Characterization of genetic interactions between cell cycle regulators and Armadillo in *Drosophila melanogaster*

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The Armadillo protein of *Drosophila melanogaster* is the pivotal transducer of the Wingless pathway, a signalling pathway that directs cell fate decisions during development. Cytoplasmic Armadillo is normally kept low by constitutive proteolysis. However, receipt of Wingless results in the stabilization of Armadillo and its transfer to the nucleus where it can activate gene transcription. Intriguingly, Armadillo is also necessary for cell-cell adhesion at epithelial adherens junctions.

In order to explore how these two functions might be regulated and effected *in vivo*, a genetic modifier screen was conducted. One interacting mutation was a recessive lethal named *l(3)neo40*, but inconsistencies regarding the nature of the interaction and the cause of lethality put a stop to its further characterization.

Armadillo 'loss-of-function' phenotypes in the adult wing are sensitive to the gene dosage of several regulators and targets of the mitotic kinase Cdk1, including Cyclin A, Cyclin B, String, Twins, and Cdk1 itself. Curiously, Dacapo and Cyclin E, negative and positive regulators of S phase entry respectively, exhibit similar interactions. The cell-biological reasons for these observations are unclear.

Altering the genetic dosage of String also modifies wing phenotypes resulting from over-expression of Armadillo. The manner of these interactions indicates that String might be a negative regulator of Armadillo function. Indeed, increased String function reduces cytoplasmic Armadillo levels in imaginal disc cells, apparently through stimulating its proteolysis. String is an activating phosphatase of Cdk1 and is rate-limiting for mitotic entry in *Drosophila* cells. Consistent with this role and the results described above, mitotic cells have relatively low levels of cellular Armadillo and experimental induction of mitosis lowers cytoplasmic Armadillo. Thus, cell cycle-intrinsic down-regulation of Armadillo may function to reduce Wingless signalling and/or intercellular adhesion during cell division.
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ABBREVIATIONS

A-P: anterior-posterior
a.c.v.: anterior cross vein
AJ: adherens junction
APC: adenomatous polyposis coli protein
APC/C: anaphase promoting complex/cyclosome
BDGP: Berkeley Drosophila Genome Project
βgal: β-galactosidase
CAD': E-cadherin intracellular domain
Cyc: Cyclin
dAPC2: Drosophila adenomatous polyposis coli protein 2
Arm: armadillo
C96>Arm: C96-GAL4 UAS-Armadillo
C96>CADi: C96-GAL4 UAS-CADi
cycA: cyclin A
cycB: cyclin B
cycB3: cyclin B3
cycD: cyclin D
cycE: cyclin E
dap: dacapo
Daxin: Drosophila axin
dCBP: Drosophila CREB-binding protein
dsh: dishevelled
Dweel: Drosophila weel
En>Arm: Engrailed-GAL4 UAS-Armadillo
En>CADi: Engrailed-GAL4 UAS-CADi

G1/G2 phase: gap phase in cell cycle
GFP: green fluorescent protein
GSK-3β: glycogen synthase kinase-3β
hs: heat shock
L1-5: longitudinal vein 1-5
LEF: lymphoid enhancer-binding factor
LRP: low density lipoprotein receptor-related protein
M phase: mitotic phase of cell cycle
NES: nuclear export sequence
NLS: nuclear localization sequence
PBS: phosphate buffered saline
PTw: PBS + Tween
PTx: PBS + Triton X-100
p.c.v.: posterior cross vein
PH3: phosphohistone H3
S phase: DNA synthesis phase of cell cycle
SOF: sensory organ precursor
TCF: T cell-specific factor
WT: wild type
UAS: Upstream Activating Sequences (for GAL4)
ZNC: zone of non-proliferating cells

fz: frizzled
fzr: fizzy-related
fzy: fizzy
gro: groucho
new: neuralized
pan: pangolin
pbl: pebble
PP2A: protein phosphatase 2A
Rbf: retinoblastoma-family protein
Real: Regulator of cyclin A1
rux: roughex
sgg: shaggy
shg: shotgun
slimb: supernumerary limbs
sig: string
trbl: tribbles
tws: twins
wg: wingless
CHAPTER 1: INTRODUCTION

"Oh my darling Armadillo, Let me tell you of my love"

Michael Flanders and Donald Swann, The Armadillo

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1.1 The cellular requirements for metazoan development

In its simplest terms, the development of a multicellular organism proceeds through the regulation of three basic, yet key processes: cell proliferation, cell-cell adhesion, and the acquisition of cell fate and function. The high degree of evolutionary conservation of the components of the cell division cycle, cell-cell adhesion complexes, and the molecular pathways that specify cell type is testament to their importance. Of course, at a less superficial level, several additional cell biological phenomena are required for normal metazoan development. For example, some cells are programmed to die rather than to live; certain cell types come together to form discrete tissues, organs and organ systems; other cells are highly motile and migrate around the developing organism; and the size and shape of cells and organs must also be controlled.

Each of these processes is regulated individually at many levels, both genetic and epigenetic, and which combine both intracellular and intercellular cues. Crucially, all these events must also be exquisitely co-ordinated and integrated during development. The identification of molecules that might perform such an integration function has long been a 'holy grail' of developmental biology, but has become particularly so in the post-genomic era. One such candidate is the β-catenin/Armadillo protein (reviewed by Miller and Moon, 1996). Investigations in the fruit fly, *Drosophila melanogaster*, initially identified Armadillo as a transducer of the Wingless signal that specifies cell fate during development. Separate studies in cultured mammalian cells identified β-catenin as a critical mediator of cell-cell adhesion at epithelial adherens junctions. Other analyses showed that Wnt/Wingless signalling promotes cell proliferation both in vertebrates and in *Drosophila*. The realization that β-catenin and Armadillo were one and the same protein therefore put it at a unique cellular hub: a single factor that could potentially regulate cell proliferation, intercellular adhesion and cell determination. This thesis describes an
additional and unexpected link between the regulation of Armadillo function and cell cycle progression in *Drosophila*.

### 1.2 Specification of cell types by the Wnt/Wingless signalling pathway

#### 1.2.1 Developmental effects of Wnt/Wingless signalling

Developmental cell fate is commonly specified by the acquisition and integration of extracellular information through interactions with other cells. This may occur either locally, via cell surface determinants, or at a distance, via secreted signalling molecules such as Wnts. Wnt signalling is notable in its ability to direct diverse cell fate choices at different developmental phases in species ranging from nematode worms to humans (reviewed by Wodarz and Nusse, 1998).

Wnt proteins comprise a large family of cysteine-rich, glycosylated secreted ligands (Wodarz and Nusse, 1998). The binding of Wnts to cell-surface receptors activates an intracellular signalling cassette (see section 1.2.2) to determine context-dependent cellular outcomes, such as differentiation, proliferation, motility or polarity. The Wnt ligands and downstream signalling components have been conserved through evolution, although research has been most extensive in the fruit fly and in various vertebrate systems.

*Wingless signalling in Drosophila*

*Drosophila* possess 7 Wnts (Adams, 2000), although the functions of Wingless (DWnt1) are by far the most studied and best characterized. Relatively early in embryogenesis, Wingless is expressed in reiterated stripes and is required for proper segmentation, the critical process that lays down the insect body plan for subsequent developmental patterning (Nusslein-Volhard, 1980; van den Heuvel *et al.*, 1989). Later in embryogenesis,
Wingless also acts at a distance to specify naked cell fates in the larval cuticle (Bejsovec and Martinez Arias, 1991). Other embryonic roles include the patterning of the gut mesoderm (Hoppler and Bienz, 1995) and the specification of coronary and neuronal precursors (Wu et al., 1995; Bhat, 1996; Bhat and Schedl, 1997).

During larval development, Wingless is required for the proper development of the imaginal discs, epithelial sacs of cells that are the primordia of the adult appendages. In the developing eye, Wingless is a negative regulator of the morphogenetic furrow, a wave of cell-type determination that produces the neural cell clusters that will form the adult ommatidia (Treisman and Rubin, 1995). In leg discs, wingless expression defines ventral-anterior leg (Theisen et al., 1996). Wingless is also required for the development of the genitalia (Chen and Baker, 1997) and the dorsal-ventral (D-V) patterning of the adult abdomen (Kopp et al., 1999).

More pertinent to this thesis are the roles of Wingless signalling in the development of the imaginal wing disc, which in fact gives rise to both the wing proper and the body wall of the mesothorax. The primary and earliest role of Wingless is in specifying the wing primordium during the second larval instar (Ng et al., 1996). Wingless is expressed anterior-ventrally within the wing pouch and its presence here is required to initiate correct D-V compartmentalization and to activate the expression of the pro-wing genes vestigial and scalloped (Williams et al., 1993). Consistent with this early role of Wingless, and as its name suggests, certain wingless mutants have no wings (Sharma and Chopra, 1976).

By the mid-third larval instar, Wingless is expressed in a narrow stripe of cells at the D-V boundary of the wing disc, which is also the presumptive wing margin (Phillips and Whittle, 1993). Here, Wingless specifies the production of bristles and sensory cells on the prospective wing margin by up-regulating the expression of the pro-neural genes achaete and neuralized in adjacent cells, and of cut in those cells expressing wingless (Couso et al., 1994; Zecca et al., 1996). This late expression of Wingless also mediates the
growth-promoting activities of the dorsal-ventral boundary, probably through the activation of *vestigial* and *distal-less* across the wing pouch (Neumann and Cohen, 1996b; Neumann and Cohen, 1997; see section 1.4.1).

*Drosophila* Wnts may also play a role in specifying tissue polarity in the adult wing (Adler *et al.*, 1997). One gene required for this polarity, *frizzled*, encodes a transmembrane receptor capable of transducing the Wingless signal (Bhanot *et al.*, 1996; see section 1.2.2), although Wingless itself does not appear to be the relevant ligand.

Wnts in other species

There are at least 14 Wnts in mammals (Dale, 1998), and each has been implicated in different developmental processes (reviewed by Wodarz and Nusse, 1998). In the mouse, for example, Wnt-7A is required for dorsal limb development, Wnt-4 has an essential role in kidney development, and Wnt-1 is required for midbrain and cerebellum development. In the frog *Xenopus laevis*, Wnt signalling has been implicated in the establishment of the primary body axis, although it appears that an endogenous Wnt may not be involved in this process (Cadigan and Nusse, 1997).

