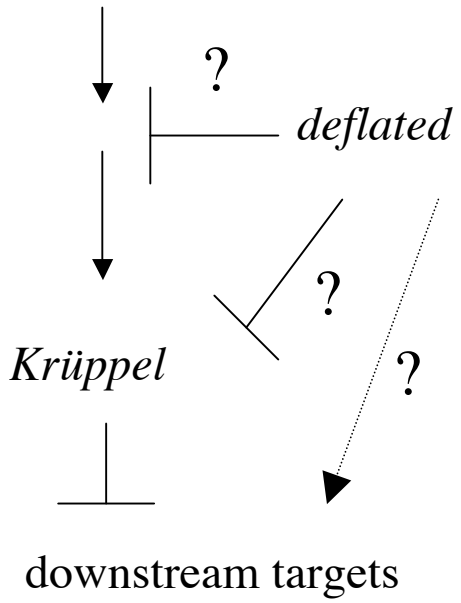


Figure 5.6 A proposed model for *deflated's* interaction with *Krüppel*.

The genetic data suggests an antagonistic role for *deflated* in *Krüppel* function. It is possible that *deflated* inhibits upstream factors or *Krüppel* itself, or activates downstream targets. The activation of targets is unlikely (see text), therefore it is shown as a dashed line.

In further support of an antagonistic role for *deflated* in *Krüppel* function is the finding that loss of *deflated* phenocopies a downstream target of Krüppel, *ken and barbie* (*ken*), which is repressed by Krüppel activity (Matyash et al., 2004). Mutations in *ken* result in the absence of external genitalia and the posterior abdominal segment, which is similar to what is seen in the *deflated* transheterozygotes (Figure 4.4). It is possible that the lack of genitalia in the *deflated* mutants is due to loss of inhibition of Krüppel, which would result in less KEN activity.

5.4.5 Genetic interaction studies provide information on how DEFLATED may be regulated

The findings that DEFLATED contains a putative 14-3-3 binding site and a putative SUMO modification site (Figure 4.2) and that overexpressed *deflated* interacts genetically with both *14-3-3* isoforms and *smt3* (Figure 5.5) indicate that DEFLATED may be directly regulated by these proteins. Furthermore, the interactions between three of the *deflated* alleles and *deflated* overexpression also provide some insight on how DEFLATED may be regulated.

The different abilities of the different *deflated* alleles to modify the overexpression of *deflated* allows for the speculation upon which regions of DEFLATED may mediate its own regulation. Many cells seem to tolerate excess *deflated* so if it is assumed that the overexpression phenotype in the wing is due to an inability of these cells to tolerate the excess *deflated* by not correctly regulating its normal activity then the genetic interactions may identify a region of negative regulation. Examination of the different alleles' abilities to modify the overexpression phenotype and the protein products that these alleles are predicted to produce should aid in identifying this regulatory region.

defl^{Z} was the only *deflated* allele that suppressed the overexpression wing phenotype. This is surprising because it is predicted to be the least truncated of the mutant proteins (Figure 5.7), only lacking 15 amino acids from the C-terminus and it might therefore be expected to provide near-wild type function. As this is was observed to not be the case it suggests that *defl*^{Z} protein behaves neither like the exogenous full length protein nor the endogenous wild type protein. Instead, the apparent activity of *deflated* is reduced, which results in an almost wild type wing. How this allele produces a seemingly antimorphic phenotype is currently unknown.

In contrast to *defl*^{Z}, *defl*^{L} was observed to not dominantly modify the *deflated* wing overexpression phenotype. This suggests that the predicted truncated protein encoded by *defl*^{L} is able to act like the wild type protein in this assay. *defl*^{P} does not reduce *deflated* function nor does it produce any more in this assay. As the DEFL^{L} protein appears to be regulated as though it were wild type endogenous protein it is likely therefore to contain the necessary regulatory region(s).

Surprisingly, *defl*^{P} was found to enhance the *deflated* wing overexpression phenotype. While is it formally possible that this surprising result may be due to an uncharacterised lesion not linked to the *deflated* locus, assuming that it is due to the *defl*^{P} suggests that the DEFL^{P} product behaves in a similar manner to the overexpressed protein. One explanation for this is that DEFL^{P} is lacking the necessary regulatory region(s) that act to hold the wild type protein in check. When these regulatory mechanisms are swamped by excess wild type protein, DEFLATED is no longer correctly regulated. When DEFL^{P} is also present there is even more protein being misregulated and the phenotype

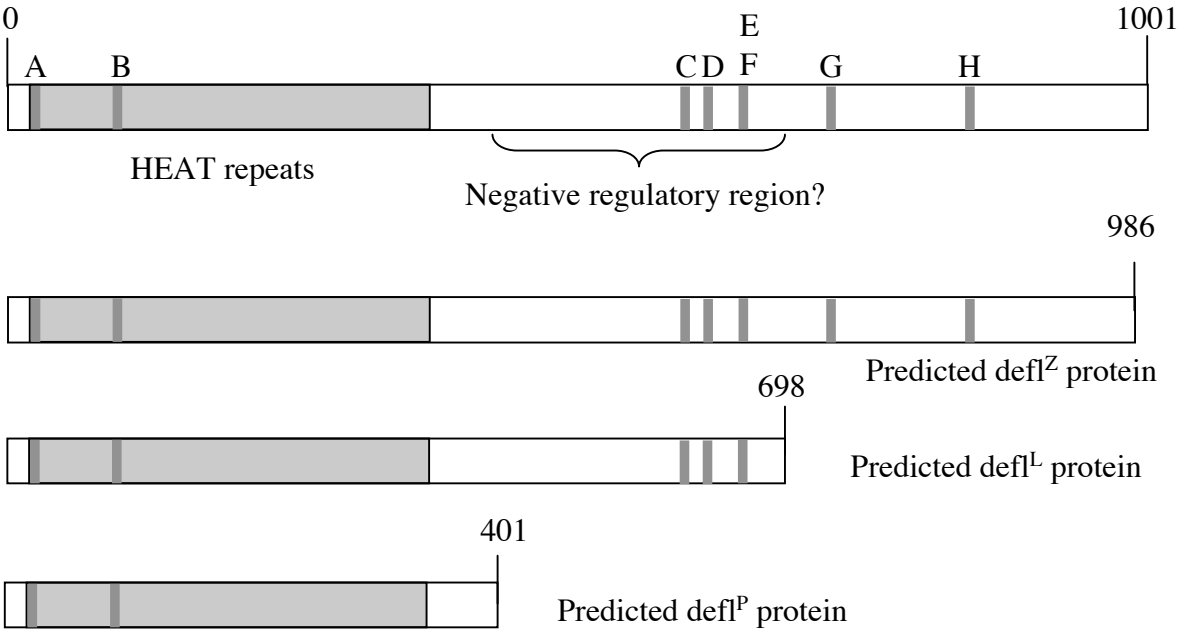


Figure 5.7 Schematic of the predicted truncated proteins for the three *deflated* alleles.

defl^{Z} is able to dominantly suppress the *deflated* overexpression phenotype despite the prediction that it encodes an almost full length protein product. *defl*^{L} does not modify, whereas *defl*^{P} enhances. This suggests that the region of the protein that is lacking in defl^{P} but present in defl^{L} (amino acids 401-698) may act to negatively regulate the protein, as shown by the bracket. The conserved motifs in this region are an adaptor protein complex interaction site (C), a CHK2 phosphorylation site (D), a 14-3-3 binding site (E), and a phosphorylation site for proline-directed kinases (F).

is worsened. Therefore, these regulatory regions are likely to lie in the region of the protein that is present in $DEFL^{L}$ (because it is still properly regulated) but are absent in $DEFL^{P}$ (because it is not properly regulated). Examination of the predicted protein products shows that this region comprises the amino acids 401-698 (Figure 5.7). Therefore, this region is likely to comprise a regulatory region. This region contains a number of conserved putative protein motifs; one of the adaptor complex binding sites, a Chk2 phosphorylation site, a 14-3-3 binding site, and a phosphorylation site for proline-directed kinases. Any of these sites, either separately or in combination, could serve to negatively regulate DEFLATED. Alternatively, the negative regulation could be due to another as yet unidentified motif.

The finding that *deflated* overexpression can be modified by halving the amount of 14-3-3 proteins indicates that the putative 14-3-3 binding site at amino acids 650-655 may be a genuine binding site that mediates this negative regulatory effect. Interestingly, it appears that the 14-3-3ε protein may act to negatively regulate DEFLATED function as *14-3-3ε* enhanced the *deflated* overexpression phenotype when heterozygous. It is possible that DEFLATED could also be positively regulated by 14-3-3ζ at this site, since halving its dose was able to suppress the excess *deflated* activity. If the 14-3-3 site is important (and sufficient) for the regulation of DEFLATED activity, then mutation at this site in an otherwise wild type protein should phenocopy the effects of *deflated* overexpression and the phenotype of the $defl^{P}$ allele.

While not recognised as being within the postulated regulatory region of amino acids 401-698, it is possible that DEFLATED may also be regulated by SUMO modification. SUMO modification generally acts to activate its targets. However, the genetic data

supports an interpretation that DEFLATED would be negatively regulated by sumoylation, as reducing the amount of SUMO resulted in a enhancement of the *deflated* overexpression phenotype. In *Drosophila,* SUMO and its conjugation and deconjugation machinery are localised to the nucleus in older embryos and S2 cells (Shih et al., 2001; Smith et al., 2004). As a result nearly all SUMO-conjugated proteins are nuclear. Recently it has been reported that sumoylation acts at the plasma membrane to regulate ion channel function (Rajan et al., 2005). Therefore, if DEFLATED is a SUMO target it is likely that it would localise to the nucleus but it is also possible that it would localise to the plasma membrane. Alternatively, the SUMO modification site may not be functional and the genetic interaction observed between *smt3* and *deflated* is caused by a reduction in the activation of some negative regulator of DEFLATED, causing the worsening of the phenotype observed. To differentiate between these alternatives biochemical analysis of DEFLATED will be required to test the involvement of SUMO conjugation in its regulation.

5.4.6 Conclusions about *deflated* function

The conclusions about *deflated* function that can be drawn from the genetic interactions described in this chapter are: (*i*) it interacts with *Cyclin E* and can act as a negative regulator of S-phase; (*ii*) it may be involved in linking DNA replication completion with M-phase entry; (*iii*) it appears to be a negative effector of Dpp signalling; (*iv*) it is an antagonist of *Krüppel*; (*v)* it may contain a negative regulatory region between amino acids 401 and 698, which may involve 14-3-3 binding at amino acids 650-655; and (*vi*) it may be modified by SUMO. Obviously these tests were not exhaustive, particularly in regards to examining the role of *deflated* in various signalling pathways. The genes and pathways examined were chosen in part because the alleles were readily

available in the lab or from the Bloomington Stock Centre and the results obtained suggest further genetic analysis is required to fully understand the role of *deflated* in these pathways. Apart from further genetic analysis, in depth cell biology and biochemical studies will be required to determine the mechanisms of *deflated* function and regulation.

Chapter 6- *deflated* is involved in Ras signalling

6.1 Introduction

Control of the cell cycle is coordinated with growth and development in multicellular organisms through signalling pathways. Some signalling pathways result in upregulation of cell proliferation, while some result in downregulation (Section 1.2). Therefore these signalling pathways play important roles during development and tumourogenesis. One pathway that has received much attention is the RTK/Ras signalling pathway since it plays important roles in differentiation and cell proliferation (Section 1.2.2). Disruption of this pathway can result in perturbation of cell differentiation as observed in *Drosophila* adult wings and eyes (de Celis, 2003; Simon et al., 1991), or it can result in the loss of cell proliferation regulation as observed in over 30% of human cancers (Chang et al., 2003). Therefore an understanding of this pathway and of its regulation will aid in understanding how cell proliferation and differentiation are coordinately controlled.

Some of the phenotypes displayed by *deflated* mutants and when *deflated* is overexpressed suggests that *deflated* may play a role in regulating cell signalling. These phenotypes include the loss of some of the L5 vein in the *deflated* transheterozgyote wings (Section 4.7.5) and the formation of extra vein material and the duplication of macrochaetae when *deflated* is overexpressed (Section 5.4.3). Furthermore, *deflated* interacts genetically with components of three different signalling cascades, Toll, Dpp, and Wg. Since *deflated* affects both cell proliferation and cell signalling, it is pertinent to know whether the role of *deflated* is primarily one of cell proliferation or cell signalling.

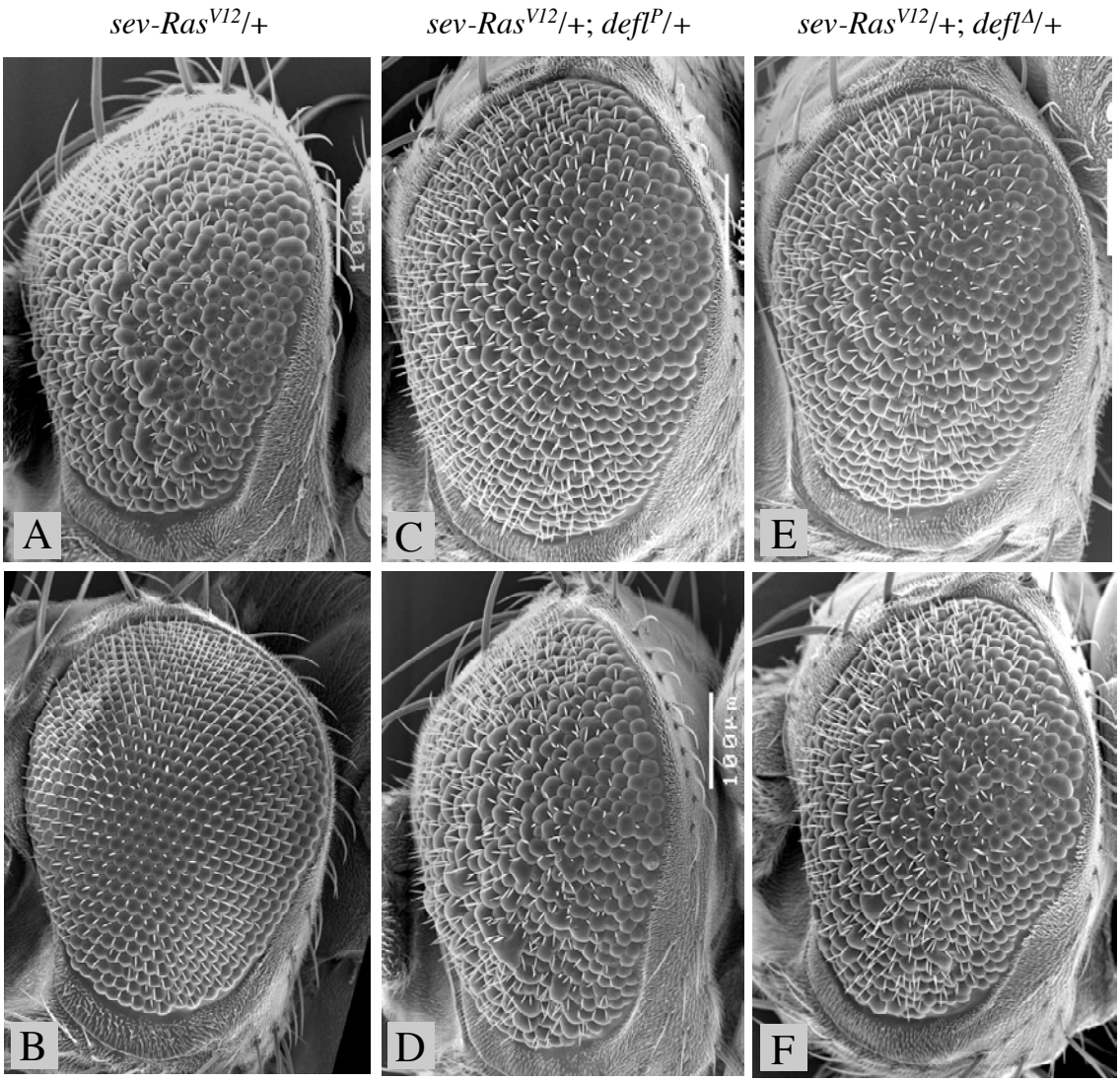


Figure 6.1 *defl*^{P} dominantly suppresses the rough eye phenotype of *sev-Ras*^{V12}.

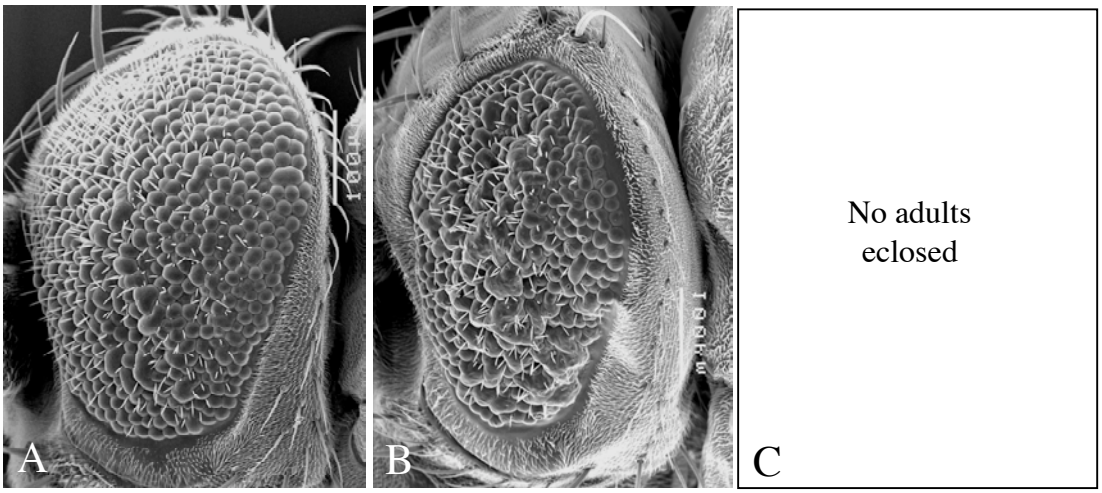
(**A**) *sev-Ras*^{V12} results in a rough eye phenotype due to increased recruitment of photoreceptor cells. The ommatidia are unevenly arrayed and some are increased in size. (**B**) Wild type eyes show an orderly array of ommatidia. (**C**) *defl*^{P}/+ is able to dominantly suppress the *Ras*^{V12} rough eye phenotype. Many ommatidia are decreased in size. The ommatidia are also more ordered. (**D-F**) *defl*^{Z}, *defl*$^{\Delta}$, and *defl*^{L} fail to dominantly modify the *Ras*^{V12} rough eye phenotype. The eyes are as disorganised and contain a similar number of enlarged ommatidia of similar severity as eyes from *sev-Ras*^{V12} individuals.

To further examine the role(s) of *deflated* in cell signalling, a *Drosophila* tumour model of activated Ras (Ras^{V12}) was used. Ras^{V12} is a constitutive allele that is unable to hydrolyse GTP, thus it remains in an active state. When Ras^{V12} is expressed in the developing eye under the *sevenless* promoter ($sev\text{-}Ras^{V12}$), a rough eye phenotype with disorganised and blistered-looking ommatidia results due to cone cells adopting an R7 photoreceptor fate (Fortini et al., 1992). The Ras^{V12} model has been used extensively to identify many of the members of the Ras signalling cascade through genetic modification of the rough eye phenotype (Chang and Rubin, 1997; Karim et al., 1996). Since in this model the only cells that are affected are post-mitotic and the outcome is differentiation, it was hoped that the function of *deflated* in signalling could be further investigated in a context where cell proliferation does not play a role.

6.2 Genetic interactions between Ras signalling components and *deflated*

6.2.1 *deflated* interacts genetically with Ras^{V12}

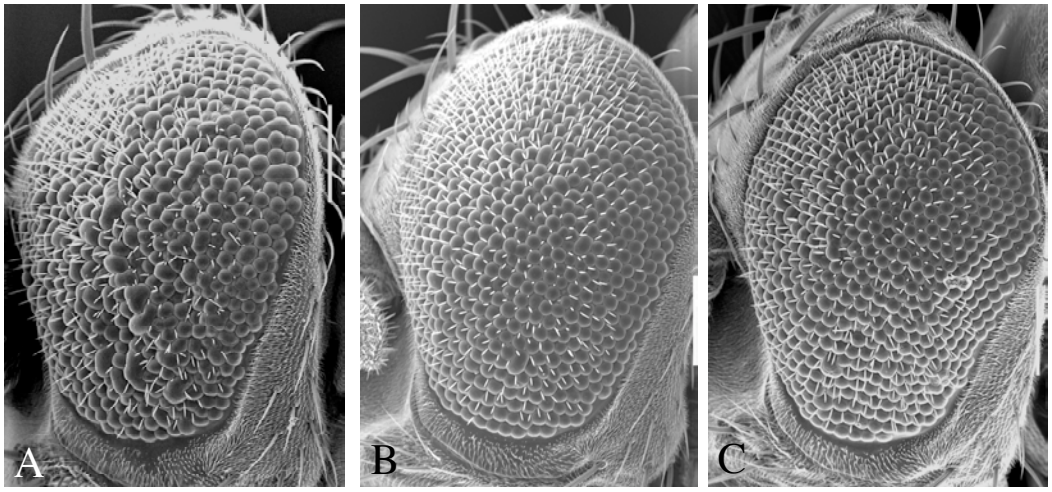
To test the effects of altered *deflated* expression on the Ras^{V12} eye phenotype, the four different *deflated* alleles ($defl^{Z}$, $defl^{L}$, $defl^{P}$ and $defl^{\Delta}$) were crossed into the Ras^{V12} background and their heterozygous effects on the Ras phenotype were assessed. $defl^{P}$ was able to suppress the rough eye phenotype producing ommatidia that were less disorganised and most were of normal size (Figure 6.1 B). Unexpectedly, $defl^{L}$, $defl^{Z}$, and $defl^{\Delta}$ failed to modify the $sev\text{-}Ras^{V12}$ rough eye phenotype (Figure 6.1 D-F). The resulting eyes were equally disorganised and contained as many enlarged ommatidia as the $sev\text{-}Ras^{V12}$ eye (Figure 6.1A). This result suggested that *deflated* may act downstream from activated Ras or in a parallel pathway that affects the Ras signalling outcome. Since the null allele of *deflated* ($defl^{\Delta}$) does not suppress the Ras phenotype



sev-Ras^{V12}/+ | *sev-Ras^{V12}*/+; *defl^{P}*/*defl^{Z}* | *sev-Ras^{V12}*/+; *defl^{L}*/*defl^{Z}*

Figure 6.2 The phenotype of *sev-Ras^{V12}* is enhanced in a *deflated* transheterozygous background.

(**A**) *sev-Ras^{V12}* results in a rough eye phenotype. (**B**) The expression of *sev-Ras^{V12}* in a *defl^{P}*/*defl^{Z}* transheterozygous background results in an enhancement of the rough eye phenotype. (**C**) *sev-Ras^{V12}*/+*; defl^{L}*/*defl^{Z}* flies fail to eclose, presumably due to strong enhancement of Ras signalling leading to lethality.



w^{1118}/+; +/+;
sev-Ras^{V12}/+

w^{1118}/+; *sev*-Gal4/+;
sev-Ras^{V12}/*UAS-defl*

w^{1118}/+; +/+;
sev-Ras^{V12}/*UAS-defl*

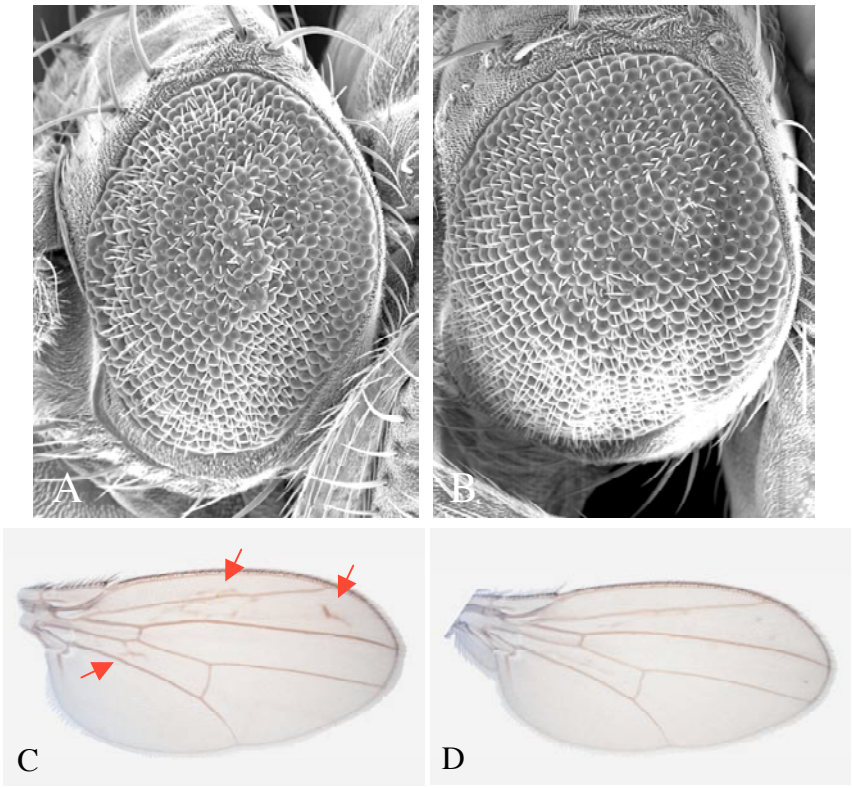
Figure 6.3 Overexpression of *deflated* suppresses the *sev-Ras*^{V12} eye phenotype.

(**A**) *sev-Ras*^{V12} results in a rough eye phenotype. (**B**) Expression of P[UAS-*defl*]AV3 by *sev*-Gal4 or (**C**) leaky expression of P[UAS-*defl*]AV3 in the absence of Gal4 suppresses the rough eye phenotype in a dose-sensitive manner.

(Figure 6.1 E), *defl*^{P} cannot be behaving as a null in this assay. Additional genetic data (see below) are consistent with *defl*^{P} being a recessive neomorphic or separation of function allele (discussed in detail in Chapter 7).

In order to understand the genetic relationship between activated Ras and the *deflated* alleles further, the effect of total loss of wild type *deflated* was assessed. As described in Section 4.4.2, 60% of the *defl*^{P}/*defl*^{Z} transheterozygotes make it to adulthood. When *sev-Ras*^{V12} is expressed in the *defl*^{P}/*defl*^{Z} background the resulting adults showed an enhancement of the *Ras*^{V12} eye phenotype (Figure 6.2 B). The *defl*^{L}/*defl*^{Z} combination is likely to provide less *deflated* function, since only 18% of the adults make it adulthood (Section 4.4.2). However, when *Ras*^{V12} was expressed in the *defl*^{L}/*defl*^{Z} background the pharate adults failed to eclose and all died as pupae, presumably due to even greater enhancement of Ras signalling. These data suggest that *deflated* may normally act as a negative regulator of Ras signalling, which is lost in the *deflated* mutant background. It is interesting to note that *defl*^{P} results in the enhancement of Ras signalling in a transheterozygous situation, whereas it is able to suppress Ras signalling when it is heterozygous.

To further extend the analysis, the effects of overexpressed *deflated* on the *Ras*^{V12} phenotype was assessed. Following on from the data above suggesting that *deflated* is a negative regulator of Ras signalling, it would be predicted that having extra DEFLATED protein may suppress the *sev-Ras*^{V12} eye phenotype. Indeed, overexpression of *deflated* did suppress the activated Ras eye phenotype in a dose-dependent manner (Figure 6.3 B and C). When P[UAS-*defl*] AV3 was overexpressed by *sev*-Gal4, driving expression in the same cells as *Ras*^{V12}, the eye was almost wild



sev-Gal4, rl^{SEM}*/ +; +/+* *sev-Gal4, rl*^{SEM}*/+; defl*^{P}*/+*

Figure 6.4 *defl*^{P} dominantly suppresses *rl*^{SEM}

(**A**) Expression of *rl*^{SEM} by *sev*-Gal results in a mild rough eye phenotype with the ommatidia showing moderate levels of disorganisation. (**B**) The ommatidial disorganisation of *rl*^{SEM} is dominantly suppressed by *defl*^{P}. (**C**) Low level expression of *rl*^{SEM} in the wings of flies results in a small amount of extra wing vein material between the longitudinal veins L2 and L3 and L4 and L5 near the anterior cross vein (arrows). (**D**) The ectopic wing vein material was also dominantly suppressed by *defl*^{P}.