Wnt genes also exist in the nematode *Caenorhabditis elegans* and appear to be involved in promoting asymmetric cell divisions (reviewed by Cadigan and Nusse, 1997 and Wodarz and Nusse, 1998). Both *mom-2* and *lin-44* encode Wnt genes that are required to polarize adjacent cells. *mom-2* is required also for the correct orientation of mitotic spindles in certain blastomeres.

1.2.2 Wnt/Wingless signal transduction

*Drosophila*

Numerous genetic and biochemical studies have led to the description of a canonical ‘signalling cassette’ for Wnt/Wingless transduction, in which the level of the β-
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catenin/Armadillo protein is the pivotal factor (Figure 1.1; reviewed by Dale, 1998). In *Drosophila*, absence of the Wingless ligand results in ubiquitin-mediated proteolysis of cytoplasmic Armadillo via the specificity factor Slimb and the proteasome (Jiang and Struhl, 1998; reviewed by Maniatis, 1999). Armadillo is tagged for ubiquitylation through phosphorylation by the serine-threonine kinase Shaggy (also called Zeste-white3) in concert with the Daxin and dAPC2 (also called E-APC) proteins (Siegfried *et al.*, 1992; Peifer *et al.*, 1994a; Peifer *et al.*, 1994c; Hamada *et al.*, 1999; Willert *et al.*, 1999a; McCartney *et al.*, 1999; Yu *et al.*, 1999). In this situation, cytoplasmic Armadillo is low and Wingless target genes are repressed through the action of the Pangolin (also called dTCF) transcription factor and its co-repressors Groucho and dCBP (Cavallo *et al.*, 1998; Waltzer and Bienz, 1998). Cells that receive Wingless do so via a seven-pass transmembrane receptor of the Frizzled family (Cadigan *et al.*, 1998; Bhanot *et al.*, 1999; Chen and Struhl, 1999). This leads to inhibition of the kinase activity of Shaggy through a largely unknown mechanism that requires the cytoplasmic protein Dishevelled (Klingensmith *et al.*, 1994; Noordermeer *et al.*, 1994; Peifer *et al.*, 1994a; Peifer *et al.*, 1994c; Theisen *et al.*, 1994; Yanagawa *et al.*, 1995). Armadillo is thus stabilized and accumulates in the cytoplasm, before ultimately translocating to the nucleus (Orsulic and Peifer, 1996; Pai *et al.*, 1997). Nuclear Armadillo binds and contributes a transcriptional transactivation domain to the Pangolin DNA-binding protein (van de Wetering *et al.*, 1997), thus forming a bipartite transcription factor that regulates expression of Wingless-responsive genes such as * engrailed* in the embryonic epidermis (DiNardo *et al.*, 1988) and * vestigial* in the larval wing primordium (Neumann and Cohen, 1996b). Several points have been simplified in this outline view of Wingless signal transduction. Upstream of the receptor, membrane-bound proteoglycans, such as Dally, have an important role in increasing the efficiency of Wingless reception (Lin and Perrimon, 1999; Tsuda *et al.*, 1999). These may assist in the presentation of the ligand or may act as co-receptors to
In a cell not receiving Wingless (left hand side), Armadillo associates with a 'destruction complex' and is phosphorylated by the Shaggy kinase. This tags the protein for ubiquitin-mediated proteolysis by Slimb and the proteasome. Consequently the cytoplasmic level of Armadillo is low and Wingless-responsive genes are repressed through the action of the Pangolin transcription factor bound to its co-repressors Groucho and dCBP. In the presence of Wingless, a signal is transmitted through a membrane receptor of the Frizzled family and the cytoplasmic protein Dishevelled. This inhibits the activity of the destruction complex, thus stabilizing Armadillo in the cytoplasm. Upon nuclear entry, Armadillo associates with Pangolin to mediate transcriptional transactivation of target genes. Several components and mechanisms have been omitted for clarity—see text. Wg, Wingless; Fz, Frizzled; Dsh, Dishevelled; Daxin, *Drosophila* Axin; Sgg, Shaggy; Arm, Armadillo; Pan, Pangolin; Gro, Groucho; dCBP, *Drosophila* CREB-binding protein; green arrow, activation; red arrow, inhibition.
facilitate the binding of Wingless to Frizzled receptors. Recently, the low density lipoprotein receptor-related protein (LRP) Arrow has been identified as an additional potential co-receptor for Wingless (Wehrli et al., 2000). Unlike the influence of proteoglycans on Wingless signalling, the role of Arrow at the cell surface is absolutely required for signal transduction. However, its mechanism of action and its functional connection with Frizzled receptors remain open questions.

While both Frizzled and DFrizzled2 can bind and transduce Wingless (Bhanot et al., 1999; Chen and Struhl, 1999), they do so with different affinities and efficacies (Boutros et al., 2000; Rulifson et al., 2000). Based on these data, DFrizzled2 appears to be the predominant transducer of the Wingless signal in vivo while Frizzled primarily regulates planar polarity.

The precise mechanism by which the Wingless-Dfrizzled2 signal is transmitted to downstream components is unresolved. Genetic epistasis experiments have placed Dishevelled downstream of the receptor and upstream of Shaggy (Klingensmith et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994; Yanagawa et al., 1995). Dishevelled lacks enzymic motifs, but does possess protein-protein interaction domains and so probably functions as a molecular adaptor, perhaps at the cell membrane (Dale, 1998).

The nature of the interactions between dAPC2, Daxin, Shaggy and Armadillo (the so-called ‘destruction complex’) have not been elucidated in Drosophila, and the contribution of each of these components to the regulation of Armadillo stability are incompletely understood. The exception is Shaggy, which has long been known to phosphorylate an N-terminal region of Armadillo and thus cause its degradation (Figure 1.2); N-terminal deleted versions of Armadillo increase to high levels and exhibit enhanced signalling ability (Peifer et al., 1994a; Peifer et al., 1994c; Zecca et al., 1996; Pai et al., 1997). More recently, phosphorylated Armadillo has been found to be ubiquitylated via Slimb, a specificity factor that is part of an ubiquitin ligase complex which targets phospho-
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Armadillo for degradation by the proteasome (Jiang and Struhl, 1998; Figure 1.2). Daxin negatively regulates Wingless signalling and binds directly to Armadillo, Shaggy and dAPC2 (Hamada et al., 1999; Willert et al., 1999a; Figure 1.2). It is thought that Daxin acts as a scaffold protein to facilitate Shaggy-mediated phosphorylation of Armadillo, although a more direct role is suggested by the finding that over-expressed Daxin alone can destabilize Armadillo (Willert et al., 1999a). While dAPC2 also functions to antagonize Armadillo activity, its mechanism of action is not clear (McCartney et al., 1999; Yu et al., 1999). One possibility is that dAPC2 shuttles Armadillo between the cytoplasm/nucleus and junctional zones, as dAPC2 can track along actin filaments and adherens junctions may be sites of Armadillo destruction (Bienz, 1999; Townsley and Bienz, 2000). Dpresenilin, the Drosophila homologue of the human Presenilin protein implicated in Alzheimer’s disease, has also been implicated in the trafficking of Armadillo between adherens junctions and the proteasome, though, like dAPC2, its precise function in Armadillo regulation remains unresolved (Noll et al., 2000).

There are also several twists to the control of gene transcription by the Armadillo-Pangolin complex. In the absence of Wingless signalling, Pangolin is thought to be bound to DNA and actively repress gene transcription through its interaction with Groucho and dCBP (Cavallo et al., 1998; Waltzer and Bienz, 1998). Groucho is a classical co-repressor and acts as such on a number of other DNA binding proteins (Cavallo et al., 1998). CBP, however, is a histone acetylation enzyme that can reconfigure chromatin and it is thus traditionally regarded as a transcriptional co-activator. Nonetheless, genetic and biochemical data demonstrate that dCBP acts as a repressor of Pangolin-mediated transcription, maybe through decreasing the binding affinity of Armadillo to Pangolin (Waltzer and Bienz, 1998). Upon Wingless stimulation, Armadillo enters the nucleus through an uncharacterized mechanism. Binding of Armadillo to Pangolin is thought to activate transcription by displacing Groucho and dCBP and by contributing a C-terminal
Figure 1.2  Armadillo functional domains and binding proteins

Armadillo is depicted as a linear protein of 843 amino acids. The turquoise boxes represent the 13 imperfect 'Armadillo repeat' motifs which mediate protein-protein interactions. The green oval marks the site of the serine/threonine residues phosphorylated by Shaggy and recognized by the F box protein Slimb. Functional domains or binding sites for components of the Wingless pathway are shown above the protein, and sites associated with adhesion are shown below. All domains and binding sites shown have been reported for Drosophila Armadillo except for dAPC2 and dAxin, which have been inferred from data on vertebrate β-catenin. Sites for several other binding proteins have been omitted for clarity. Shaggy, Slimb (Pai et al., 1997); dAPC2, dAxin (Dale, 1998); Pangolin, transactivation domain (van de Wetering et al., 1997); Teashirt (Gallet et al., 1999); α-catenin, Drosophila E-cadherin (Pai et al., 1996; Orsulic and Peifer, 1996).
transcriptional transactivation domain (van de Wetering et al., 1997; Cavallo et al., 1998; Waltzer and Bienz, 1998; Figure 1.2).

Other nuclear factors that appear to affect Wingless-mediated transcription include the zinc-finger protein Teashirt, which can bind the C-terminus of Armadillo and mediate Wingless-induced transcriptional repression (Gallet et al., 1999; Waltzer et al., 2001; Figure 1.2); the nuclear proteins dPontin and dReptin (Bauer et al., 1998a), which may form tripartite complexes with Armadillo and the TATA-box binding protein to modulate DNA unwinding; and Eyelid, a DNA-binding protein that antagonizes Wingless-mediated transcription (Treisman et al., 1997). As a final complication, it has been reported that the Armadillo-Pangolin complex can also repress gene transcription in certain contexts (Payre et al., 1999).

Vertebrates

Vertebrates possess the same conserved Wnt signalling cassette as Drosophila, although the situation is made more complex by the existence of multiple homologues and additional regulatory mechanisms (reviewed by Dale, 1998). Nevertheless, studies in vertebrate systems have uncovered novel mechanisms of Wnt signal transduction which may be applicable to fruit flies, in addition to finding similarities between the two systems.