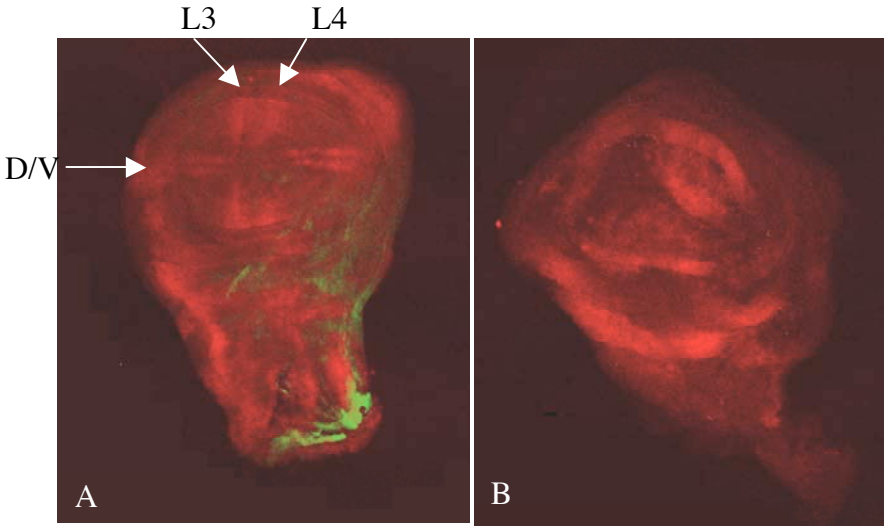
type and shows very mild ommatidial disorganisation (Figure 6.3 B). Leaky expression of the P[UAS-*defl*] AV3 transgene in the absence of a Gal4 driver also suppressed the rough eyes, though to a lesser extent (Figure 6.3 C). These data implicate *deflated* as a negative regulator of Ras signalling.

6.2.2 $defl^{P}$ dominantly suppresses the MAPK allele rl^{SEM}

If *deflated* acts as a negative regulator of Ras signalling then it be predicted to also interact with other Ras signalling cascade components such as MAPK (Section 1.2). To test this, $defl^{P}$ was crossed into a rl^{SEM} background. rl^{SEM} is a hypermorphic allele of *rolled*, the gene that encodes MAPK. This allele results in both a mild eye and wing phenotype. Increased Ras signalling in the eyes, caused by rl^{SEM}, results in enhanced recruitment of R7 cells, resulting in a rough eye phenotype (Figure 6.4 A). Increased Ras signalling in the wing results in ectopic vein formation between L2 and L3 and around the anterior cross vein (Figure 6.4 C). $defl^{P}$ was found to be able to suppress both the eye and wing phenotypes of the rl^{SEM} allele. The eyes were more organised (Figure 6.4 B) and the wings no longer contained the ectopic wing veins (Figure 6.4 D). Since the rl^{SEM} allele is still capable of responding to upstream signals, the suppression observed does not allow *deflated* to be placed either upstream or downstream of MAPK. However, the ability of $defl^{P}$ to suppress the rl^{SEM} phenotypes shows that the effects of *deflated* are not restricted to activated Ras and that *deflated* can consistently modify the Ras signalling pathway.

6.3 Overexpression of *deflated* affects the activation of MAPK

Since *deflated* can affect Ras signalling it was possible that the ectopic vein material observed in wings overexpressing *deflated* (Figure 5.3) was due to perturbation of Ras



MS1096-Gal4; CyO(Act-GFP) *MS1096-Gal4; 2x defl*

Figure 6.5 *deflated* ovexpression disrupts MAPK activation

Third instar wing imaginal discs stained with antibody to pMAPK (red; Sigma) and anti-GFP (green; Molecular Probes). (**A**) Wing imaginal disc from a control sibling larva expressing the GFP balancer. pMAPK is expressed flanking the dorso-ventral boundary (D/V) and the presumptive longitudinal veins L3 and L4. (**B**) Wing imaginal disc from a larva expressing P[UAS-*defl*]BB1 and P[UAS-*defl*]BA2 driven by MS1096-Gal4. The wing disc shows abnormal morphology. The characteristic pMAPK staining pattern is also lost, replaced by staining in rough concentric circles.

signalling. To examine this, third instar wing discs from larvae overexpressing two copies of the wild type *deflated* cDNA (*MS1096-Gal4*; *P[UAS-defl]BB1*, *P[UAS-defl]BA2*/+) and their control siblings (*MS1096-Gal4*; *CyO (Actin-GFP)*/+) were simultaneously fixed and stained for activated MAPK. The antibody used (di-phosphorylated ERK-1 and 2; Sigma) only recognises the di-phosphorylated activated form of MAPK so is an excellent marker for activation of Ras signalling. Wing imaginal discs expressing normal levels of *deflated* were distinguished from their *deflated* overexpressing siblings by co-staining with anti-GFP. In wild type (GFP-positive) third instar wing discs, pMAPK staining was found to occur in a characteristic pattern flanking the dorso-ventral boundary and along the presumptive veins L3 and L4 (Figure 6.5 A).

Wing discs in which *deflated* was overexpressed (GFP-negative) showed abnormal morphology and an altered pattern of pMAPK (Figure 6.5 B). pMAPK fluorescence was found in a pattern of rough concentric circles, but as the morphology of the discs was abnormal it was difficult to ascertain which regions were expressing pMAPK. It is clear that when *deflated* is overexpressed, MAPK is not strongly activated in the presumptive margin or veins. These observations suggest that the overexpression of *deflated* blocks the Ras signalling pathway upstream of MAPK, resulting in no MAPK activation in the presumptive wing margin and vein cells. Instead, MAPK activation occurs in other inappropriate cells. It is interesting to note that for such a severe effect upon disc morphology and Ras signalling, the resulting adult wing phenotype appears to be mild (Figure 5.5 B).

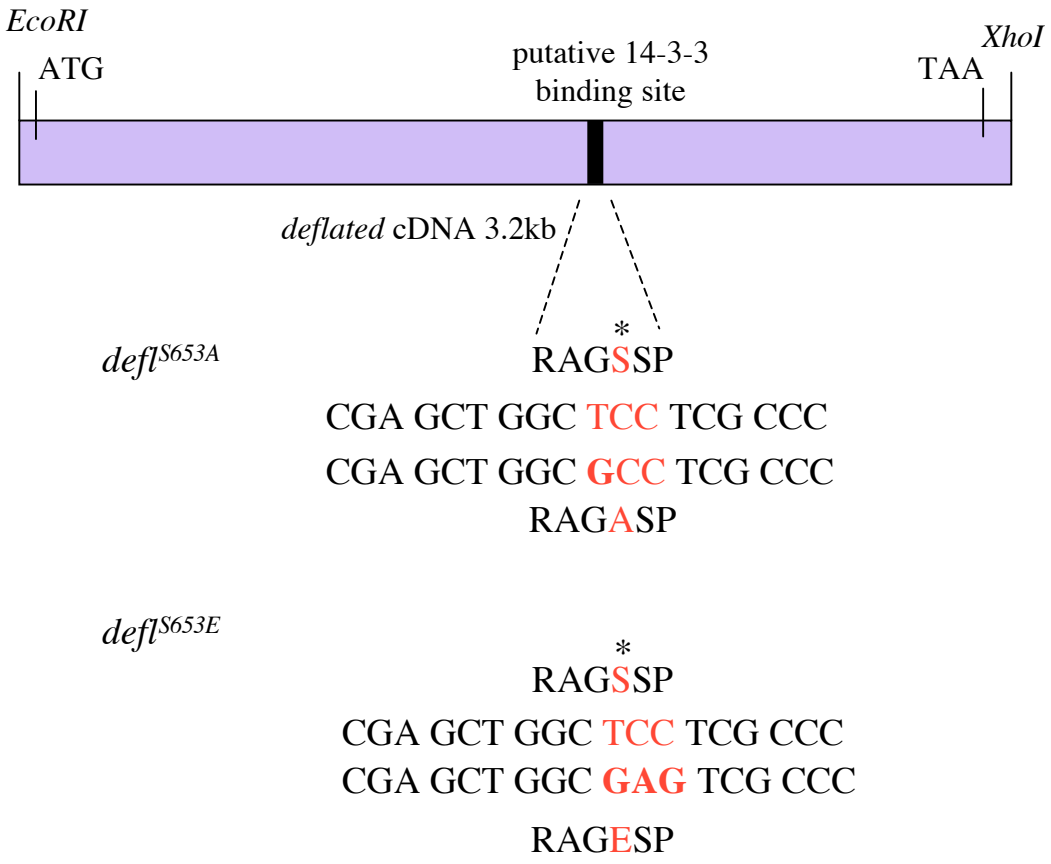


Figure 6.6 Scheme of the amino acid substitutions within the conserved putative 14–3-3 binding site at 650-655.

The position and amino acids of the putative 14-3-3 binding site encoded within the *deflated* cDNA are shown. Amino acids substitutions and their required nucleotide changes are shown. The serine predicted to be phosphorylated in the consensus (shown in red) was mutated to either an alanine or a glutamic acid by introducing the nucleotide changes (shown in bold) by site directed mutagenesis. Both changes resulted in loss of a *BseR1* restriction site, thus allowing for easy identification of mutagenised plasmids.

6.4 The role of the putative 14-3-3 binding site of DEFLATED in Ras signalling

In the previous chapter evidence was presented for a putative negative regulatory region within DEFLATED between amino acids 401 and 698 (Figure 5.7). Since *defl*^{P} is the only *deflated* allele that can suppress the *Ras*^{V12} phenotype and DEFL^{P} is predicted to lack this region, it is possible that this regulatory region may contribute to the genetic interactions with *Ras* and *rl* described above. In terms of Ras signalling, the two motifs that seem most likely to be capable of mediating the interactions with Ras and MAPK are the putative 14-3-3 binding site at 650-655 and the putative MAPK phosphorylation site at 651-657 (Figure 4.2 and Figure 5. 7). The putative 14-3-3 binding site was of interest because 14-3-3 alleles interact genetically with *deflated* (Figure 5.5) and these proteins have an established role in Ras signalling (1.2.1).

6.4.1 Mutation of S653 to A causes a dominant phenotype when overexpressed

14-3-3 proteins generally bind phospho-serine or threonine residues within the consensus RSXpS/TXP (mode 1) or RXXXpS/TXP (mode 2). However, 14-3-3 proteins have been reported to bind to other sites, including those that are not phosphorylated (Dougherty and Morrison, 2004). The putative 14-3-3 binding site in DEFLATED is 650 RAGSSP 655 (Figure 4.1). Therefore S653 was predicted to be the crucial phosphorylatable residue in mediating 14-3-3 binding.

To test whether DEFLATED's conserved putative 14-3-3 binding site has an effect on Ras signalling, *in vitro* site-directed mutagenesis of S653 was performed. Two constructs were generated. In one the serine at 653 was replaced by an alanine to potentially prevent phosphorylation at this site and prevent 14-3-3 binding (*defl*^{S653A}; Figure 6.6). The second construct generated, a glutamic acid substituted for serine 653,

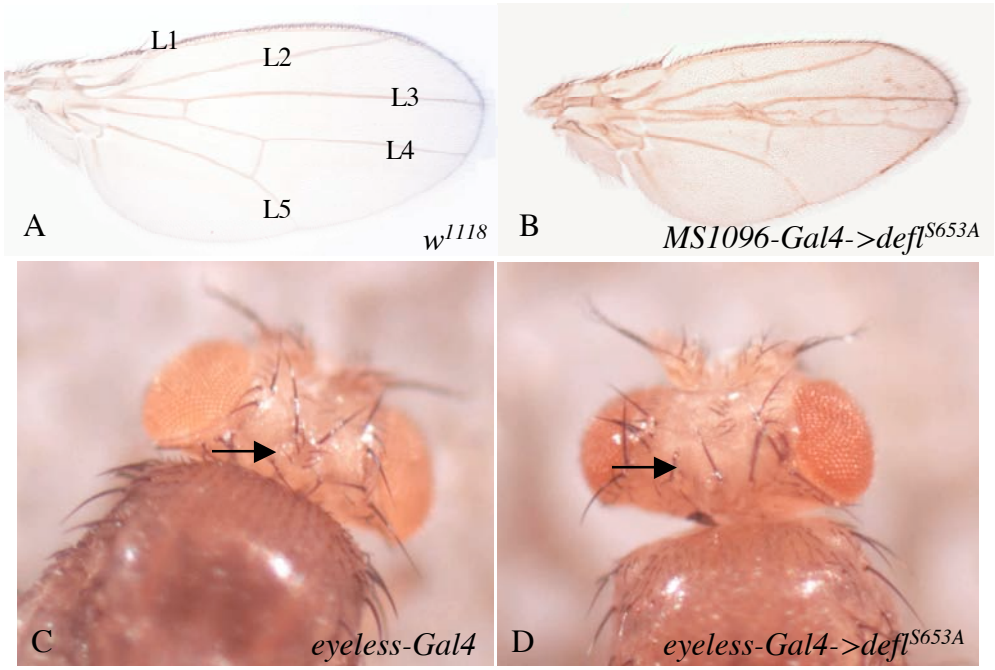


Figure 6.7 Overexpression of *defl*^{S653A} results in dominant phenotypes

(**A**) Wild type wing with the longitudinal veins labelled L1-L5. (**B**) Expression of P[UAS-*defl*^{S653A}]AB1 by MS1096-Gal4 results in a reduced wing with loss of intervein region between L3 and L4 and the formation of ectopic veins. (**C**) Wild type head, showing the tripartite formation of ocelli at the back of the head (arrow) and the normal bristle morphology. (**D**) Expression of P[UAS-*defl*^{S653A}]AB1 by *eyeless*-Gal4 results in a reduction of head volume, loss of occelli (arrow), and malformed bristles, a phenotype referred to as “hammerhead”.

which would potentially mimic constitutive phosphorylation (*defl*^{S653E}; Figure 6.6). These mutations were made via site-directed mutagenesis and cloned into the Gal4 responsive pUAST vector (Section 2.16.3). Transgenic flies containing both of these constructs were generated. Thirteen independent transgenic lines of P[UAS-*defl*^{S653A}] and five independent transgenic lines of P[UAS-*defl*^{S653E}] were generated.

To characterise the effects of *defl*^{S653A} and *defl*^{S653E} expression compared to wild type expression, a series of Gal4 drivers were used. Most tissues were tolerant of excess wild type DEFLATED since two copies were required for a phenotype to be readily observed (Section 5.3.1). In general, tissues were more sensitive to *defl*^{S653A} than to wild type *deflated*. One copy of P[UAS-*defl*^{S653A}] expressed at 29°C resulted in phenotypes in the wing, head, and thoracic bristles in at least two of the seven independent lines tested. When P[*UAS-defl*^{S653A}]AR1 or AB1 were used to overexpress *defl*^{S653A} in the wing using MS1096-Gal4, extra wing veins, a reduction in intervein regions, and a slight curl was observed (Figure 6.7 B). Overexpression of AR1 or AB1 by *eyeless*-Gal4 resulted in an abnormal 'hammerhead' phenotype, which was more severe in males. This phenotype was characterised by a marked decrease in head volume at the back of the head, a lack of occelli, and a disruption and thickening of the macrochaetae (Figure 6.7 D). When P[UAS-*defl*^{S653A}]AB1, T2, or AJ1 were overexpressed by *nos*-Gal4, thoracic bristles were frequently duplicated (data not shown), similar to what was observed when two copies of wild type *deflated* was overexpressed (Figure 5.3). No perturbations in eye development was observed when *defl*^{S653A} was overexpressed using GMR-Gal4 or *sev*-Gal4 and no other Gal4 driver lines were tested. These indicate *defl*^{S653A} behaves as a dominant allele since overexpression caused phenotypes that were either more severe or not observed when wild type *deflated* was overexpressed. These

data also show that S653 is an important residue in normal *deflated* function and is likely to be required for regulation of DEFLATED.

In contrast to the effects of $defl^{S653A}$, overexpression of one copy of $defl^{S653E}$ using *nos*-Gal4, *eyeless*-Gal4, *sevenless*- Gal4, or GMR-Gal4 29ºC did not produce any abnormal phenotypes (three independent lines tested). P[UAS-$defl^{S653E}$]H2 did show duplication of thoracic macrochaetae, but as this was the only line that produced this phenotype, it is difficult to ascertain whether the effect is general or due to transgene position effects. These data imply that $defl^{S653E}$ is likely to act as a recessive allele and that the S to E substitution mimicking phosphorylation at this site has little or no effect on endogenous DEFLATED protein function.

6.4.2 Mutation of S653 to A or E produces a protein that cannot provide the necessary function throughout development

To test whether the $defl^{S653A}$ and $defl^{S653E}$ encoded proteins could provide full wild type function, transgenes carrying these mutant alleles were assessed for their ability to rescue the second instar lethality of $defl^{L}/defl^{L}$ and $defl^{P}/defl^{P}$ individuals. Since there was no need to drive expression of the *deflated* cDNA transgenes (Section 4.5), two $defl^{S653A}$ and one $defl^{S653E}$ second chromosome lines were crossed into the homozygous $defl^{L}$ and $defl^{P}$ backgrounds in the absence of a Gal4 driver. All three transgenes failed to rescue the second instar lethality of homozygous $defl^{L}$ or $defl^{P}$ individuals. Therefore, mutating the serine at position 653 to either an alanine or a glutamic acid results in a protein that seems to be unable to complement $defl^{P}/defl^{P}$ or $defl^{L}/defl^{L}$ lethality. Taken together, these data show that the S653 is a crucial residue for normal DEFLATED function.

6.5 The subcellular localisation of DEFLATED::GFP

6.5.1 Generation and analysis of flies expressing DEFLATED::GFP

To examine DEFLATED's subcellular localisation, a construct was generated in which the Green Fluorescent Protein (GFP) coding sequence was fused in frame to the C-terminus of wild type *deflated* cDNA (Section 2.16.2). This gene fusion was cloned into both pUAST and pUASP to allow for high levels of expression in both somatic and germline tissues, respectively. Nine independent transgenic lines of pUAST-*defl::GFP* and five independent lines of pUASP-*defl::GFP* were established. These lines were characterised to ensure that they retained the same function as the untagged wild type transgenes. Each transgene was first overexpressed using various Gal4 driver lines at 29°C. In all instances phenotypes that were observed were of the same type and severity as when untagged *deflated* cDNA was overexpressed. These transgenes were then crossed into homozygous $defl^{\Delta}$ and $defl^{P}$ backgrounds to assess their ability to rescue the second instar homozygous lethality of these alleles. All transgenes tested were capable of rescue. Two independent pUAST-*defl::GFP* lines (AF2 and C2) were found to fully rescue $defl^{L}/defl^{L}$, so that a healthy stock without the TM6B balancer could be established. One pUASP-*defl::GFP* line (BI1) could rescue $defl^{\Delta}/defl^{\Delta}$ to the pupal stage, lethality at which stage would most likely be due to homozygosity of the *nbs* deletion (Section 4.5). These data show that tagging the wild type DEFLATED protein C-terminally with GFP results in a fusion protein that seems not to differ from the wild type protein in function.

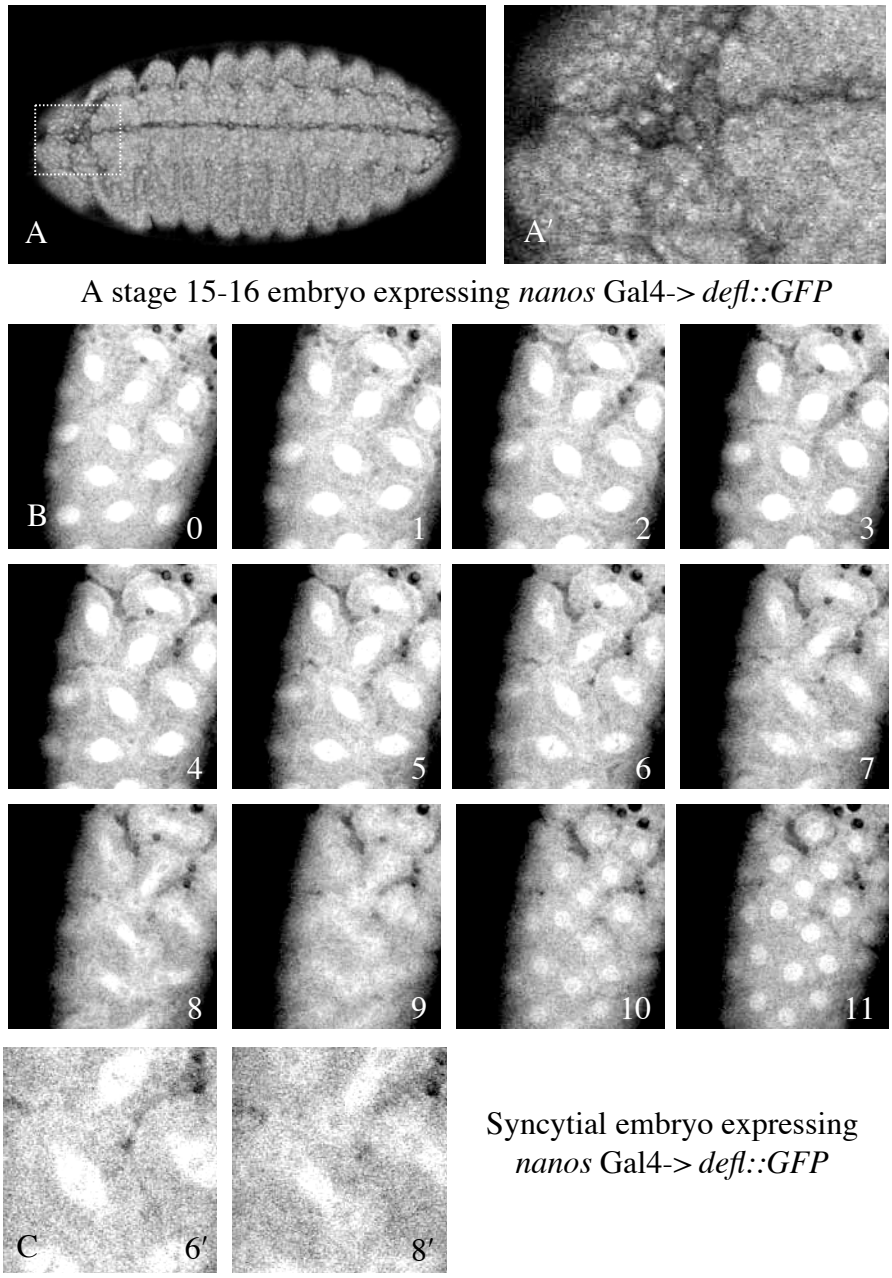


Figure 6.8 Subcellular localisation of DEFLATED::GFP in embryos.

(**A**) DEFL::GFP, expressed by *nanos*-Gal4::VP16 induced expression of P[UAS-*defl::GFP*]AH1, localises throughout the cell, with some cells showing strong nuclear localisation. (**A′**) A higher magnification of the region boxed in A. (**B**) During mitosis DEFL::GFP localises to the mitotic spindle and to the midbody after the metaphase to anaphase transition. DEFL::GFP was not found localised to chromosomes, seen as the black shadow at the metaphase equator (6 min timepoint). The images are a series of time lapse images. The number in the bottom right hand corner indicates the number of minutes after the first image was captured. (**C**) Higher magnification showing DEFL::GFP localisation to mitotic spindles but not to chromosome and to the spindle midbody in late anaphase.

6.5.2 DEFLATED::GFP shows strong nuclear and mitotic spindle localisation in embryos

To examine the intracellular localisation of DEFLATED::GFP, embryos expressing P[*UASP-defl::GFP*] AH1 driven by *nanos*-Gal4 were collected, manually dechorionated, mounted on double sided tape in halocarbon oil and examined by live confocal microscopy. In the embryos examined, DEFLATED::GFP was found not perturb development. The fusion protein localised strongly to the nucleus and less strongly to the cytoplasm at all stages of embryonic development (Figure 6.8 A and B).

Examination of the intracellular dynamics of DEFLATED during syncytial and cellularised mitoses revealed that DEFLATED::GFP localisation varied with cell cycle stage (Figure 6.8 B). During interphase the fusion protein was mainly nuclear, and upon nuclear envelope breakdown a large percentage of the protein became cytoplasmic. A proportion of the protein, however, localised to the mitotic spindle in prometaphase and metaphase and was excluded from the condensed chromosomes (seen as a dark shadow at the metaphase equator (Figure 6.8 B and C, 6 min timepoint) . During anaphase, DEFLATED::GFP localised to the midbody (Figure 6.8 B and C, 8 min timepoint). Attempts to fix these embryos and detect the DEFLATED::GFP with an anti-GFP antibody were unsuccessful, which indicates that DEFLATED is very sensitive to fixation conditions.

6.5.3 DEFLATED::GFP localisation in wing imaginal discs

The localisation of DEFLATED::GFP was also examined in third instar imaginal discs. In light of the sensitivity of DEFLATED to fixation conditions, wing discs expressing DEFLATED::GFP (driven by MS1096-Gal4) were dissected and mounted live in PBS

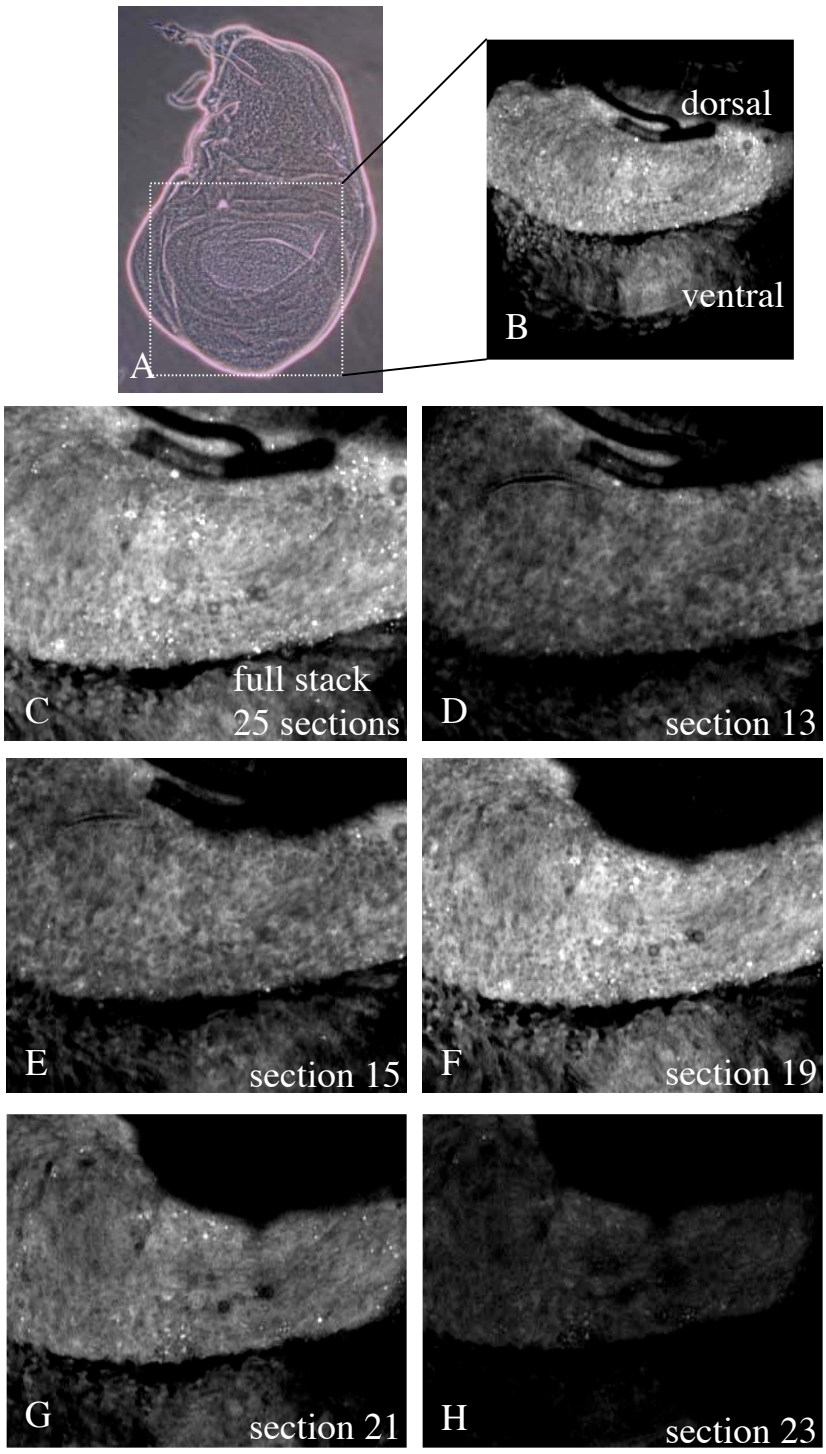


Figure 6.9 DEFLATED::GFP localises to the apical surface in third instar wing imaginal discs

(**A**) A wild type wing imaginal disc (from Figure 4.5) showing the analogous region of the wing pouch magnified in B. (**B**) A third instar wing imaginal disc showing the subcellular localisation of DEFLATED::GFP. The image is a projection of 25 optical sections through the disc. (**C**) The same wing disc shown increased in size to show the localisation in the dorsal compartment more clearly. (**D-H**) Individual optical sections of the disc shown in C. The basal membrane was at section 1 and the apical membrane was at section 25. Most of the bright foci were found in sections 17-21, indicating that they were found just under the apical surface.

and rapidly examined by confocal microscopy. Multiple optical sections of the wing disc were taken and then projected to form a single image (Figure 6.9 B and C). Expression driven by MS1096 Gal4 occurs predominantly in the wing pouch (which forms the adult wing blade, data not shown) and this is where DEFLATED::GFP expression was observed. Unlike embryos, the localisation of DEFLATED::GFP was not strongly nuclear with most cells showing a diffuse pattern of localisation. Fluorescence was stronger in the dorsal compartment of the wing disc, and optical sections showed puncta of strong fluorscence close to the apical surface of the disc (Figure 6.9 D-I).