At the level of Wnt reception, vertebrate studies have confirmed the importance of members of the LRP family as co-receptors with Frizzled proteins (Pinson et al., 2000; Tamai et al., 2000; Mao et al., 2001). Furthermore, LRP-5 has been shown to bind directly to Axin and transduce Wnt signals (Mao et al., 2001). Another study has found that the rat Frizzled1 is likely a G protein-coupled receptor and that G proteins form part of the Wnt signal transduction pathway (Liu et al., 2001). These two reports are particularly important in that they establish erstwhile missing links between the Wnt receptor and intracellular signalling components.
Analogous to *Drosophila*, vertebrate Axin, APC and the homologue of *Drosophila* Shaggy, GSK-3β, are implicated in the down-regulation of β-catenin. The ‘scaffold’ protein Axin binds directly to APC, β-catenin and GSK-3β, while GSK-3β has been shown to not bind directly to β-catenin (Hart *et al*., 1998; Ikeda *et al*., 1998; Sakanaka *et al*., 1998). Thus in unstimulated cells, GSK-3β phosphorylates Axin, which in turn promotes binding of Axin to β-catenin, and this facilitates GSK-3β-mediated phosphorylation of β-catenin, targeting it for degradation (Hart *et al*., 1998; Ikeda *et al*., 1998). Wnt-induced inhibition of GSK-3β induces rapid dephosphorylation of Axin, perhaps via protein phosphatase 2A whose catalytic subunit binds directly to Axin (Hsu *et al*., 1999; Willert *et al*., 1999b). This effect decreases the affinity of Axin for β-catenin and releases it from the destruction complex (Willert *et al*., 1999b). These findings provide a partial explanation for the mechanism of how Wnt signalling regulates the activity of the destruction complex and identifies GSK-3β-mediated phosphorylation of β-catenin as a two-step process. Vertebrate Dishevelled appears may also be part of the destruction complex as it too binds to Axin (Kishida *et al*., 1999; Li *et al*., 1999; Smalley *et al*., 1999; Salic *et al*., 2000).

As in *Drosophila*, the precise role of APC in vertebrate Wnt signalling has not been firmly established, though it clearly antagonizes β-catenin accumulation (Dale, 1998). For example, APC binds β-catenin directly within the destruction complex, and mutation of the β-catenin regulatory domains correlates strongly with the upregulation of β-catenin (Polakis, 2000), while transfection of APC can decrease the level of β-catenin (Munemitsu *et al*., 1995). APC appears to have numerous roles and many subcellular sites of expression, only some of which may be involved in β-catenin turnover (Reinacher-Schick and Gumbiner, 2001). For example, in confluent polarized epithelial cells, the vast majority of APC is associated with the plasma membrane and probably does not take part in β-catenin degradation (Reinacher-Schick and Gumbiner, 2001). APC also binds to microtubules and the microtubule binding protein EB1, which may reflect a subcellular
shuttling role of APC (see below) or a role in cell migration (Smith et al., 1994; Munemitsu et al., 1994; Mimori-Kiyosue et al., 2000a; Su et al., 1995; Mimori-Kiyosue et al., 2000b).

Recently, APC has been shown to be a nucleocytoplasmic shuttling protein, containing both nuclear localization and nuclear export sequences (Henderson, 2000; Neufeld et al., 2000a; Rosin-Arbesfeld et al., 2000; Zhang et al., 2000). APC has therefore been heralded as a β-catenin chaperone, modulating both its nuclear abundance and its relative stability (Bienz, 1999; Henderson, 2000). In contrast, a different study examining β-catenin distribution under more physiological conditions found that export did not depend on a classical export pathway and that β-catenin may cross the nuclear pore freely in either direction (Wiechens and Fagotto, 2001). This view is supported by the finding that β-catenin lacks a recognizable nuclear localization sequence and rather enters the nucleus on its own via direct interaction with the nuclear pore complex (Fagotto et al., 1998). Although the nucleocytoplasmic transport of β-catenin may be a largely non-directional process, its import is nonetheless likely to be regulated by cytosolic factors (Fagotto et al., 1998).

Consistent with the findings in Drosophila, numerous reports show that phosphorylated β-catenin associates with and is regulated by β-TrCP and FWD1, the homologues of Drosophila Slimb in humans and mice respectively (Orford et al., 1997; Marikawa and Elinson, 1998; Hart et al., 1999; Liu et al., 1999; Winston et al., 1999; Kitagawa et al., 1999). These studies confirm that N-terminal phosphorylated β-catenin/Armadillo is specifically recognized and ubiquitylated by the ubiquitin ligase complex SCFβ-TrCP/Slimb in order to render it a target for degradation by the proteasome (Aberle et al., 1997; Maniatis, 1999). Furthermore, it has been reported that β-catenin-TCF signalling induces β-TrCP expression and activity in a human cell line, thus accelerating the degradation of β-catenin and creating a negative feedback loop (Spiegelman et al., 2000).
Several additional regulatory mechanisms have been uncovered in the vertebrate system that may or may not be applicable to *Drosophila*, and include the following. Secreted Frizzled related proteins have been identified that bind Wnt ligands to antagonize their signalling activity (reviewed by Polakis, 2000). Casein kinase II binds to and phosphorylates Dishevelled, an event that correlates with membrane-localization of Dishevelled and may thus facilitate Wnt signal transduction (Willert *et al.*, 1997). The Frat-1/GBP protein can inhibit GSK-3β activity, perhaps by competing with Axin for binding to GSK-3β (Li *et al.*, 1999; Salic *et al.*, 2000; Thomas *et al.*, 1999; Yost *et al.*, 1998). At the level of transcriptional control, the CtBP protein acts as a co-repressor of Wnt-mediated transcription (Brannon *et al.*, 1999), and, in contrast to *Drosophila*, P300/CBP is a transcriptional co-activator (Sun *et al.*, 2000).

### 1.2.3 The pivotal role of β-catenin/Armadillo in Wnt/Wingless signalling

As discussed above, Wnt/Wingless signalling is regulated in many ways and at several steps during signal transduction. Nearly all of these regulatory mechanisms are centred on controlling the protein stability of β-catenin/Armadillo. Control at this level presumably facilitates a rapid response to changes in Wnt/Wingless stimulation. Indeed, the transcription of *armadillo* is constitutive (Riggleman *et al.*, 1989) and regulated proteolysis is thought to be the major control step (Dale, 1998).

Despite this clear correlation between β-catenin/Armadillo stability and signalling, it is not known if increased protein levels are either necessary or sufficient for Wnt transduction. Thus it remains quite possible that other post-translational modifications are also important. In support of this view, analyses during the early development of the frog *Xenopus laevis* found that mutation of the N-terminal GSK-3β phosphorylation sites enhanced β-catenin signalling without significantly increasing its cellular levels (Guger and Gumbiner, 2000). Other observations that β-catenin’s signalling activity does not always
correlate with its levels have also been reported (Korinek et al., 1997; Young et al., 1998; Nelson and Gumbiner, 1999). Drosophila Armadillo is phosphorylated on serine, threonine and tyrosine residues separate from the N-terminal Shaggy target sites (Peifer et al., 1994a). These phosphorylation events were found to be both tissue- and developmental stage-specific, indicating that they had roles apart from the control of protein stability. Therefore, although control of β-catenin/Armadillo turnover is critical in controlling its signalling activity, it may not be the only regulatory step. These modifications could potentially affect the nuclear translocation or transcriptional activity of β-catenin/Armadillo.

1.2.4 β-catenin/Armadillo also functions in cell-cell adhesion

β-catenin/Armadillo is an essential component of epithelial cell-cell adherens junctions (AJs) in metazoans (Figure 1.3; Gumbiner, 2000). The core components of these junctions are classical E-cadherins, single pass transmembrane proteins that function as dimers and mediate homotypic adhesion between neighbouring cells via their extracellular domains. β-catenin/Armadillo binds to the cadherin cytoplasmic tail and to α-catenin. α-catenin then links to cortical actin, either directly or via α-actinin or vinculin, so as to connect AJs to the cytoskeleton and provide strong intercellular adhesion. In vertebrates, p120ctn and plakoglobin (also known as γ-catenin) are also present in AJs. Plakoglobin and β-catenin are 70% identical in sequence and each binds directly to E-cadherin in a mutually exclusive fashion to mediate adhesion. p120ctn binds to E-cadherin at a juxtamembrane site that is distinct from that of β-catenin and plakoglobin, though its function is unknown.

Adhesive interactions between cells are dynamic during tissue development (for example, cell sorting and morphogenesis) and regulation of cadherin-based adhesion at AJs is thought to underlie these changes (reviewed by Gumbiner, 2000 and McNeill, 2000). It has long been known that the level of cadherin expression can modulate the strength of
Figure 1.3 Components and regulation of adherens junctions in vertebrate cells

E-cadherin molecules form apical cis-dimers and mediate cell-cell adhesion by binding in trans to similar dimers on adjacent cells (not shown). Intracellularly, β-catenin and plakoglobin (not shown) bind to the cytoplasmic tail of E-cadherin in a mutually exclusive manner, while p120\textsuperscript{ct} binds to a separate juxtamembrane region. β-catenin links to the actin cytoskeleton through α-catenin and α-actin or vinculin (not shown). Receptor and Src family tyrosine kinases are thought to phosphorylate components of the adherens junction and so reduce adhesion. For example, tyrosine phosphorylation of β-catenin may result in its dissociation from junctions, though it is not clear whether it remains bound to other junctional components. Additionally, members of the G12 subfamily of heterotrimeric G proteins have been shown to bind the cytoplasmic tail of E-cadherin and cause release of β-catenin into the cytoplasm. β-cat, β-catenin; α-cat, α-catenin, RTKs, receptor tyrosine kinases; G12, heterotrimeric G protein.
adhesion, while the type of cadherin can determine the specificity of cell-cell interaction (Gumbiner, 2000). However, the rapid assembly and disassembly of AJs that accompany certain morphogenetic and homeostatic processes are more likely to be regulated by post-translational modifications of the components of the AJ complex (Figure 1.3).