6.6 Discussion

In this chapter a number of genetic approaches were taken to further investigate the role that *deflated* plays in cell signalling. The effects of *deflated* on Ras signalling was examined. These data show that *deflated* has a negative role in regulating Ras signalling. Since the *deflated* wing overexpression phenotype is reminiscent of perturbations of Ras signalling, the effect that *deflated* has on MAPK activation was assessed. Taken together, these data suggest that DEFLATED is likely to act downstream of Ras but upstream of MAPK. Examination of the role that the putative 14-3-3 binding site of DEFLATED indicates that the conserved serine 653 is crucial for proper function. To further understand the roles that *deflated* plays in cell proliferation and cell signalling, a fusion protein with GFP was generated and this fusion protein was used to observe the subcellular localisation of DEFLATED.

6.6.1 The role of *deflated* in Ras signalling

The genetic interactions observed between *deflated*, Ras^{V12} and $rolled^{SEM}$ demonstrate that *deflated* is involved in the Ras signalling pathway. The data obtained was unable to distinguish between the possibility that DEFLATED is a component of the pathway, or has a role in another cellular process that affects the outcome of Ras signalling. The data are consistent with DEFLATED acting as a negative regulator of Ras signalling since the Ras^{V12} phenotype was enhanced in $defl^{P}/defl^{Z}$ transheterozygotes (Figure 6.2) and suppressed by *deflated* overexpression (Figure 6.3). While these data are consistent with a role as a negative regulator, they do not categorically show that DEFLATED is a negative regulator acting in the Ras pathway. To address this, mitotic clones of $defl^{\Delta}$ would be required to properly assess the role of DEFLATED as a negative regulator. These clones would contain no DEFLATED protein and therefore Ras signalling could be examined in these cells. If DEFLATED is normally a negative regulator of Ras signalling then it would be expected that Ras signalling would be enhanced in these clones. If DEFLATED does not normally act upon the Ras pathway then Ras signalling should be unaffected.

In the absence of data obtained from mitotic clones it is still likely that DEFLATED is required to negatively regulate Ras signalling. The findings that both $defl^{P}$ and *deflated* overexpression suppress Ras signalling, indicates that Ras signalling is negatively regulated under these circumstances. While the expression and stability of $DEFL^{P}$ still requires confirmation, the genetic data indicate that the protein encoded by $defl^{P}$ and the exogoneous DEFLATED protein appear to behave as neomorphs, separation of function, or dominant negative proteins. Assuming that the genetic interaction observed is not due to another unlinked lesion on the $defl^{P}$ chromosome, it is unlikely that $defl^{P}$

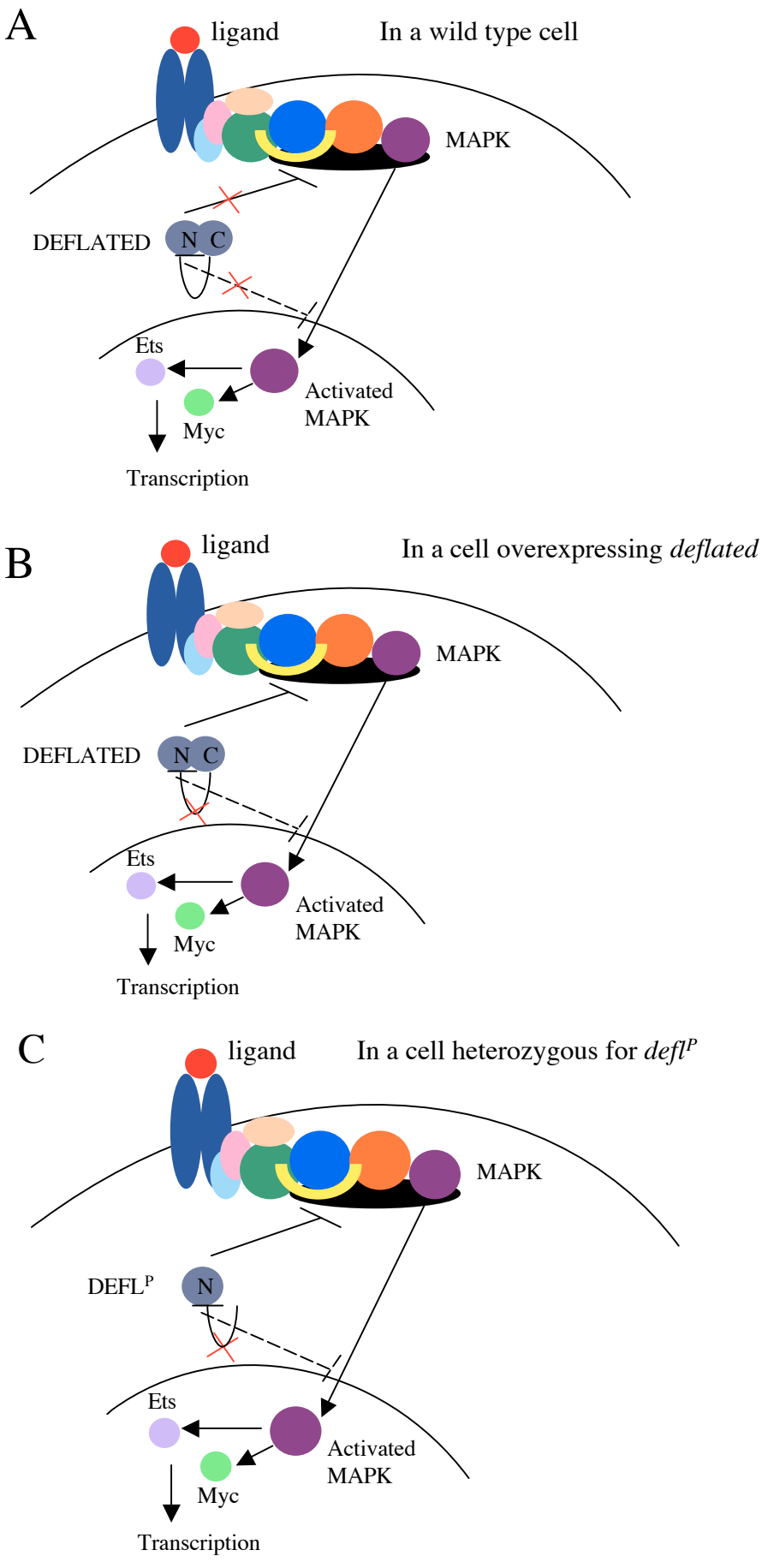

A
ligand
In a wild type cell
MAPK
DEFLATED
N
C
Ets
Activated
MAPK
Myc
Transcription
B
ligand
In a cell overexpressing *deflated*
MAPK
DEFLATED
N
C
Ets
Activated
MAPK
Myc
Transcription
C
ligand
In a cell heterozygous for *defl*^{P}
MAPK
DEFL^{P}
N
Ets
Activated
MAPK
Myc
Transcription

Figure 6.10 A hypothetical model for DEFLATED function in Ras signalling

(**A**) In a wild type cell Ras signalling is not affected by DEFLATED, due to the regulatory region in the C-terminus (a.a. 401-968) negatively regulating the N-terminus. (**B**) When *deflated* is overexpressed the excess DEFLATED is no longer negatively regulated and the protein can suppress Ras signalling at either the plasma membrane (or endosome) and prevent the subsequent activation of MAPK or by preventing activated MAPK translocating into the nucleus. This second possibility is less likely for reasons explained in the text and therefore is represented by the dashed line. (**C**) When $DEFL^{P}$ protein is present it cannot be negatively regulated as it lacks the C-terminus. This truncated protein therefore is capable suppressing Ras signalling. See Figure 1.6 for an explanation of the proteins involved in the Ras signalling cascade.

acts in a dominant negative manner as the associated lethality is easily rescued by both wild type and GFP tagged protein. Therefore, $DEFL^{P}$ and exogenous DEFLATED have either new functions or misregulation of the normal DEFLATED function. This implies that the N-terminus of DEFLATED encodes a functional domain and the C-terminus a regulatory domain. When DEFLATED is truncated and no longer negatively regulated by the N–terminus it may be free to perform its normal function, which in the case of Ras signalling is to negatively regulate the pathway. Therefore, the protein domains or motifs that mediate DEFLATED's interaction with the Ras signalling cascade must lie in the N-terminus.

From the data obtained DEFLATED could affect the Ras pathway at two different points in the signalling cascade. The first is at the plasma membrane where Raf, MEK, and MAPK are recruited and activated (Section 1.2.1). The second is when activated MAPK is translocated into the nucleus and activates its target transcription factors. These two alternatives are shown in the hypothetical model in Figure 6.10.

Since *defl*^{P} and wild type *deflated* overexpression can suppress activated Ras, it can be concluded that *deflated* is genetically downstream from *Ras*. The overexpression of *deflated* also results in an abnormal patterning of pMAPK activation, consistent with *deflated* acting upstream of pMAPK. Therefore, DEFLATED is likely to affect the activation of Raf, MEK, or MAPK at the plasma membrane. This interpretation is supported by the finding that DEFLATED::GFP localises to the apical surface of wing imaginal discs. The activation (and endocytosis) of EGFR (the receptor upstream of Ras in most *Drosophila* cells) occurs at the apical surface of wing imaginal discs (Weber et al., 2003). Activated Ras and activated downstream effectors are found on

endosomes (Di Guglielmo et al., 1994; Oksvold et al., 2000), indicating that endocytosis and signalling are closely linked. Endocytosis involves the recognition of cargo proteins by the adaptor protein complex, which then recruits clathrin to form clathrin coated pits and vesicles (Mousavi et al., 2004). DEFLATED could be involved in endocytosis since it contains an evolutionarily conserved clathrin box in its N-terminus and two putative binding sites for the μ subunit of the adaptor protein complex (Figure 4.2). The reasoning that the N-terminus must mediate the interaction with Ras signalling is consistent with a model of DEFLATED function where DEFLATED has a role in endocytosis and it is in this role that DEFLATED can act to suppress Ras signalling.

An alternative model for DEFLATED function arises from the finding that the HEAT repeats located in the N-terminus of DEFLATED are predicted to be structurally similar to those known to bind Ran (Section 4.2), which suggests another mechanism for the involvement of DEFLATED in the Ras signalling pathway. The Ran-GTPase pathway is involved in the nuclear import and export of proteins (Moore, 2001; Pemberton and Paschal, 2005), including activated MAPK. In the case of activated MAPK, importin 7 (encoded by *moleskin* in *Drosophila*) and importin β (encoded by *ketel*) are required for nuclear import (Lorenzen et al., 2001). It is therefore possible that DEFLATED may act as a nuclear import factor. Like importin 7, DEFLATED contains a putative Ran binding site in the N-terminus, and is about the same size as importin 7 (1001 a.a. for DEFLATED compared to 1049 a.a. for importin 7). DEFLATED could be an import factor for MAPK and therefore loss of regulation results in it being unable to import MAPK into the nucleus. On the other hand, DEFLATED may not normally bind MAPK at all, but upon loss of its wild type regulation it is able to bind MAPK and

prevent it from being imported into the nucleus by importin 7 / importin β. A second alternative may involve DEFLATED as a conditional binding partner of importin β regulated by DEFLATED's putative regulatory region (amino acids 401-698). Loss of this normal regulation could then cause DEFLATED to constitutively bind importin β, preventing it from binding its other partners. In the case of Ras signalling, this could prevent the import of MAPK and therefore suppress both the Ras^{V12} and rl^{SEM} phenotypes. Although this last model is plausible, it is not entirely consistent with the pMAPK pattern observed in the wing imaginal discs. If DEFLATED is involved in the nuclear translocation of MAPK then the pattern of staining should be similar to wild type, with the possibly of a greater intensity of staining because MAPK is not translocating into the nucleus and pMAPK staining is thought to reflect its cytoplasmic localisation (Kumar et al., 2003). Since *deflated* overexpression causes a change in the pattern of MAPK activation, it is more likely that the effect occurs upstream of MAPK. Therefore the hypothesis that DEFLATED affects Ras signalling by regulating endocytosis is at this point more plausible.

6.6.2 Possible regulation of DEFLATED function in Ras signalling

In the previous chapter it was reasoned that there is a putative negative regulatory region between amino acids 401-698, which contains the conserved putative 14-3-3 binding site (Section 5.4.5). Since $defl^{P}$ was able to enhance *deflated* overexpression, $DEFL^{P}$ is likely to not be regulated correctly, therefore the absence of this region in the $DEFL^{P}$ protein and presence in $DEFL^{L}$ identified this region as a putative regulatory region. A similar finding was found for the effects of *deflated* upon Ras signalling. When overexpressed or when $defl^{P}$ was a heterozygote, *deflated* was capable of suppressing Ras signalling. Therefore, it is possible that the loss of the negative

regulatory region at 401-698 or saturation of the regulatory mechanisms that act on this region causes the suppression of the Ras signalling pathway.

There are three pieces of data that suggest that the regulation of DEFLATED in this region may be mediated by 14-3-3 binding. The first is that *deflated* overexpression is dominantly modified by both 14-3-3 isoforms (Figure 5.5). While this modification may indicate a role for DEFLATED in M-phase entry (Section 5.4.2), it may alternatively reflect the role of DEFLATED in Ras signalling. Secondly, it is likely that DEFLATED affects the Ras signalling pathway at the plasma membrane or on the endosomes where Raf, MEK and MAPK are activated. This is also where 14-3-3 proteins are known to act in Ras signalling (Section 1.2.1). So it is possible that DEFLATED is a target of 14-3-3 regulation at the plasma membrane. Thirdly, genetic interactions show that 14-3-3 proteins act positively in Ras signalling. If 14-3-3 binds DEFLATED to keep it inactive (and therefore prevent it from inhibiting Ras) then this scenario is consistent with the positive roles of 14-3-3 proteins.

While it is unknown whether DEFLATED can physically bind 14-3-3, the predicted phosphorylated serine (S653) predicted to mediate 14-3-3 binding has is likely to be essential for DEFLATED function. Since expression of $defl^{S653A}$ acted dominantly while $defl^{S653E}$ did not, it is possible that phosphorylation at this site is required for negative regulation of DEFLATED function. If this site is phosphorylated then it is likely to bind 14-3-3 proteins. This raises two questions. The first is what is the identity of the kinase that phosphorylates DEFLATED at S653? This site was not recognised as a known phosphorylation site of any currently characterised kinase as identified by protein motif predictions. The second question is how would binding of 14-3-3 at this site negatively

regulate DEFLATED function? Generally 14-3-3 proteins form dimers that regulate their targets' function by forming either intra- or inter- molecular interactions. Three additional putative 14-3-3 binding sites were identified in DEFLATED but as they did not show strong conservation amongst DEFLATED homologues they were not tested by site-directed mutagenesis. It is possible that one or more of these sites are functional and therefore DEFLATED could be regulated intra-molecularly. If only the 650-655 14-3-3 binding site is functional, then DEFLATED may be regulated by 14-3-3 bringing it into contact with a second protein. While most known cases of 14-3-3 binding involves 14-3-3 dimers, there is at least one case in which a protein is regulated by a 14-3-3 monomer. The protein SLOB is bound by a 14-3-3 monomer and when bound is able to negatively regulate the calcium-dependent potassium channel, SLOWPOKE (Zhou et al., 2003). Therefore it is feasible that DEFLATED binds one 14-3-3 protein and this is sufficient to negatively regulate its function.

6.6.3 DEFLATED at the mitotic spindle

The findings that DEFLATED::GFP localises to nuclei and to the mitotic spindle support a direct role for DEFLATED in cell proliferation. Its localisation to the mitotic spindle may involve the potential binding of Ran or clathrin, two proteins that also localise to mitotic spindles and have essential roles in its formation and function.

The Ran pathway functions by generating a gradient of high Ran-GTP in the nucleus and high Ran-GDP in the cytoplasm. It would be expected that this gradient would be disrupted during mitosis when the nuclear envelope breaks down. However, the gradient in maintained by the localisation of RCC1 on the chromatin during mitosis (Carazo-Salas et al., 1999), thereby generating a high concentration of Ran-GTP in the

vicinity of the chromosomes. A high concentration of Ran-GTP is sufficient to nucleate microtubules (Carazo-Salas et al., 1999; Wilde et al., 2001) and it is thought that the generation of a high concentration of Ran-GTP at the chromatin is important for proper spindle formation and function. In accordance with this model, various components of the Ran pathway are localised along mitotic spindles. In mammalian cells, RanGAP1 and RanBP2 localise to both spindles and microtubule associated kinetochores, with higher localisation at the kinetochores (Joseph et al., 2004; Joseph et al., 2002; Matunis et al., 1996). In *C. elegans,* Ran localises along the length of the chromosomes at points of microtubule attachment (Bamba et al., 2002). In *Drosophila,* Ran (in GTP bound form) localises around, but not directly on, the spindles. RCC1 localises to the chromatin and RanGAP is occasionally found along the outside edge of microtubules (Trieselmann and Wilde, 2002). Therefore, the findings that DEFLATED::GFP localises to the spindle and is a potential Ran binding protein suggests that DEFLATED may form a complex with Ran or may be involved in the Ran mediated regulation of spindle formation and function.

Aside from its well known role in endocytosis, clathrin also localises to the mitotic spindle (Okamoto et al., 2000; Royle et al., 2005) and its localisation is dependent on the N-terminus of the heavy chain, which is the region that binds proteins that contain a clathrin box. The localisation of clathrin occurs as a triskelion and is important for normal spindle formation. In cells lacking the clathrin heavy chain, chromosomes do not congress to the metaphase plate correctly and, as a result, the cells arrest due to activation of the spindle checkpoint. As DEFLATED contains a well conserved clathrin box, it is a good candidate for recruiting clathrin to the mitotic spindle. It is possible that DEFLATED may bind both Ran and clathrin, acting as a bridge between them.

The localisation of DEFLATED::GFP to nuclei and to spindles during mitotis provides further support for a role in regulating cell proliferation. Although this localisation may be an artifact of overexpression, since *deflated* mRNA was not detected in syncytial or cellularised embryos, post cellularised cells also revealed a nuclear localisation. As the attempts to fix DEFLATED::GFP embryos were unsuccessful (Section 6.5.4), it could not be confirmed that DEFLATED::GFP localised to spindles (by co-staining with anti-tubulin) in older embryos, where it is known that *deflated* is expressed. However, the localisation of DEFLATED::GFP to spindles does appear to reflect real DEFLATED localisation for a number of reasons. Firstly, DEFLATED is likely to bind Ran and/or clathrin, two proteins that localise to mitotic spindles and have roles in regulating spindle function (Royle et al., 2005; Triselmann and Wilde, 2002). Secondly, the overexpression of *deflated::GFP* does not result in abnormal phenotypes, which indicates that this localisation does not interfere with normal cellular function. Although confirmation of a genuine spindle localisation during mitosis will require DEFLATED antibodies to be raised, the presence of clathrin and Ran binding motifs in DEFLATED argues strongly that the spindle localisation is not an artefact.

Localisation of DEFLATED to the mitotic spindle supports the genetic findings that *deflated* may link DNA synthesis with M-phase entry (Section 5.4.2). Furthermore spindle localisation indicates that DEFLATED is involved in regulating spindle formation or function. Since DEFLATED::GFP localises to the midbody in anaphase, there is also a possibility that DEFLATED has a role in mitotic exit and/or cytokinesis. DEFLATED may regulate the function of the spindle by physically binding Ran and/or clathrin.

In conclusion, the findings of this chapter support a role for DEFLATED in regulating Ras signalling, which could explain its effects on cell cycle progression. However, the finding that DEFLATED localises to mitotic spindles also supports a direct role for DEFLATED in regulating aspects of M-phase progression. These results support the genetic findings presented in Chapter 5 that suggest DEFLATED links DNA replication with M-phase entry.

Chapter 7- Towards a model of DEFLATED function

This thesis describes the initial characterisation of the novel *Drosophila* gene, *deflated.* Due to high co-expression with the S-phase genes *Ctf4* and *Dp, deflated* was hypothesised to be a regulator of cell proliferation. The mRNA expression patterns and the phenotypes of homozygous, transheterozygous, and rescued *deflated*$^{-}$ mutant individuals were consistent with *deflated* having a role in regulating cell proliferation. Genetic interaction analysis with cell cycle regulators suggested that the role of *deflated* in cell proliferation may be in linking DNA synthesis with entry into M-phase. The finding that DEFLATED::GFP localises to the mitotic spindle implicates DEFLATED in spindle formation and function, further supporting this role.

The results presented in the previous chapters demonstrate that *deflated* interacts genetically with signalling pathways, including the Ras pathway, and that the wing and bristle phenotypes produced by *deflated* overexpression, indicate that *deflated* may play a role in regulating cell signalling. This suggests that the effect *deflated* has on cell signalling may in part be due this role in cell signalling. In Chapter 6, the effect of *deflated* on Ras signalling was examined in some detail. It was shown that DEFLATED is likely to play a role near the beginning of the Ras signalling cascade, perhaps at the plasma membrane (Figure 6.10). Since DEFLATED was found to have an evolutionarily conserved motif known to bind clathrin (Figures 4.1 and 4.2) and DEFLATED::GFP was observed to have an interphase localisation pattern that could reflect clathrin coated vesicles or endosomes (Figure 6.9), it is possible that DEFLATED may function at the points in which signalling pathways intersect with endocytic pathways. The presence of a putative Ran binding domain (Section 4.2)

suggests that DEFLATED may also function in the Ran pathway. This last alternative may explain *deflated*'s antagonistic role in Krüppel function. The questions this chapter addresses are: is it feasible that DEFLATED could function within each of these pathways? What roles do these pathways have in regulating cell proliferation? Is it likely that DEFLATED could function separately in all three pathways or can it be a molecule that acts at the intersection of these three pathways?

7.1 Upon the genetic natures of the *deflated* alleles

The *deflated* alleles generated in this study through P-element mutagenesis all resulted in deletion of the entire coding region or a portion thereof (Figure 3.8). Three of the alleles, $defl^{P}$, $defl^{L}$, and $defl^{Z}$, are predicted to generate proteins that lack the C-terminus, while $defl^{\Delta}$ produces no protein (Figure 5.7). Throughout this study all alleles have behaved consistently in each of the assays and therefore conclusions can be drawn about their natures. $defl^{\Delta}$ is certainly a null allele, yet it was not used in many of the assays as this allele is also null for *nbs*. Therefore, any data obtained using $defl^{\Delta}$ could be confounded by loss of *nbs* function, which is expected to have a role in DNA replication and cell cycle arrest (Section 1.1.4). Recently, the sequencing of a piggyBac insertion ($defl^{c106100}$, also known as $nbs^{c106100}$) shows that this transposon has inserted 49 bp downstream of the *deflated* start codon (Gene Disruption Project Members, 2005; Thibault et al., 2004). This indicates that this insertion is likely to be null for *deflated*, but how it affects *nbs* function still needs be determined.

The three different partial deletion alleles, $defl^{L}$, $defl^{P}$, and $defl^{Z}$ showed different genetic interactions and therefore are different types of alleles. $defl^{L}$ consistently behaved as a hypomorphic allele. In *trans* to $defl^{Z}$, it produced a stronger phenotype

than *defl*^{P}, a phenotype similar to *defl*$^{\Delta}$, and was less easily rescued by cDNA expression than *defl*^{P} (Section 4.5). On the basis of these data, *defl*^{L} appears to be a stronger allele (retaining less function) than *defl*^{P}. In constrast, *defl*^{P} appears to be a recessive neomorph or a separation of function mutation rather than a hypomorphic mutation, based on the genetic interactions observed between *defl*^{P} and activated Ras and *defl*^{P} and *deflated* overexpression in the wing (Sections 5.4.5 and 6.6.2). In both of these situations, *defl*^{P} behaves similarly to wild type overexpression. *defl*^{Z} behaved consistently as a weak hypomorphic allele, however this allele was not used in many of the analyses due to the presence of a second mutation elsewhere on the chromosome, which could potentially confound any observations made (Section 3.4). Finally, the phenotypes observed from the overexpression of wild type *deflated* are likely to occur due to loss of the normal checks on *deflated* function. This conclusion can be made due to the fact that high levels of DEFLATED protein are required to induce abnormal phenotypes. This suggests that the regulators controlling DEFLATED function are normally in excess of DEFLATED and it is only within the wing imaginal disc that these putative regulatory mechanisms become rate limiting when *deflated* is overexpressed (Section 5.3.1).

7.2 Possible functional domains of DEFLATED

DEFLATED is predicted to have functional domains and motifs indicative of a role in cell cycle regulation, cell signalling, endocytosis and/or the Ran pathway (Section 4.2). All of these predictions are based on evolutionarily conservation of primary amino acid sequence or 3-D structure predictions based on primary amino acid sequence, and therefore require confirmation within a biochemical context. Within this study, none of these domains or motifs were fully experimentally tested, however, for some evidence

was obtained to suggest that they may be functional. Furthermore, this study has inferred the presence of a putative regulatory region between amino acids 401-698 based on genetic data, and this contains a number of these conserved motifs (Section 5.4.5).

Two genetic interaction studies indicated that DEFLATED contains a putative negative regulatory region between amino acids 401 and 698. These studies included the genetic interactions observed between the *deflated* alleles and *deflated* overexpression and the genetic interactions between the *deflated* alleles and activated Ras (Sections 5.4.5 and 6.6.2). From these studies it appears that this region of DEFLATED is required to regulate the activity of the N-terminus, which is predicted to encode a putative clathrin box, a putative Ran binding domain, and a putative cyclin binding site. Although speculative, these data are consistent with the N-terminus of DEFLATED encoding its functional domain and the C-terminus encoding a regulatory domain.

Support for potential regulation of DEFLATED by 14-3-3 proteins arose from the finding that mutation of the conserved serine at position 653 to an alanine results in a protein that acts dominantly when overexpressed and cannot provide DEFLATED function necessary for normal development (Section 6.4.3). While this site has not yet been shown to bind 14-3-3 proteins, it is clear that this residue and its potential phosphorylation are essential for normal DEFLATED function.

Support for possible regulation of DEFLATED by SUMO modification arose from the finding that *smt3* (encoding SUMO) dominantly enhanced *deflated* overexpression (Section 5.4.5). This indicates that if the SUMO modification site in DEFLATED is

genuine then it serves to negatively regulate DEFLATED function. However, it remains possible that the genetic interaction observed between *deflated* overexpression and *smt3* reflects an indirect biochemical interaction, where SUMO modifies a regulator of DEFLATED.

Support for the functionality of several motifs and domains within the N-terminus of DEFLATED arose through more circumstantial evidence. The localisation of DEFLATED::GFP to both mitotic spindles and to the apical surface of wing imaginal discs is consistent with the clathrin, adaptor binding and/or the Ran binding site being functional. These localisation patterns may reflect the primary function of DEFLATED as a regulator of M-phase, or involvement in endocytosis and Ras signalling (Section 6.6).

7.3 *deflated's* role in regulating cell proliferation

deflated was initially hypothesised to be a putative regulator of cell proliferation due to a high correlation of evolutionarily conserved co-expression with the S-phase genes *Ctf4* and *Dp* (Section 1.4.2). In accordance, *deflated* mRNA is expressed in cells that are proliferating, and expression ceases once developmentally controlled proliferation has ceased (Section 4.7.2). In many ways, the *deflated* mutants phenocopy mutants of the *E2f*, *Dp*, *Rbf* S-phase regulatory pathway, thereby suggesting a role for DEFLATED in regulating cell proliferation at various stages of development, and in cells that have differential regulatory requirements, such as the endocycle and gene amplification in follicle cells (Section 4.7.3).

Examination of the cell cycle status of neuroblasts in brains from homozygous larvae also demonstrated the requirement for wild type *deflated* function in regulating both S- and M-phase (Section 4.7.6). Further evidence for a role in S-phase came from the finding that overexpressed *deflated* could attenuate the ectopic S-phases induced by overexpressed *Cyclin E* (Section 5.2.2). While these data at first seems contradictory, with $defl^{P}$ homozygotes showing a decrease of the number of cells in S-phase per brain, it is likely that *deflated* actually has a negative role in S-phase as $defl^{P}$ seems to be a separation of function allele rather than a hypomorph. This hypothesis is supported not only by the interaction observed with *Cyclin E* overexpression but also by the finding that $defl^{L}$ homozygous brains show a small increase of cells in S-phase, indicating that loss of function results in loss of S-phase inhibition.