Many studies in vertebrate cells indicate a key role for tyrosine phosphorylation of cadherin-catenin complexes in modulating cell adhesiveness, mediated through receptor tyrosine kinases or Src family kinases that localize to AJs (Figure 1.3; reviewed by Daniel and Reynolds, 1997). For example, v-Src-mediated tyrosine phosphorylation of cadherin, β-catenin, plakoglobin, α-catenin or p120<sup>cm</sup> correlates with reduction or inhibition of cadherin-mediated adhesion, with β-catenin being the most common target (for recent examples see Lampugnani et al., 1997; Muller et al., 1999; Ohsugi et al., 1999; Roura et al., 1999). The underlying biochemical mechanism that results in decreased adhesion is not known, but could involve dissociation of the cadherin-catenin complex or conformational changes that abrogate adhesive functions (Daniel and Reynolds, 1997; Figure 1.3). However, other studies found that phosphorylation of β-catenin by v-Src did not disrupt the cadherin-catenin complex (Papkoff, 1997), and that an E-cadherin-α-catenin fusion protein, which functions without any β-catenin in the complex, was still regulated by v-Src (Takeda et al., 1995). In summary, the jury is still out on the significance of reversible tyrosine phosphorylation of β-catenin in regulating cadherin-mediated cell-cell adhesion in vivo.

The small GTPases, Rac, Rho, and Cdc42 have been shown to affect cadherin-mediated adhesion, possibly through regulating the actin-α-catenin connection at AJs (Kaibuchi et al., 1999). It has also been demonstrated recently that members of the G12 subfamily of heterotrimeric G proteins can bind the cytoplasmic domain of E-cadherin to cause release of β-catenin into the cytoplasm (Meigs et al., 2001; Figure 1.3).

In Drosophila, Armadillo binds to Drosophila E-cadherin and α-catenin and these components co-localize to the apical zone of cell-cell junctions (Peifer and Wieschaus,
1990; Oda et al., 1993; Oda et al., 1994; Figure 2). Armadillo is the single β-catenin/plakoglobin homologue in Drosophila (Peifer and Wieschaus, 1990) and is required for AJ formation throughout development, from the very earliest stages (Peifer et al., 1993; Cox et al., 1996). The dynamic regulation of adherens junction formation in Drosophila has received relatively scant attention. For example, although Armadillo is tyrosine-phosphorylated in vivo, the significance of this to the regulation of cell adhesion has not been addressed (Peifer et al., 1994a). However, differential expression of Drosophila E-cadherin between migrating and surrounding cells has been shown to be required for cell migration during oogenesis (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998; Niewiadomska et al., 1999).

1.2.5 How are the two primary roles of β-catenin/Armadillo connected?

The fact that β-catenin/Armadillo is required both for cell-cell adhesion at the plasma membrane and for Wnt/Wingless transduction in a nucleocytoplasmic fraction raises obvious questions regarding the interdependency of these two pools during Wnt/Wingless signalling (Ben-Ze'ev and Geiger, 1998). Structure-function analyses of Armadillo have demonstrated both separate and overlapping regions of the protein that are required for cell-cell adhesion and signalling (Figure 2; Orsulic and Peifer, 1996): the α-catenin binding site, Shaggy target sites and transcriptional transactivation domain map to distinct regions, while the Daxin, dAPC2, E-cadherin and Pangolin binding sites overlap. This mutual exclusivity presumably restricts the association of any single Armadillo molecule to either an adhesion or transcription or degradation complex at any given time, and also explains why the membrane pool is stable while the cytoplasmic pool can be subject to dAPC2-mediated proteolysis. Nevertheless, the functions of β-catenin/Armadillo in adhesion and signalling can be completely separated: β-catenin/Armadillo mutant proteins in Xenopus or
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*Drosophila* that cannot bind cadherin can still function in signalling, and *vice versa* (Cox *et al.*, 1996; Orsulic and Peifer, 1996; Sanson *et al.*, 1996; Funayama *et al.*, 1995).

The corollary of the mutual exclusivity in the binding partners of β-catenin/Armadillo is that, under limiting conditions, its binding to one partner prevents its participation in other processes (Heasman *et al.*, 1994; Cox *et al.*, 1996; Fagotto *et al.*, 1996; Sanson *et al.*, 1996; Torres *et al.*, 1996). For example, over-expression of E-cadherin in *Drosophila* can sequester Armadillo at the membrane and decrease the pool available for signalling (Sanson *et al.*, 1996). However, it is not yet known how readily β-catenin/Armadillo exchanges between cadherin-bound and free cytoplasmic pools under Wnt/Wingless stimulation during normal development. The most parsimonious explanation of the present data is that cadherin-bound β-catenin/Armadillo is unavailable for signalling and functions solely in adhesion, while newly produced, cadherin-free β-catenin/Armadillo can be stabilized upon Wnt/Wingless stimulation and participate in signalling (Miller and Moon, 1996; Cox *et al.*, 1999; Orsulic *et al.*, 1999).

As alluded to above, tyrosine phosphorylation of junctional β-catenin and the action of certain heterotrimeric G proteins can cause the dissociation of β-catenin from adherens junctions and its release into the cytoplasm (Daniel and Reynolds, 1997; Meigs *et al.*, 2001). However, the consequences of this relocalization for Wnt/Wingless signalling are not clear, although some have suggested that tyrosine phosphorylated β-catenin might be stable and thus facilitate signalling (Daniel and Reynolds, 1997).

The cellular response to Wnt/Wingless can involve distinct effects on cell adhesion in transcriptionally-dependent and independent manners. For example, Wnt signalling can induce adhesion by increasing the stability of complexed cadherin-β-catenin (Bradley *et al.*, 1993; Hinck *et al.*, 1994b; Toyofuku *et al.*, 2000). This indicates that cadherin-bound β-catenin may be involved in adhesive changes even if it cannot participate in Wnt/Wingless signalling. Other studies have found that Wnt/Wingless elevates transcription of E-
cadherin, which has a LEF1 site within its promoter (Huber et al., 1996; Yanagawa et al., 1997). Perhaps in contrast to these findings, over-expression experiments in flies with wild type and dominant-negative E-cadherin indicated that regulation is not the major readout of Wingless signalling (Sanson et al., 1996).

Co-localization analyses have also suggested intimate links between the role of β-catenin/Armadillo in adhesion and signalling: APC/dAPC2 and vertebrate Axin localize to apical adhesive junctions (Yu et al., 1999; Reinacher-Schick and Gumbiner, 2001; Fagotto et al., 1999), while Dishevelled associates with the plasma membrane upon Wnt/Wingless stimulation (Yang-Snyder et al., 1996). These observations have inspired a model in which AJs are the cellular intersections of β-catenin/Armadillo function in adhesion and Wnt/Wingless signalling. According to this model, APC/dAPC2 binds to β-catenin/Armadillo in the cytoplasm or nucleus and shuttles it through the cytoplasm to junctional zones (Bienz, 1999). Once there, β-catenin/Armadillo can be incorporated into AJs or may be delivered to an apically localized destruction complex. Wnt/Wingless signals may act by inhibiting the activity of this complex, perhaps by causing the dissociation of APC/dAPC2 (Bienz, 1999). Indeed, in the Drosophila embryo, association of dAPC2 with junctional compartments appears to be critical for the destabilization of Armadillo (McCartney et al., 1999; Yu et al., 1999).

In summary, the function of β-catenin/Armadillo in signalling is distinguishable from its role in adhesion. However, the use of a common molecule in signalling and adhesion is probably significant (Hinck et al., 1994a; Ben-Ze'ev and Geiger, 1998; Ben-Ze'ev et al., 2000).

1.2.6 Is β-catenin/Armadillo involved in other processes?

β-catenin/Armadillo has been implicated in several non-Wnt/Wingless pathways. For example, in worms, the Tak1 and Nemo-like kinases act in a pathway that phosphorylates
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WRM1, a divergent β-catenin/Armadillo homologue, and affects its nuclear localization (Rocheleau et al., 1997; Ishitani et al., 1999; Meneghini et al., 1999). In another study, activation of the integrin-linked kinase was shown to induce nuclear translocation of β-catenin and activate the transcription of Wingless-responsive genes (Novak et al., 1998). A third publication reported that insulin-like growth factor 1 stimulation caused tyrosine phosphorylation and stabilization of β-catenin (Playford et al., 2000). β-catenin has also been shown to bind the EGF receptor (Hoschuetzky et al., 1994), the Alzheimer's disease gene product Presenilin (e.g. Murayama et al., 1998), and the actin-bundling protein fascin (Tao et al., 1996).

Intriguingly, there have also been several connections made between Wnt/Wingless signalling or cadherin-mediated cell adhesion and cell cycle control. Before describing these relationships, it is necessary to introduce the components and regulatory mechanisms of the cell division cycle.

1.3 Cell proliferation and the cell cycle

1.3.1 An introduction to the cell cycle

The cell cycle comprises the duplication of the genome and its subsequent equal division into two cells at mitosis and thus results in cell proliferation over time (reviewed by Murray and Hunt, 1993). The cycle is conventionally split into four sequential phases: a G1 'gap' phase, S phase (DNA replication), a second gap phase (G2), and mitosis (M). The term 'interphase' describes the combined non-mitotic phases of the cell cycle, while G0 is defined as a quiescent state that occurs when cells withdraw from cycling during early G1.

A series of cyclin-dependent kinases (Cdks) and their obligate activating Cyclin subunits from the basic eukaryotic cell cycle machinery (Nigg, 1995). The activity of
specific Cdk-Cyclin complexes oscillates through the cell cycle, and promotes entry into and progression through distinct cell cycle phases by phosphorylating appropriate cellular substrates. For example, in mammalian cells, Cdk4/6-Cyclin D promotes G1 progression, Cdk2-Cyclin E complexes regulate entry into S phase, Cdk2-Cyclin A activity is required for DNA replication, and the action of Cdk1 (Cdc2)-Cyclin A/B drives mitotic entry and progression.

Cdns are regulated at a number of levels (Nigg, 1995). First, the expression, stability and subcellular location of their cognate Cyclin subunit determine both the time and place of Cdk activation through the cell cycle (Miller and Cross, 2001). For example, mammalian Cyclin B1 is expressed during interphase but Cdk1-Cyclin B complexes translocate to the nucleus only upon entry into mitosis (Takizawa and Morgan, 2000). A second type of Cdk regulation is imparted by both inhibitory and activating phosphorylation. The classical example is Cdk1, where phosphorylation of a specific threonine residue is required for kinase function, while phosphorylation of a second threonine and of a tyrosine residue inhibits activity (Nigg, 2001). Third, the activity of Cdns is controlled by their binding to discrete inhibitory proteins. For example, at the G1-S transition, members of the Cip/Kip family, such as p21 and p27, bind Cdk2-Cyclin E/A heterodimers and inhibit their activity, probably by causing structural changes in the complex (Sherr and Roberts, 1999).