The hypothesis that *deflated* may negatively regulate S-phase is consistent with the speculation that *deflated* may link DNA synthesis with M-phase entry. If *deflated* is required to prevent re-replication and to signal to M-phase regulators that DNA synthesis has occurred and therefore M-phase can occur, then it would be expected that disrupting its function may cause re-replication and/or M-phase to occur prematurely. In brains from both $defl^{P}/defl^{P}$ and $defl^{L}/defl^{L}$ homozygous larvae cells were less likely to be in M-phase (Figure 4.6), indicating that *deflated* is required for M-phase entry. This hypothesis is further supported by the genetic interactions observed in Chapter 5. Nearly all the interactions observed were consistent with overexpression of *deflated* causing premature M-phase entry, possibly in the absence of DNA synthesis completion (Section 5.4.2). Furthermore, DEFLATED::GFP was found to localise to the mitotic spindle and midbody, indicating that it may play additional roles in M-phase progression and M-phase exit. Therefore, it appears that the main role of DEFLATED

in the cell cycle is to link the progression of S-phase with M-phase progression. It is unknown whether it does this by directly regulating the cell cycle regulators themselves or whether it is involved in the intricate sensing and signalling pathways that orchestrate cell cycle progression. The latter alternative is supported by the findings that DEFLATED affects Ras signalling and that it may bind Ran and/or clathrin.

7.4 The role of *deflated* in cell signalling

One way in which DEFLATED activity could affect cell cycle progression is via cell signalling. A number of alleles from different cell signalling pathways were shown to modify the *deflated* overexpression phenotype (Section 5.3.5), suggesting that DEFLATED may be involved in their regulation or that they may regulate DEFLATED. These alleles were tested on the basis that the extra wing vein phenotype is characteristic of signalling perturbation. Further genetic interactions with Ras^{V12} and rl^{SEM} indicated that *deflated* might be a negative regulator of Ras signalling (Section 6.6.1). Ras signalling is required for cells to enter S-phase and is also required for differentiation of many cell types (reviewed in Chang et al., 2003; Kurada and White, 1999). In the Ras model used in this study, activated Ras was expressed in post-mitotic cells that were differentiating, not cycling. Therefore these observations establish that the role of *deflated* in this pathway is not as a cell cycle regulator. The genetic interactions with Ras^{V12} place *deflated* downstream of Ras and the staining of wing discs overexpressing *deflated* for pMAPK indicated that DEFLATED is likely to be upstream of MAPK. Therefore, DEFLATED is likely to act on the plasma membrane or on endosomes where Raf, MEK and MAPK are recruited and activated. This is consistent with the postulated 14-3-3 regulation of DEFLATED, as 14-3-3 proteins are known to act at this point in Ras signalling.

7.4.1 DEFLATED as a component of the Ras signalling pathway?

If DEFLATED is an integral component of the Ras signalling pathway it would be expected that loss of function caused by the *defl*$^{\Delta}$ allele would dominantly modify the *Ras*^{V12} phenotype. The fact that *defl*$^{\Delta}$ does not modify *Ras*^{V12} may mean three things: (i) either there is still sufficient wild type protein to provide normal function, (ii) that loss of *nbs* is confounding the phenotype, or (iii) that DEFLATED is not actually a component of the pathway, but rather has another role that affects the Ras pathway. This last alternative may also explain the interactions observed with other signalling pathways, as even though the pathways are interconnected and do affect one another, it appears that DEFLATED affects different signalling pathways individually. Therefore, it is only when this other presumptive pathway is significantly disrupted (i.e. in the transheterozygotes or when *deflated* is overexpressed) is an effect on Ras signalling observed. If it is that *deflated* is acting in another pathway that impinges upon Ras signalling, the interaction with Ras is worthy of further study, since it would enable an understanding of the function of this other pathway and how it affects Ras signalling. Furthermore, since *deflated* appears to be a negative regulator of Ras signalling, *deflated* is also likely to be a candidate tumour suppressor, further study of which could lead to a better understanding of tumourogenesis.

7.4.2 *deflated*, Ras signalling, and spindle function

Apart from the well established role of Ras/Raf/MAPK pathway in inducing G1-S phase progression (Section 1.2), there is mounting evidence to suggest that the Raf-MAPK pathway also regulates M-phase entry and exit, the spindle checkpoint, and spindle dynamics. In *Xenopus* and mammalian cells, active MAPKK and MAPK are

required for the G2-M transition and for maintaining a mitotic state (Guadagno and Ferrell Jr., 1998; Hayne et al., 2000; Wright et al., 1999). These proteins are then required to be inactivated for the exit of mitosis, a process that is independent of mitotic cyclin destruction (Chau and Shibuya, 1999). During mitosis both MAPK and MEK localise to microtubules, spindle poles and the midbody, whereas MAPK also localises to the kinetochores of prometaphase and unnattached chromosomes (Shapiro et al., 1998; Zecevic et al., 1998), which suggests a role in maintaining spindle function and signalling to the spindle checkpoint. In support of this, the Raf/MAPK pathway is upregulated in response to microtubule-destabilising drugs (Hayne et al., 2000) and abnormal spindle structures are observed in the absence of MAPK (Horne and Guadagno, 2003).

Like *deflated* homozygous tissues, absence of wild type MAPK (encoded by *rl*) in *Drosophila* caused a decrease in the number of cells in mitosis in third instar larval brains (Inoue and Glover, 1998). Cells in M-phase in rl^{-} brains were more frequently in metaphase than anaphase and these cells were unable to arrest in the presence of colchicine, indicating a failure of the spindle checkpoint. It is possible that the defects in M-phase observed in *deflated* mutant cells and the genetic interactions observed in Chapter 5 could be explained by *deflated* perturbing has on Ras/Raf/MAPK signalling. If *deflated* is a negative regulator of the pathway then the $defl^{P}$ allele would still provide this negative regulation and therefore there would be less cells in M-phase. When overexpressed in the wing, *deflated* changes the pattern of MAPK activation, which could result in the loss or inhibition of the spindle checkpoint, thereby possibly explaining the non-modification of the wing phenotype by *wee1* alleles (Table 5.1). As *rl* has been shown to genetically interact with *abnormal spindles* (Inoue and Glover,

1998), it would be interesting to determine if *deflated* could also interact with known spindle and mitotic regulators to test whether the spindle checkpoint is perturbed in the absence of *deflated* or when *deflated* is overexpressed.

7.4.3 Cell signalling and eggshell formation

A role for *deflated* in cell signalling could also be invoked to explain the eggshell phenotypes observed in eggs laid by transheterozygous or cDNA rescued homozygous mothers. The thin chorion phenotype observed is likely to be due to a defect in endoreplication or gene amplification in the follicle cells, which, is controlled by the S–phase regulatory network of E2F, Cyclin E, and Rbf (Sections 1.3.4 and 4.7.3). This network has been shown to be under the developmental control of the Notch pathway at the mitotic-to-endocycle transition (Shcherbata et al., 2004), whereas the developmental switch regulating the switch from endocycle to gene amplification is still unknown. It is possible that the role of *deflated* in chorion production is to ensure correct developmental timing of S-phase regulation within the follicle cells through cell signalling regulation of cell cycle regulators.

The second obvious eggshell defect observed in eggs laid by *deflated* transheterozygous and cDNA rescued homozygous females was malformation of the dorsal appendages (Sections 4.4.2 and 4.5). The dorsal appendages are formed by dorsal follicle cells with the developing oocyte that are specified by Grk-Egfr signalling. These cells migrate anteriorly over the nurse cells, forming tubular structures and secreting chorion, which forms the dorsal appendages (Schnorr et al., 2001). The migration of the follicle cells is also controlled by Egfr signalling (Dammai and Hsu, 2003). Given that *deflated* affects Ras signalling, it is tempting to speculate that the *deflated* dorsal appendage phenotype

is due to perturbation of Egfr-activated Ras signalling. This conclusion is not well supported, as the defects seen in the *deflated* mutants are not as severe as those seen the in Egfr/Ras pathway mutants. Even mild Ras alleles show a severe dorsal appendage defect, where no appendages are formed, or only a single appendage forms (Schnorr and Berg, 1996). As dorsal appendage phenotypes were only observed in mild allelic combinations of *deflated*, it is possible that in a situation where stronger alleles could be examined, such as in germline clones, the defects would be more severe and therefore more similar to those seen in Egfr/Ras pathway mutants.

The finding that *deflated* transheterozygotes show mild dorsal appendage defects may also reflect a role for *deflated* in other signalling pathways, as hinted by the genetic interactions observed in the wing (Section 5.4.3). Indeed, TGFβ signalling is required in a more limited role than Egfr signalling for formation of the dorsal appendages (Twombly et al., 1996) and *deflated* has been shown to interact with *dpp* in the wing (Section 5.4.3). Other signalling pathways that are involved in dorsal appendage formation include the JNK pathway and a pathway including *bullwinkle* and *shark* (Tran and Berg, 2003). However, none of the dorsal appendage defects observed in mutants from any of these pathways resemble the "holly leaf" appearance of the *deflated* dorsal appendages. This may mean that *deflated* may define a novel pathway in dorsal appendage formation or that *deflated* has a role within a known pathway that results in a novel phenotype.

7.5 The role of *deflated* in the Ran pathway

Since DEFLATED contains HEAT repeats that are most like those found in Ran binding proteins, it is possible that DEFLATED may be a component of the Ran

pathway. If a role in the Ran pathway is confirmed biochemically then much of the data obtained in this study (with the exception of its role upstream of MAPK and its punctate localisation pattern in wing discs) can be explained. The Ran pathway regulates nuclear import and export, mitotic spindle formation, nuclear envelope formation and cell cycle progression (Moore, 2001; Pemberton and Paschal, 2005). Since the Ran pathway can regulate both S- and M-phase progression, which appears to be partially independent of its role in regulating nuclear import, the effects of *deflated* on cell cycle progression could be due to an effect within or upon this pathway.

7.5.1 The Ran pathway and direct regulation of cell cycle progression

If DEFLATED is a Ran binding protein then its role in regulating S-phase and M-phase could be via this pathway. The RanGEF, RCC1, was first identified as a regulator of chromosome condensation. Incubation of a hamster cell line containing a temperature sensitive mutation in RCC1 (tsBN2) resulted in premature entry into mitosis, irrespective of completion of DNA synthesis (Nishitani et al., 1991). This is similar to the proposed role for DEFLATED in linking DNA synthesis with M-phase entry (Section 5.4.2 and 7.3). In the tsBN2 cells cultured at the restrictive temperature, the inhibitory phosphorylation of cdk1 is lost and Cyclin B/cdk1 can induce premature M-phase entry. However, a dominant negative mutation of Ran (Ran T24N), which is thought to act by sequestering RCC1, inhibits M-phase entry (Clarke et al., 1995; Kornbluth et al., 1994). This block can be relieved by okadaic treatment, which inhibits the protein phosphatase PP2A (PP2A normally prevents the activation of cdc25 by phosphorylation (Clarke et al., 1993)), which places the Ran pathway upstream of PP2A in regulating M-phase entry with S-phase completion (Clarke et al., 1995).

In addition, RCC1 is required for DNA replication (Dasso et al., 1992) and the expression of Ran T24N also blocks DNA synthesis (Kornbluth et al., 1994; Ren et al., 1994), which implies a role for the Ran pathway in S-phase as well as M-phase. A role for RCC1 and Ran in S-phase appears to be linked to the inability of nuclei to grow in these mutants. The inability for the nuclei to grow is not dependent on nuclear transport, so the Ran pathway may be involved in generating a suitable nuclear structure for DNA synthesis (Kornbluth et al., 1994). The premature M-phase phenotype is also not dependent on nuclear transport, or even nuclear formation, as the activation of cdc2 by Ran T24N expression is observed in nuclear free extracts (Clarke et al., 1995; Kornbluth et al., 1994).

Since the Ran pathway directly affects both DNA replication and linking the completion of DNA synthesis with M-phase entry, a potential role for DEFLATED in this pathway is consistent with the hypothesised role for DEFLATED in M-phase entry. If DEFLATED is a component of the Ran pathway, a speculative role consistent with the data is that DEFLATED aids in preventing RanGTP from either forming or signalling to PP2A that M-phase can occur.

7.5.2 Nuclear import and the regulation of cell proliferation

In addition to its nuclear-independent role in regulating mitotic entry, the Ran pathway also has a nuclear import dependent role in regulating cell cycle progression. DEFLATED could therefore be involved in this aspect of Ran function. Nuclear import plays an important role in regulating the entry into M-phase by causing CyclinB-cdc2 and Cdc25C/String to be actively exported out of the nucleus during interphase (Section

1.1.3). Nuclear import may also play a role in regulating S-phase entry and preventing re-replication and ensuring S-phase exit.

Different regulators of the cell cycle are imported into the nucleus in different ways. Therefore, if DEFLATED is involved in the Ran pathway then the genetic interactions observed with cell cycle regulators (Sections 5.4.1 and 5.4.2) could reflect a role for DEFLATED in their import and export, rather than in their direct activation or activity. For example, Cyclin E and Cyclin B are imported into the nucleus in different ways. Cyclin E is imported as though it contains a classical nuclear localisation signal (NLS). It is bound by importin α, which is bound by importin β and translocated into the nucleus. Cyclin B, on the other hand, binds importin β directly at a site distinct from its importin α binding site (Moore et al., 1999). Interestingly, Cyclin A does not even need to be imported into the nucleus. Mitotis (and ectopic S-phases) can occur even when Cyclin A is tethered to the plasma membrane, however, mitotic exit does not occur properly because the Cyclin A is not degraded properly when tethered at the plasma membrane (Dienemann and Sprenger, 2004). These different requirements for nuclear import may explain the different abilities of overexpressed *deflated* to modify the eye phenotypes caused by overexpressed *Cyclin E* and *Cyclin A*. It is feasible that the ability of *deflated* to suppress *Cyclin E* could be due to it interfering with the import of Cyclin E into the nucleus, possibly by interfering with the importin α/β complex. Since the importin α/β complex does not seem to be required nor essential for Cyclin A activity, *deflated* would be expected to have no effect on its overexpression, as was observed (Figure 5.1).

The Ran pathway also has a role in preventing re-replication, which is partially dependent on nuclear transport. RanGTP and Crm1 are needed to prevent the reassociation of MCM helicase with chromatin once S-phase has been initiated. This inhibition is not dependent on nuclear export as it can occur in the absence of a nuclear membrane (Yamaguchi and Newport, 2003). On the other hand, Crm1-dependent nuclear export of Cdc6 and Orc1 is also important for preventing re-replication in metazoans (Jiang et al., 1999; Laman et al., 2001; Petersen et al., 1999). If DEFLATED is involved in the Ran pathway then it is possible that the effects it has on S- phase (and also M-phase) may be due to improper regulation of the DNA replication licensing factors. This speculation is supported in part by the finding that overexpressed *deflated* is enhanced by both *double parked*, which is a pre-replication complex component, and *cdc2*, which is required to prevent endoreplication. These data suggest that DEFLATED may play a role in regulating re-replication.

7.5.3 The *deflated* and *Krüppel* interaction and nuclear transport

A role for DEFLATED in nuclear transport could also explain the genetic interactions observed with *Krüppel*. The model proposed in Chapter 5 (Figure 5. 6) indicates that *deflated* is acting upstream, along with, or downstream of *Krüppel*. For reasons explained earlier (Section 5.4.4), a downstream effect seems unlikely, thereby inferring either an upstream or direct interaction with *Krüppel*. If DEFLATED does play a role in the Ran pathway, either directly or indirectly, it could prevent nuclear import or upregulate export of Krüppel, thereby acting as a negative regulator. Not much is known about the nuclear transport of Krüppel. In mammals the Krüppel-like factors GKLF/KLF4 and EKLF/KLF1 localise to the nucleus due to the presence of a classical NLS or due to an NLS present within the zinc finger domain (Pandya and Townes,

2002; Quadrini and Bieker, 2002; Shie and Tseng, 2001). EKLF/KLF1 binds both importin β and importin α in vitro so Krüppel is likely to be translocated via a Ran-dependent mechanism in vivo (Quadrini and Bieker, 2002).

Although compelling, a potential role for DEFLATED in the Ran pathway can not readily explain the punctate apical localisation of DEFLATED::GFP nor the finding that DEFLATED is likely to be upstream of MAPK activation in the Ras signalling cascade, if all the effects of the Ran pathway occur in the nucleus. However, there is precedence for a non nuclear role for a Ran binding protein. RanBPM localises to the plasma membrane and plays a role in signal transduction. RanBPM was first discovered as a Ran binding protein in a yeast two hybrid assay, even though it does not contain a classical Ran binding domain as found in importin β (Nakamura et al., 1998). Initially it was found that RanBPM had a role in regulating microtubule nucleation. However, subsequent studies have not substantiated this role. Instead there is increasing data in the literature to suggest that RanBPM may be a scaffold protein integrating signalling molecules, such as receptor protein-tyrosine kinases and integrins, with their downstream effectors, such as the Ras/MAPK signalling pathway (Denti et al., 2004; Wang et al., 2002). Even though the putative Ran-binding domain in DEFLATED was recognised due to its similarity with domains found in proteins such as importin β, and is therefore different to RanBPM, it is possible that DEFLATED is a Ran binding protein that has functions in the nucleus and at the plasma membrane, analogous to RanBPM.

7.6 The role of deflated in endocytosis

Endocytosis is the regulated uptake of molecules at the plasma membrane, and can take place by four different methods; clathrin-dependent endocytosis, caveolae-mediated endocytosis, macropinocytosis, and dynamin- and clathrin- independent endocytosis (reviewed in Mousavi et al., 2004; Seto et al., 2002). The best studied is clathrin-dependent endocytosis. It has been well documented that clathrin-mediated endocytosis plays an important role in regulating cell signalling (Seto et al., 2002). What is not so clear is the precise role endocytosis plays in cell signalling. Initially it was thought that the internalisation of receptors following their activation by ligand binding was required to desensitise or down-regulate signalling. While this does seem to occur, particularly for G protein-coupled receptors (Scott et al., 2002), it is becoming clear that in some contexts endocytosis may in fact increase signalling, and that there is substantial cross-talk between endocytic and signalling pathways.

7.6.1. Endocytosis and Ras signalling

Endocytosis plays a role in the regulation of RTK/MAPK, Notch, Wg/Wnt, TGFβ/Dpp, and Hedgehog signalling pathways. The RTK/Ras/Raf/MAPK pathway is a good example to illustrate the interactions between signalling and endocytosis since the mechanisms are reasonably well understood. Moreover, to understand how DEFLATED may act in either Ras signalling or endocytosis, an examination of the two processes is required. Proteins involved in endocytosis that are phosphorylated as a result of RTK activation include clathrin (Wilde et al., 1999), Eps15 (Confalonieri et al., 2000), and Hrs (Komada and Kitamura, 1995). The phosphorylation of these proteins is necessary for the internalisation of the receptor and for the signal to be transduced. In the case of EGFR internalisation, the debate about whether

internalisation attenuates or enhances signalling has been addressed. Signalling is enhanced in situations where recycling back to the membrane is favoured over degradation, due to the presence of receptor heterodimers rather than homodimers (Lenferink et al., 1998). Signalling also is enhanced and fine tuned by the formation of endosomes, in which receptors associate with their targets, since the majority of the phosphorylated forms of these targets are found in endosomal fractions (Di Guglielmo et al., 1994; Oksvold et al., 2000). Furthermore, it is possible that internalisation allows selective activation of targets as it was found that MAPK is not activated when internalisation was prevented, but other targets were activated as normal (Kranenburg et al., 1999).

Endosomes form multivesicular bodies (MVBs) where it is thought that receptors are sorted and either recycled to the cell surface or are sent to the lysosome for degradation. Two known inhibitors of Ras signalling play a role in the sorting to lysosomes, Cbl and Hrs. Cbl is a ubiquitin ligase and has been shown to ubiquitinate RTKs (Levkowitz et al., 1998). Hrs can bind ubiquitin through its ubiquitin-interacting motif (Lloyd et al., 2002). Both proteins result in the upregulation of receptor degradation, with ubiquitin possibly acting as the sorting signal. Therefore, while internalisation of the receptor is needed for signalling to occur, it is in the regulation of the downstream events, such as how long the receptor and their targets remain on endosomes and whether the receptors are recycled or sent to lysosomes, that determines whether the overall outcome of internalisation is one of attenuation or enhancement. It is clear that the cross talk between cell signalling and endocytosis serves to fine-tune the signalling process, by allowing regulation to occur at various points, therefore allowing for subtle signalling outcomes dependent on cell type or developmental stage to take place.

7.6.2 Is DEFLATED an endocytic component or a Ras signalling component?

The findings that DEFLATED contains a well conserved clathrin box and adaptor protein binding sites, is localised to the apical surface in wing discs, displays genetic interactions with the Ras signalling pathway, and that *deflated* overexpression prevents spatially-correct MAPK activation, all support a role for DEFLATED in endocytosis. DEFLATED might regulate signalling through endocytosis or it may be a protein that is part of a signalling cascade that is regulated by endocytosis. Genetically, *deflated* is downstream of *Ras,* so it is unlikely that it plays a role in regulating the initial internalisation of receptor tyrosine kinases. However, since cell signalling may be increased by concentrating active receptors and their downstream effectors on the surface of the endosome (Seto et al., 2002) it is possible that DEFLATED plays a role at the endosome.

If DEFLATED is either involved in regulating the activation of Raf, MEK, or MAPK at the plasma membrane or on endosomes, or is an endocytic component involved in creating these endosomes, then DEFLATED should be found in endosomes. The punctate localisation pattern of DEFLATED::GFP in the wing imaginal discs is similar to that of endosomes, supporting this speculation. Double labelling with known endosomal components would be required to confirm that the punctate localisation pattern is indeed endosomal. Since it appears that DEFLATED is a negative regulator of Ras signalling it is tempting to speculate on how it may function. If DEFLATED is an endocytic protein then it may negatively regulate clathrin-coated pit or vesicle formation or it may promote the disassembly of clathrin. If DEFLATED is endocytosed

then it may compete with activated receptors for adaptor complex binding sites and prevent the internalisation of receptors.

7.6.3 Endocytic proteins have potential roles in the nucleus

At first a role for DEFLATED in endocytosis may seem implausible, considering the localisation of DEFLATED to the nucleus and to mitotic spindles. As already discussed (Section 6.6.3), clathrin also localises to the spindle and has a necessary function in chromosome segregation. Surprisingly, other endocytic proteins demonstrate nucleo-cytoplasmic shuttling (reviewed in Benmerah et al., 2003). Their ability to localise to the nucleus was generally only observed when nuclear export is shut down by the Crm1 inhibitor, LMB (Vecchi et al., 2001). These data show that, at steady state, export of these proteins is favoured over import. In support of this, the nuclear export signals (NES) for the endocytic proteins Eps15 and β-arrestin2 have been determined (Poupon et al., 2002; Scott et al., 2002). None of the nuclear shuttling endocytic proteins contain a classical NLS, so the mechanism of their import into the nucleus is still unknown (Benmerah et al., 2003). Eps15 and CALM can act as positive regulators of Gal4 transcription (Vecchi et al., 2001) and epsin binds to the transcription factor PLZF via its ENTH domain, which is structurally similar to HEAT and ARM repeats (Hyman et al., 2000). This suggests that their nuclear role may be to regulate transcription.

Like endocytic proteins, DEFLATED does not contain a classical NLS. If DEFLATED is primarily involved in endocytosis then its nuclear localisation could be due to its overexpression, which would saturate the export pathway and possibly imply a role for DEFLATED in regulating transcription. However, this seems unlikely. In the cases of the endocytic proteins the nuclear localisation observed was never greater than 30% of

the total protein, despite long incubations in LMB (Vecchi et al., 2001). In the case of DEFLATED::GFP, over 50% of the protein showed nuclear localisation in embryos. In addition, no abnormal phenotypes were observed in flies where DEFLATED::GFP was overexpressed with *nanos*-Gal4, which indicates that the localisation observed was not an abnormal event. Therefore, if DEFLATED is an endocytic protein its role in the nucleus may differ from other proteins that display nucleo-cytoplasmic shuttling and is more likely to reflect the role clathrin plays at the mitotic spindle.

7.7. *deflated* function: many roles or a central hub?

In this study it has been established that *deflated* is required for normal cell proliferation. DEFLATED is likely to regulate M-phase entry and progression and it may have a role in ensuring that M-phase only occurs after DNA synthesis is completed. Despite this central role in cell cycle progression, it is unlikely that DEFLATED is a core cell cycle regulatory protein. It is more likely that it is involved in one or more of the many monitoring and signalling pathways that ensure correct progression through the cell cycle. At this stage there is evidence to suggest that DEFLATED may be a component of three separate pathways, that have all been shown to regulate cell cycle progression. These three pathways are the Ras/Raf/MAPK pathway, the Ran pathway, and endocytosis.

The Ras signalling pathway has a well documented role in the G1-S phase transition and a less understood role in the G2-M phase transition and spindle assembly. The data are inconclusive at this stage as to whether DEFLATED is actually a member of the Ras signalling pathway, but it appears that its function does negative regulate Ras signalling. Therefore the effects DEFLATED has on Ras signalling may account for the

effects DEFLATED has on cell proliferation. If DEFLATED is primarily a component of the Ran pathway is effects on cell proliferation could be due to misregulation of nuclear transport of cell cycle regulators. Alternatively, DEFLATED could influence the direct role Ran has in regulating nuclear envelope formation, S-phase progression, M-phase progression, or spindle function. Lastly, if DEFLATED is primarily an endocytic protein, then it could affect cell proliferation in two ways. The first way is through regulation of Ras signalling, since signalling and endocytosis are tightly co-regulated. The second way is DEFLATED may function physically with clathrin at the mitotic spindle and thus have a direct role in cell cycle progression.

It is formally possible that DEFLATED does play separate roles in the Ras, Ran and endocytic pathways but it is more likely that it has a primary role in only one of the pathways. The genetic and physical interactions with the other two pathways would then be a reflection of the crosstalk between these pathways. This interpretation that DEFLATED is primarily a Ras signalling component, or a Ran pathway component, or an endocytic protein does not explain all the available data. The findings that DEFLATED::GFP shows multiple subcellular localisations and that it contains domains and motifs indicative of physical interactions with different proteins, including clathrin, Ran, and MAPK indicate that DEFLATED is unlikely to be a protein functioning in only one pathway.

A different interpretation, which highlights the findings that DEFLATED may physically interact with many different proteins and that DEFLATED genetically interacts with components of different cellular pathways, is that DEFLATED acts as an adaptor protein. Therefore DEFLATED is not an integral component of the Ras, Ran,

or endocytic pathways, but instead functions to mediate interactions between components of these pathways and regulate their crosstalk.

This suggested model of DEFLATED as an adaptor can account for all the findings of this study. In particular, the postulated role of DEFLATED in regulating M-phase entry may involve the binding of clathrin, Ran, and MAPK to regulate M-phase entry and the function of the mitotic spindle. This model would predict that DEFLATED is involved in their localisation to the spindle and that these proteins may possibly interact through binding to DEFLATED. Since DEFLATED also localises to the nucleus and possibly to endosomes during interphase, this model would also predict that DEFLATED may bind and regulate some interactions between endocytic, Ran, Ras, and possibly other signalling pathways by binding some components in different subcellular compartments.

If DEFLATED acts as an adaptor its roles in regulating cell proliferation would be both direct, by acting at the mitotic spindle, and indirect, by regulating the signals that feed into the core cell cycle machinery. Therefore DEFLATED is hypothesised to be a key regulator of cell proliferation by aiding in the integration of various monitoring and signalling pathways that result in correct cell cycle progression.

7.8 Final comments

The development of a multicellular organism requires the coordination of cell proliferation with cell growth and differentiation. The finding that DEFLATED does regulate cell proliferation and it may do so by integrating various signals from extracellular cues (Ras signalling pathway and endocytosis) and intracellular cues (Ran

pathway and possibly other as of yet unknown pathways) indicates that it plays an important but complex role.

The genetic observations described in Chapter 5 support a role for DEFLATED in regulating S- to M-phase progression. This role would require the sensing of the state of DNA replication and signalling to the M-phase machinery. Since DEFLATED is likely to be an adaptor protein it is predicted to act by integrating or modulating these signals. The proper regulation of S- to M-phase progression is crucial to ensure correct development and to prevent tumourogenesis, so further study of DEFLATED is likely to increase our understanding of this regulation.

The role of DEFLATED as an adaptor is likely to extend to other signalling pathways, including those involving Ras and Ran. Therefore, further study of *deflated* is warranted to to understand how these pathways are regulated and how they influence cell proliferation. The further study of *deflated* will no doubt contain some surprises, but will also yield insight not only into the primary function of DEFLATED but also how various cellular processes intersect to facilitate one of those crucial decisions for a cell- to divide or not divide.