The critical role of regulated protein destruction in the control of cell cycle transitions should also be emphasized as the levels of several key cell cycle regulators are controlled by ubiquitin-mediated proteolysis by the proteasome (Koepp et al., 1999). The formation of polyubiquitin-protein conjugates requires three components: an activating enzyme (E1), a conjugating enzyme (E2), and an ubiquitin ligase that is also thought to act as a specificity factor (E3). E3s provide the key regulatory step, and two such are known to regulate cell cycle transitions. First, the SCF complex, which comprises at least three
components (Skp1, Cdc53 and an F-box protein), targets the G1 Cyclins D and E, the Cdk inhibitors p21 and p27, and the E2f transcription factor (Patton et al., 1998; Koepp et al., 1999). Second, the anaphase-promoting complex/cyclosome (APC/C), which comprises at least 10 subunits, targets the mitotic Cyclins A and B, the APC/C activator Cdc20/Fizzy, and the Securin proteins that inhibit the separation of sister chromatids at mitosis (Koepp et al., 1999; Zachariae and Nasmyth, 1999). Thus SCF targets mainly G1/S factors while the APC/C targets G2/M regulators. Together, they mediate irreversible proteolysis of cell cycle proteins in order to promote directionality through the proper sequence of cell cycle phase transitions.

1.3.2 The Drosophila cell cycle

Drosophila exhibits different modes of cell cycle regulation with respect to tissue type and developmental stage, with cycles becoming progressively more complex as development proceeds (Edgar, 1995; Edgar and Lehner, 1996; Vidwans and Su, 2001). The cleavage cycles of early embryogenesis occur in a syncytium and lack gap phases and cytokineses. After cellularization, a G2 phase is acquired, and later, towards the end of embryogenesis, cells enter a G1/G0 phase for the first time.

Two different modes of cell cycle regulation exist during larval development. The true larval tissues undergo endoreduplication cycles that consist of repeated rounds of S phase without intervening mitoses and so lead to an increase in ploidy. Cells of the imaginal discs, which are the precursors of the adult appendages, proliferate with a canonical mitotic cell cycle and thus remain diploid (Figure 1.4). Curiously, groups of non-clonally related cells in imaginal wing discs undergo synchronized mitoses, despite having variable G1, S and G2 phases at first (Milan et al., 1996). An explanation for this phenomenon is still wanting.
The targets and time of action of the major cell cycle regulators are indicated. Cyclin D-Cdk4 does not have a critical cell cycle role, though it may still phosphorylate Rbf in vivo. Functional E2f is actually a heterodimer of E2f and Dp subunits. Rca1 may function in G2, though this has not been formally demonstrated. Cyclins A, B and B3 have distinct roles during mitosis (not shown). The inhibitory action of Dweel on Cdk1 appears to be redundant or insignificant in vivo. Polo may have multiple mitotic functions (not shown). Rux appears to function both in the later stages of mitosis and during early G1. It is not known whether all the regulatory events depicted here operate in all mitotic cycles throughout Drosophila development. Fzr, Fizzy-related; Cdk, Cyclin-dependent kinase; Cyc, Cyclin; Rbf, Retinoblastoma family member; Dap, Dacapo; Rca1, Regulator of Cyclin A1; Stg, String; Dweel, Drosophila Weel; Trbl, Tribbles; Tws, Twins; Fzy, Fizzy; Rux, Roughex; Pbl, Pebble. Green arrow, activation; red arrow, inhibition; no arrow, general promotion of cell cycle progression. See text for details.
Remarkably *Drosophila* Cdk4-Cyclin D function is not required for cell cycle progression or even viability (Datar et al., 2000; Meyer et al., 2000). Furthermore, cell cycle phasing remains wild type after increasing or reducing Cdk4-Cyclin activity (Datar et al., 2000; Meyer et al., 2000). Therefore, Cdk4-Cyclin D has a minimal role in cell cycle progression in *Drosophila*.

The G1-S transition in *Drosophila* is sensitive to nutrition, cellular growth, and tissue-specific regulatory mechanisms (Britton and Edgar, 1998; Johnston et al., 1999; Jones et al., 2000; Prober and Edgar, 2000; Figure 1.4). These parameters appear to impinge on the protein levels of Cyclin E, which binds Cdk2 and is ultimately the limiting factor for G1-S in most tissues (Knoblich et al., 1994; Johnston et al., 1999; Prober and Edgar, 2000). Cdk2-Cyclin E complexes induce and can then be specifically inhibited by Dacapo, a Cdk-inhibitor that appears to be the single representative of the Cip/Kip-family of inhibitors in flies, and is instructive in introducing G1 arrest during embryogenesis (de Nooij et al., 1996; Lane et al., 1996; de Nooij et al., 2000; Figure 1.4). Cdk2-Cyclin E phosphorylates the *Drosophila* Rb homologue, Rbf, to derepress E2f activity, and is likely to be the major Rbf kinase in vivo (Du et al., 1996; Meyer et al., 2000; Figure 1.4). As in mammals, *Drosophila* E2f activates the transcription of factors required for DNA synthesis (Duronio et al., 1995; Duronio et al., 1998), although in contrast to mammalian dogma, *Drosophila* Cyclin E appears to have an essential role in DNA replication separate from inducing E2f activity (Duronio et al., 1995; Duronio et al., 1996; Royzman et al., 1997). Indeed, studies in the fruit fly have clearly demonstrated that Cyclin E is a downstream target of E2f and that Cyclin E activation is both necessary and key for the G1-S transition in cells undergoing canonical cycles (Knoblich et al., 1994; Duronio et al., 1996; Duronio et al., 1998). Thus ectopic expression of Cyclin E alone can bypass E2f and induce S phase in proliferating cells of the embryo and imaginal discs (Duronio et al., 1995; Neufeld et al., 1998).
Control of S phase progression in *Drosophila* is not well understood, although E2f-dependent ORC1 (origin recognition complex 1) levels appear to govern replication origin activity (Asano and Wharton, 1999). It is also clear that Cyclin A does not function normally with Cdk2 to control DNA synthesis, which is in contrast to mammalian cell cycles (Sprenger et al., 1997).

Metazoan mitosis is conventionally divided into four stages: prophase, metaphase, anaphase, and telophase (Murray and Hunt, 1993). During prophase, duplicated chromosomes condense, the nuclear envelope breaks down, and a microtubule-based mitotic spindle begins to radiate from two bipolar microtubule organizing centres called centrosomes. Spindle formation is completed by metaphase, during which chromosomes attach to the microtubules and position themselves midway between the two poles. Anaphase involves the breakage of the link between sister chromatids and their subsequent separation to opposite poles of the spindle. Finally, during telophase, cytokinesis occurs, the chromosomes decondense and acquire a new nuclear envelope, and the microtubule array regains its interphase state. Entry into mitosis is triggered by activation of the evolutionarily conserved mitotic kinase, Cdk1, bound to one of three mitotic Cyclin types (Nigg, 2001). Mitotic exit occurs via Cyclin proteolysis and the consequent inactivation of Cdk1 (Zachariae and Nasmyth, 1999).

*Drosophila* Cdk1 couples to one of three mitotic Cyclins (A, B or B3) to coordinate progression into and through mitosis (Lehner and O'Farrell, 1990; Stern et al., 1993; Knoblich and Lehner, 1993; Jacobs et al., 1998; Figure 1.4). Although eukaryotic Cdk1 targets are known to include nuclear lamins (to cause nuclear lamina disassembly), histone H1 (to induce chromosome decondensation) and c-Src (possibly to coordinate cytoskeletal rearrangements), the precise functions of each Cdk1-Cyclin complex are not well-characterized (Moreno and Nurse, 1990). In fruit flies, the extent of this knowledge amounts to differences in Cyclin subcellular localization (Lehner and O'Farrell, 1989;
Lehner and O'Farrell, 1990; Jacobs et al., 1998), that Cyclin B associates with and organizes spindle microtubules (Maldonado-Codina and Glover, 1992; Huang and Raff, 1999), and that there may be functional redundancy between the B and B3 Cyclins (Jacobs et al., 1998). Of the three mitotic Cyclins, only Cyclin A is essential for mitosis, and is considered the most potent inducer of mitosis in Drosophila (Lehner and O'Farrell, 1989; Jacobs et al., 1998). Consistent with such ascendancy, there exists a protein, aptly named Regulator of Cyclin A1, that specifically promotes Cyclin A accumulation, and could do so in late G2 to facilitate G2-M (Dong et al., 1997).

Genetic and biochemical evidence from Drosophila implicates the multi-target serine/threonine protein phosphatase 2A (PP2A) in the dephosphorylation of Cdk1 substrates (Mayer-Jaekel et al., 1994). The PP2A holoenzyme typically comprises a core heterodimer of catalytic (C) and scaffold (A) subunits bound to a third variable regulatory subunit (B) that may impart target specificity to the complex (Millward et al., 1999). twins (also called abnormal anaphase resolution) encodes a regulatory B subunit of Drosophila PP2A (Uemura et al., 1993). Mutations in twins can yield mitotic defects, notably a metaphase-anaphase delay and abnormal anaphase figures, that are associated with a reduced phosphatase activity towards Cdk1-Cyclin B substrates (Gomes et al., 1993; Mayer-Jaekel et al., 1994). The true in vivo targets of PP2A<sup>Twins</sup> in this case are not apparent; it could plausibly activate Cdk1 itself, or might independently dephosphorylate Cdk1 substrates. Perhaps in favour of this second possibly, data from other systems are consistent with a negative regulatory role of PP2A complexes towards Cdk1-Cyclin B function (reviewed by Millward et al., 1999).

The A- and B-type Cyclins are destroyed at different times during mitosis (Whitfield et al., 1990; Lehner and O'Farrell, 1990; Maldonado-Codina and Glover, 1992; Sigrist et al., 1995; Jacobs et al., 1998; Huang and Raff, 1999). A recent study in Drosophila has linked this sequential destruction to distinct mitotic events (Parry and...
O'Farrell, 2001). Cyclin A is destroyed first, prior to the metaphase-anaphase transition, and its degradation is required for chromosome disjunction at anaphase. The majority of Cyclin B is also degraded at metaphase-anaphase, and this is necessary for later events of anaphase such as the directional movement of disjoined chromosomes. Lastly, Cyclin B3 is degraded during anaphase, and this is required for proper reorganization of the spindle and for chromosome decondensation. Thus, over-expression of non-degradable Cyclin A, B or B3 results in metaphase, early anaphase, or late anaphase arrest, respectively (Rimmington et al., 1994; Sigrist et al., 1995; Parry and O'Farrell, 2001). Taken together, these data support a model in which destruction of individual Cyclins controls late mitotic events, and that general inactivation of Cdk1 induces the G1 interphase state.

During mitosis, the APC/C is activated by Cdk1 and can associate with the activating subunit Fizzy (also known as Cdc20; Dawson et al., 1993; Dawson et al., 1995; Sigrist et al., 1995; Zachariae and Nasmyth, 1999). This complex is then responsible for the degradation of the mitotic Cyclins A, B, and B3, and fizzy mutants are consequently blocked at the metaphase-anaphase transition (Dawson et al., 1993; Dawson et al., 1995; Sigrist et al., 1995). Cyclin B and Cyclin B3 are recruited to the APC/C via a short and conserved 'destruction box' motif, while the destruction of Cyclin A is mediated by a longer and more complex signal (Jacobs et al., 2001; Kaspar et al., 2001). Recently, the Cdk inhibitor Roughex was shown to inhibit Cdk1-Cyclin A activity during metaphase and to contribute to mitotic exit (Foley and Sprenger, 2001). The biochemical mechanism of Roughex action is not known, but its inhibition of Cdk1-Cyclin A was found to be independent of Cyclin A proteolysis (Foley and Sprenger, 2001).

Cyclin A function must be completely eliminated during late mitosis in order for a cell to enter a stable G1 phase (Sprenger et al., 1997). If this doesn’t occur, residual Cyclin A is able to form a productive complex with Cdk2 and promote an ectopic S phase, as seen in fizzy-related mutant embryos and roughex mutant imaginal eye discs (Sigrist and Lehner,
1997; Thomas et al., 1994). Fizzy-related is an alternative APC/C subunit that activates the APC/C during late mitosis and G1 when it targets Cyclins A, B and B3 for degradation (Sigrist and Lehner, 1997). Roughex inhibits Cdk1-Cyclin A complexes in G1 as well as during mitosis, and so contributes to the down-regulation of Cyclin A activity (Thomas et al., 1997). In these two ways, residual Cyclin A is prevented from functioning with Cdk2 to initiate an untimely S phase, and the stability of G1 is ensured until the regulated induction of Cyclin E expression (Sprenger et al., 1997).

**String and regulation of G2-M**

Although mitotic events are co-ordinated by the conserved Cdk1-Cyclin machinery, the Cdc25 phosphatase homologue, String, has been shown to be rate-limiting for the G2-M phase transition by three criteria (reviewed by Skaer, 1998). First, string transcription anticipates mitosis by a matter of minutes and stops when mitosis ends (Edgar and O'Farrell, 1989). Second, ectopic expression of string drives G2 cells into mitosis (Edgar and O'Farrell, 1990; Neufeld et al., 1998). Finally, removal of string function arrests the cell cycle in G2 phase (Edgar and O'Farrell, 1989).

String removes inhibitory phosphotyrosine from Cdk1 to trigger mitotic entry (Edgar and O'Farrell, 1989; Kumagai and Dunphy, 1991). In diverse species, this action is competed by the Wee1/Mik1/Myt1 group of tyrosine kinases (Murray and Hunt, 1993). *Drosophila* Wee1 (Dweel) has a maternal function during the early embryonic syncytial cycles, and is also implicated in a cell cycle checkpoint (Campbell et al., 1995; Price et al., 2000). However, Dweel function appears to be dispensable for most cell cycles as zygotic null mutations produce viable flies and loss of zygotic Dweel function does not bypass the requirement for String activity (Price et al., 2000). These findings suggest a non-essential role for Dweel in regulating Cdk1 activity, though a more significant function may have
been masked by perdurance of maternal protein or by the existence of redundant Cdk1 inhibitory kinases.

The function of String itself is also regulated through the action of the Tribbles protein which specifically promotes the turnover of String in a proteasome-dependent manner (Mata et al., 2000). Consistent with this, tribbles mutants undergo precocious mitoses, while over-expression of Tribbles blocks cell division (Mata et al., 2000; Grosshans and Wieschaus, 2000; Seher and Leptin, 2000). Tribbles function was primarily characterized in the embryonic mesoderm, and it is not currently known if it is involved in cell cycle regulation in other tissues or developmental stages (Grosshans and Wieschaus, 2000; Seher and Leptin, 2000). Neither is its precise mechanism of action understood.

Activation of Cdc25C, the vertebrate counterpart of Drosophila String, requires phosphorylation of several sites in its amino-terminal domain. This is known to be catalysed by at least two kinases: the Cdk1-Cyclin B1 complex and a Polo-like kinase (reviewed by Glover et al., 1998). In the former case, this leads to a positive feedback loop of Cdk1 activation, which is enhanced further by the ability of Cdk1 to phosphorylate and inactivate the Wee1/Myt1 kinases. Furthermore, vertebrate Polo-like kinases can also phosphorylate Cyclin B1 and target it to the nucleus during prophase (Toyoshima-Morimoto et al., 2001). Although analogous roles for Drosophila Polo are yet to be demonstrated, this kinase is implicated in controlling mitotic entry in fruit flies through its regulation of centrosome function and spindle formation (Sunkel and Glover, 1988; Llamazares et al., 1991). Indeed, Polo-like kinases appear to take part in multiple mitotic events, as they also activate the APC/C at the metaphase-anaphase transition and regulate cytokinetic events in diverse species (Glover et al., 1998).

14-3-3 proteins have been identified as spatial regulators of vertebrate Cdc25C function (reviewed by Takizawa and Morgan, 2000). During interphase, Cdc25C is phosphorylated at a particular Serine residue, distinct from the activating phospho-sites
alluded to above, and binds to cytoplasmic 14-3-3 proteins that inhibit its nuclear translocation. A comparable mechanism has not been described in *Drosophila*, despite the presence of 14-3-3 homologues in the genome (Adams, 2000). However, as *Drosophila* String is not expressed during interphase, control of nuclear import may not be a relevant form of regulation.

Bursts of *string* transcription determine the timing of cell division in almost all *Drosophila* cells. But how are these bursts controlled? Several studies have found that patterning signals regulate *string* expression and that extensive *cis*-regulatory sequences can integrate such positional information (Edgar et al., 1994a; Johnston and Edgar, 1998; Lehman et al., 1999). Indeed, the current view is that developmental inputs, and not intrinsic cell cycle regulation, control the timing of *string* expression and thus cell division (Neufeld et al., 1998; Johnston et al., 1999; Prober and Edgar, 2000).

### 1.4 Co-ordination of Wnt/Wingless signalling and cell-cell adhesion with cell proliferation

#### 1.4.1 Control of cell cycle progression by Wnt/Wingless signalling

*Drosophila*

*Drosophila* Wingless has been shown to be necessary and sufficient for cell proliferation in the anlage of the Malpighian tubules, and the hinge and blade regions of the imaginal wing disc (Skaer and Martinez Arias, 1992; Neumann and Cohen, 1996a; Diaz-Benjumea and Cohen, 1995). That is, removing Wingless function caused loss of tissue, while over-expression of Wingless resulted in excess proliferation. However, Wingless apparently promotes proliferation indirectly in the developing wing blade through respecification of
‘growth control centres’ (Diaz-Benjumea and Cohen, 1995). Specific cell cycle regulatory targets in these cases are not known.

Somewhat paradoxically, *Drosophila* Wingless can also induce cell cycle arrest (Johnston and Edgar, 1998). Towards the end of larval development, Wingless is expressed in a strip of cells at the D-V boundary of imaginal wing discs, and its expression here is required for the establishment of a broad ‘zone of non-proliferating cells’ (ZNC) either side of the D-V border (O’Brien and Bryant, 1985; Phillips and Whittle, 1993). The ZNC in the posterior half of the disc is composed of G1-arrested cells, while the anterior has a central domain of G1-arrested cells sandwiched by areas of G2-arrested cells (Johnston and Edgar, 1998). These G2-arrested cells are fated to become the large sensory bristles of the wing margin (Usui and Kimura, 1992). Wingless is thought to cause G2 arrest indirectly via induction of the pro-neural genes *achaete* and *scute*, which in turn repress *string* transcription and thus co-ordinate cell specification with cell cycle stoppage (Johnston and Edgar, 1998). The mechanism of Wingless-mediated G1-arrest is not clear-cut, but the data are consistent with a direct inactivation of the G1-S phase inducer E2f (Johnston and Edgar, 1998).

In summary, *Drosophila* Wingless can induce G1 arrest, G2 arrest or cell cycling, and is thus a context-dependent regulator of cell cycle progression. However, the exact contextual conditions that determine these different outcomes remain to be determined.

**Vertebrates**

Inappropriate activation of Wnt signalling has long been linked with hyperplasia and human tumorigenesis (Polakis, 2000). In all cases, the common denominator is deregulated expression of β-catenin. For example, mutations in β-catenin that abrogate its degradation are found in a wide variety of human cancers, while inactivating mutations of the APC gene are associated with abnormally high levels of β-catenin and are found frequently in familial
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and spontaneous colon carcinomas (Polakis, 2000). Over-expressed cytoplasmic β-catenin also stimulates cell proliferation in non-cancerous cultured human keratinocytes and MDCK cells (Orford et al., 1999; Zhu and Watt, 1999). Moreover, endogenous cytoplasmic β-catenin levels were observed to increase significantly from late G1 through S phase in MDCK cells, and high levels of endogenous cytoplasmic β-catenin correlate with high proliferative potential in keratinocytes (Orford et al., 1999; Zhu and Watt, 1999). These data are most consistent with Wnt signals normally promoting cell cycle entry at G1-S. Indeed, it was reported recently that cyclin D1 and the proto-oncogene c-myc are Wnt-target genes, both of which encode factors that promote entry into S phase (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). Increased levels of endogenous cytoplasmic β-catenin may contribute to progression through the entire cell cycle as β-catenin-LEF/TCF signalling was found to increase from G1-S to reach a maximum at the G2-M border in a non-transformed mammary epithelial cell line (Orford et al., 1999).

GSK-3β has been shown to phosphorylate Cyclin D1 and promote its nuclear export and proteasomal degradation during S phase (Diehl et al., 1998; Alt et al., 2000). During G0/G1 however, GSK-3β activity must normally be repressed in order for Cdk4/6-Cyclin D1 to function and promote G1 progression, a process that could involve Wnt-mediated inhibition of GSK-3β. Alternatively, a non-Wnt stimulus could inhibit GSK-3β during late G1/early S phase and this might fortuitously increase the stability of β-catenin and promote Wnt-target gene activation during this time. However, the degree of cross-talk, regulated or not, between the different pathways in which GSK-3β is involved is unknown (Ferkey and Kimelman, 2000).

Intriguingly, GSK-3β has been also been implicated in G2 arrest in Xenopus oocytes (Fisher et al., 1999). GSK-3β blocks the G2-M transition of the first meiotic cell cycle by inhibiting Cdk1-Cyclin B complexes. It is also interesting to note that GSK-3β blocked oocyte maturation independently of β-catenin, as expression of non-degradable β-


catenin did not overcome the G2-M block (Fisher et al., 1999). Nevertheless, β-catenin protein levels were low in the G2-arrested oocytes and increased significantly during the oocyte maturation. Thus, in this case, regulation of GSK-3β inactivation appears to have untargetted consequences on β-catenin stability.

Although GSK-3β exhibits specific links with cell cycle regulation, it is not clear whether these connections are directly relevant to Wnt/Wingless signalling as this kinase is implicated in several cellular pathways (Ferkey and Kimelman, 2000). Nonetheless, it remains an open question as to how GSK-3β or β-catenin activity/levels are changed through the cell cycle: are they regulated by Wnts/Wingless, or are there cell cycle-intrinsic regulatory mechanisms? Additionally, it is a curious fact that GSK-3β/Shaggy is actually related to the Cdk subfamily of proline-directed serine/threonine kinases (Bourouis et al., 1990), and there is even some evidence that both Cdk1 and GSK-3β can phosphorylate the same proteins and residues (Baumann et al., 1993; Cornell et al., 1995).

1.4.2 Parallel regulation of Wnt/Wingless signalling and cell cycle progression?

While Wnt/Wingless signal transduction and cell cycle progression share a number of general and specific regulatory components, it is far from clear whether these facilitate cross-talk or whether they are not normally relevant in vivo. For example, the major regulatory step in Wnt/Wingless signalling is the proteolysis of β-catenin/Armadillo via the ubiquitin-proteasome pathway (Miller and Moon, 1996; Aberle et al., 1997; see section 1.2.2). The levels of many cell cycle regulatory proteins are also controlled in this manner (Koepp et al., 1999; see section 1.3.1). Thus it is possible that the activation of degradation of one class of proteins might fortuitously target the other, or conversely that degradation of one could interfere with that of the other during times of a high rate of destruction. Such a 'titration phenomenon' has been described recently: over-expression of β-catenin resulted in the accumulation of p53 apparently through interference with its proteolytic degradation.
(Damalas et al., 1999). Competition for the proteolytic machinery might occur at the level of the proteasome itself or at the level of particular proteolytic components. For example, the ubiquitin ligase SCF is a specificity factor that targets G1 Cyclins and Cdk inhibitors for ubiquitylation in addition to β-catenin/Armadillo (Koepp et al., 1999). Moreover, a recent report suggested that the specific SCF^\text{Slimb} complex might target cell cycle components in addition to its previously known substrate, Armadillo (Wojcik et al., 2000).

Another potential intersection between Wnt/Wingless signalling and cell cycle control is the regulation of nucleocytoplasmic transport as β-catenin/Armadillo and several cell cycle factors are rapidly located to the nucleus upon activation (Miller and Moon, 1996; Yang and Kornbluth, 1999). Transit of proteins between the nucleus and cytoplasm occurs via nuclear pores. Proteins containing specific nuclear localization/export sequences (NLS/NES) must first interact with soluble import/export factors (importins/exportins), followed by the docking of this complex to the nuclear pore and its subsequent translocation. Interestingly, importins are structurally related to β-catenin because they both contain a series of so-called ‘arm repeats’ (Fagotto et al., 1998; Peifer et al., 1994b; see Figure 1.2). Indeed, β-catenin lacks a classical NLS and apparently enters the nucleus on its own via direct interaction of its arm repeats with the nuclear pore complex (Fagotto et al., 1998; Yokoya et al., 1999). It is therefore possible that nuclear import of β-catenin competes with cell cycle regulators that are transported via the classical importin pathway (e.g., Cdc25C, the orthologue of Drosophila String), or that β-catenin could act as an importin itself to transport cell cycle factors that are imported through non-canonical mechanisms (e.g. Cyclin B1; Takizawa and Morgan, 2000).

Crm1 is an exportin that binds to classical leucine-rich NESs and has been shown to mediate nuclear export of several cell cycle regulatory factors including Cyclin D1, Cyclin B1, Cdc25C and the Cdk inhibitor p27 (Yang et al., 1998; Alt et al., 2000; Lopez-Girona et al., 1999; Tomoda et al., 1999). Nuclear export of APC is also dependent on Crm1.
(Henderson, 2000; Neufeld et al., 2000a). Some authors have proposed that β-catenin is exported along with APC (Henderson, 2000; Neufeld et al., 2000b; Rosin-Arbesfeld et al., 2000), although a more thorough study concluded that β-catenin is exported independently of Crml (Wiechens and Fagotto, 2001).

Other regulatory components shared between the cell cycle and Wnt/Wingless transduction include Dally and PP2A. As discussed previously (section 1.2.2), the membrane-bound proteoglycan Dally is implicated as a co-receptor for Wingless (Lin and Perrimon, 1999; Tsuda et al., 1999), but it was originally identified in Drosophila as being required for timely mitoses during development of larval tissues (Nakato et al., 1995). Twins is a Drosophila regulatory B subunit of PP2A, and PP2A<sup>twins</sup> has mitotic functions (Gomes et al., 1993; Mayer-Jaekel et al., 1994). Additionally, the PP2A catalytic C subunit has been shown to bind vertebrate Axin (Hsu et al., 1999), while a regulatory B' subunit was found to directly bind APC and its expression in human cancer cells resulted in the down-regulation of β-catenin (Seeling et al., 1999). However, as the regulatory B subunit is different in each case, there is probably no overlap of the functions of PP2A in Wnt signalling and mitosis in vivo.

1.4.3 Cell cycle-dependent regulation of β-catenin/Armadillo in adherens junctions

During mitosis, epithelial cells undergo substantial changes in their cytoskeletons and overall morphology, generally rounding up. This suggests that cell-cell adhesion may be modulated at mitosis and data from mammalian cell culture studies indicate that E-cadherin-catenin complexes might be relevant targets. First, the c-Src tyrosine kinase is activated by Cdk1 at mitosis (Moreno and Nurse, 1990) and c-Src-mediated phosphorylation of β-catenin has been correlated with decreased adhesion at adherens junctions (Daniel and Reynolds, 1997). Second, cells expressing an E-cadherin-α-catenin fusion protein did not show a rounded morphology and reduced cell-cell interaction at
mitosis, but rather retained a flat interphase cell-like appearance (Nagafuchi et al., 1994). This is consistent with post-translational modifications of β-catenin being essential for modulation of adherens junction function during mitosis. Finally, a direct biochemical analysis of the E-cadherin-catenin complex in mitotic MDCK cells found that for each of E-cadherin, β-catenin, plakoglobin, α-catenin and p120ctn, a small proportion was translocated to the cytosol during mitosis, and that mitotic β-catenin was less tyrosine phosphorylated than in interphase cells (Bauer et al., 1998b). Somewhat unexpectedly, the overall complex composition appeared unaffected, suggesting that the E-cadherin-catenin complex as a whole was internalized at mitosis. (Interestingly, in a separate study in cycling MDCK cells, cytoplasmic β-catenin was also found to increase from G1-S without appreciable variation in total β-catenin levels (Orford et al., 1999)). The study of Bauer and colleagues establishes that induction of mitosis results in changes in the tyrosine phosphorylation and the subcellular distribution of β-catenin. While the fate of the displaced β-catenin is not clear, it does not appear to be immediately available for signalling as it remained bound to cadherin and the ‘free’ β-catenin pool did not increase (Bauer et al., 1998b).

Intercellular adhesion is often associated with decreased proliferation (Aplin et al., 1999; Christofori and Semb, 1999). The best example of this is the ‘contact inhibition of growth’ seen when adherent cells grown in culture until confluence stop dividing despite the presence of mitogens and matrix attachment. The basis of the phenomenon has begun to be characterized and the Cdk inhibitors p27 and p16, along with transcriptional repression via the Retinoblastoma protein, have each been implicated in effecting cell cycle arrest mediated by E-cadherin (Levenberg et al., 1999; St Croix et al., 1998; Wieser et al., 1999; Zhang et al., 1999). However, it is also very clear that E-cadherin can antagonize β-catenin-mediated cell cycle progression (Gottardi et al., 2001). This is thought to work by sequestration of β-catenin at the membrane so as to reduce its nuclear accumulation and
transcriptional activation of cell cycle promoters such as Cyclin D1 and c-Myc. Indeed, the growth suppressor activity of E-cadherin in colorectal tumour cells has been shown to require the β-catenin binding region but not adhesive activity (Gottardi et al., 2001). In this way, E-cadherin may also act as a regulatory ‘sink’ for free β-catenin during normal development that must be overcome for productive Wnt signalling to occur.

1.5 A screen to identify factors that interact genetically with Armadillo in

*Drosophila*

1.5.1 Basis of the screen

While the many and varied studies discussed above have shed significant light on the multifunctionality, binding partners, and regulation of β-catenin/Armadillo, several questions remain regarding how the different roles of β-catenin/Armadillo are individually controlled and yet integrated together during development and homeostasis.

In order to address these types of question, a genetic screen was undertaken in the fruit fly *Drosophila melanogaster* so as to identify novel interactions with Armadillo (Greaves, 1999). The approach taken was to create Armadillo-sensitized phenotypes in the adult wing of *Drosophila* and to search for mutations that could modify these phenotypes in the heterozygous condition. Such ‘dominant modifier’ screens have been widely and successfully used in *Drosophila* to identify dosage-sensitive factors that act in a variety of signalling pathways and morphogenetic events (for recent examples see Go and Artavanis-Tsakonas, 1998; Rebay et al., 2000).

For this screen, one of two different proteins that act to either increase or decrease the ‘signalling’ pool of Armadillo was over-expressed (Figure 1.5). In one case, a
Figure 1.5  Proposed effects of over-expression of CADi or Armadillo in epithelial cells

Stylized epithelial cells are shown receiving the Wingless ligand.  
A, In *wild type* cells, Armadillo (Arm) localizes to the apical-lateral membrane, bound to E-cadherin homodimers at adherens junctions. Cytoplasmic Armadillo is stabilized upon receipt of Wingless and can activate target genes.  
B, CADi protein localizes to junctions and binds Armadillo, but cannot homodimerize or form productive cell-cell adhesion complexes. Over-expressed CADi thus titrates cytoplasmic Armadillo at the membrane, where it is stable but unavailable for signal transduction, and transcription of Wingless target genes is consequently reduced (small green arrow in nucleus).  
C, Over-expression of full-length wild type Armadillo results in increased levels of cytoplasmic Armadillo in Wingless-receiving cells, which translocates to the nucleus to hyper-activate target genes (large green arrow). Over-expressed Armadillo is presumably degraded by proteolysis in cells that don’t receive Wingless (not shown).  
D, CADi and Armadillo were over-expressed in the fly wing using the GAL4-UAS system. The transcriptional activator GAL4 was expressed under the control of *engrailed* regulatory sequences (*Engrailed-GAL4*). In this way, CADi or Armadillo expression from multimerized upstream activation sequences (UAS) was activated throughout the posterior of the developing wing.
E-cadherin
membrane
cytoplasm
nucleus

WT

UAS-CADi

UAS-Arm

D

Engrailed

GAL4

GAL4

GAL4

GAL4

Armadillo

UAS(x10)

40
truncated form of *Drosophila* E-cadherin (CADi) was expressed (Figure 1.5B; Sanson *et al.*, 1996). This protein lacks the extracellular domain, and so cannot form productive cell-cell contacts, but retains the transmembrane and cytoplasmic regions and so is able to bind Armadillo. Over-expressed CADi can sequester Armadillo and act in a dominant-negative manner to reduce the pool of Armadillo that is available for Wingless signalling (Figure 1.5B; Sanson *et al.*, 1996). For the second sensitized background, wild type Armadillo was over-expressed (Figure 1.5C; White *et al.*, 1998). This increases the amount of Armadillo that is available to take part in signalling upon Wingless stimulation and thus potentiates the pathway.

The GAL4-UAS system was used to over-express CADi or Armadillo so as to create Armadillo-sensitized phenotypes (Figure 1.5D; Brand and Perrimon, 1993). In this system, a given regulatory sequence drives expression of the yeast transcriptional activator GAL4 in specific spatiotemporal domains. GAL4 then binds to Upstream Activation Sequences (UAS) that have been cloned upstream of a gene of choice and directs the ectopic expression of this gene. Flies bearing *UAS-CADi* or *UAS-Armadillo* transgenes were generated and used to make recombinant chromosomes with the *Engrailed-GAL4* transgene, a GAL4 driver that is expressed in the posterior half of the wing throughout its development (Hama *et al.*, 1990).

*Engrailed-GAL4 UAS-CADi (En>CADi)* adult wings display a loss of the posterior wing margin and adjacent blade material, a defect reminiscent of a reduction in Wingless signalling at late larval stages (Figure 1.6C; Couso *et al.*, 1994; Sanson *et al.*, 1996; Greaves *et al.*, 1999). In contrast, *Engrailed-GAL4 UAS-Armadillo (En>Arm)* wings display a typical Wingless gain-of-function phenotype as they produce ectopic supernumerary margin bristles in the posterior blade of the wing (Figure 1.6D Blair, 1992; Greaves *et al.*, 1999; White *et al.*, 1998). Note that, while these two Armadillo-sensitized
Figure 1.6 Changing the gene dosage of canonical members of the Wingless pathway modifies Armadillo mis-expression wing phenotypes

A, Cartoon of a wild type wing. The wing blade consists of anterior (A) and posterior (P) compartments in which are occupied four longitudinal veins (L2-L5) and an anterior and posterior cross vein (a.c.v. and p.c.v. respectively). The margin is decorated with large sensory bristles in the anterior margin (L1) and smaller uninnervated bristles in the posterior margin. B, wild type control. The posterior aspect is shown. C, En>CADi^+/+ control. Much of the posterior margin and some adjacent wing blade is deleted. A few tufts of margin bristles remain (arrow). D, En>Arm^16/+ control. Ectopic bristles form near to the posterior margin (arrows) and occasionally farther into the blade (arrowhead). E, G, Heterozygosity for armadillo^xp33 enhances while co-expression of Armadillo suppresses the En>CADi^ phenotype. Compare with C. F, H, Heterozygosity for wingless^cx4 or shaggy^p127 reduces or increases the number of En>Arm^16-induced ectopic bristles respectively. Compare with D. All mutant alleles shown here are amorphic for their function in Wingless signalling. Female wings are shown and are all at the same magnification. Anterior is up and distal is to the right in these and all subsequent wing pictures.
A

B

C

D

E

F

G

H

WT

En>CADi/+  

En>Arm/+  

arm[XP33]/+; En>CADi/+  

En>Arm/wg[CX4]  

En>CADi/UAS-Arm  

sgg[D127]/+; En>Arm/+
lines may also suffer from deregulated cell-cell adhesion, the adult wing phenotypes indicate that the primary defects are in Wingless signalling.

In order for these phenotypes to be useful in a dominant modifier screen, they ought to be sensitive to subtle changes in the level or activity of Armadillo. This has been shown to be the case by reducing the gene dosage of components of the Wingless signalling cassette in these contexts (Figure 1.6E-H; Greaves et al., 1999). Moreover, the observed phenotypic enhancement (being made more severe) or suppression (being made more wild type) is consistent with the known function of these regulators. These examples demonstrate the validity of using the \textit{En>CADi} and \textit{En>Arm} wing phenotypes as the basis for conducting a dominant-modifier screen to detect novel interactors with \textit{Drosophila} Armadillo. It was hoped that these would include novel regulators of adherens junctions and the Wingless pathway, as well as other modifiers of known or unknown function that might affect Armadillo activity by unexpected mechanisms.

1.5.2 Methodology and results of the screen

The two complementary wing phenotypes described above were used in parallel in the screen, the underlying postulate being that \textit{bona fide} interactors ought to suppress one phenotype (e.g. \textit{En>CADi}) while concomitantly enhancing the other (\textit{En>Arm} in this example; Greaves et al., 1999). Rather than examining single genes by mutagenesis, Greaves et al. (1999) evaluated large genomic regions by making Armadillo-sensitized flies heterozygous for chromosomal deletions that simultaneously remove many genes. The advantage of this approach is that about 70% of the euchromatic genome can be rapidly assessed for genetic interaction with Armadillo. The disadvantages are that the individual mutations responsible for phenotypic modifications are inherently difficult to identify, and that some potential interacting loci are inevitably missed by virtue of deletions sometimes containing both enhancers and suppressors.
Chapter 1: Introduction

A candidate gene approach was used to single out particular interacting mutations in the genomic regions delineated by the deficiency chromosomes (Greaves et al., 1999). In this way, several interacting loci were identified and classified into five discrete functional groups that included both predicted and unexpected interactors. These groups were: Wingless pathway members, adherens junction components/regulators, EGF receptor signalling components, cell cycle regulators, and a class of genes of unknown identity or function defined by P element insertions (Greaves et al., 1999).

1.5.3 Aims of this thesis

Chapter 3 recounts my characterization of the interaction between Armadillo and a P insertion named l(3)neo40. Unfortunately, several molecular and genetic analyses yielded inconsistent results and these investigations were consequently terminated.

A number of cell cycle regulators were found to interact genetically with Armadillo. I describe my characterization of these interactions in various Armadillo-sensitized backgrounds in Chapter 4. CADi-associated phenotypes were found to be sensitive to the gene dosage of several different cell cycle factors, including key regulators of G1-S and G2-M. In contrast, phenotypes resulting from Armadillo over-expression proved to be relatively insensitive to changes in the level of cell cycle regulators, although a robust interaction was attributed to the string gene, which encodes the Cdc25 phosphatase that is rate-limiting for mitosis in Drosophila.

In Chapter 5, I relate my concentrated analysis on the genetic interaction between Armadillo and String in imaginal wing discs, the larval primordia of the adult wings. I find that String acts as a negative regulator of Armadillo function and acts to decrease the cytoplasmic levels of over-expressed Armadillo. This appears to result from altered rates of proteolysis of Armadillo as the expression of non-degradable forms of Armadillo is not sensitive to changes in string dosage. Consistent with its cell cycle regulatory role, mitotic
cells show relatively low levels of cellular Armadillo both in sensitized and wild type discs. These data reveal a novel mechanism of Armadillo regulation that may have important consequences for Wingless signalling and/or cell-cell adhesion in proliferating cells in general and at mitosis in particular. Plausible reasons for coupling Armadillo degradation to mitosis are discussed.
CHAPTER 2: MATERIALS AND METHODS

"You know my methods. Apply them."

Sir Arthur Conan Doyle, The Sign of Four (1890)

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2.1 Fly stocks

'SJM' denotes rebalanced or recombinant stocks that I generated. '(II)' or '(III)' identifies the chromosome of UAS transgene insertion where appropriate. References that apply solely to fly stocks are not given in full in the References section but can be found at Flybase. BL or UM denote Bloomington or Umea Stock Centre numbers respectively (correct at 3/01). Contact details for people who donated flies are given in the Acknowledgements.

<table>
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<td>J.-P. Vincent</td>
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<td>w; TM3, Sb/ TM6, Tb</td>
<td>-</td>
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<td>A. Carpenter</td>
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### Chapter 2: Materials and Methods

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<td>Df(3R)ry619/ MKRS, kar^{2} ry^{l} Sb^{l}</td>
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<table>
<thead>
<tr>
<th>Genotype</th>
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<td>( P(\text{ry}^{+7.2}=\text{PZ})\mid (3)05137^{317}/\text{TM3, ry}^{6K} \text{Sb}^1 )</td>
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<td>Hilliker et al., 1980</td>
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</table>

**'Cell cycle mutants':**

- \( w; \text{cdc2}^{2877} \text{ cn bw}/\text{CyO, P[w*, ftz-lacZ]} \) by Stern et al., 1993, C. Lehner
- \( Dm\text{cdc2}^{2877} \text{ b pr cn}/\text{CyO, dp [v1] pr cn}^3 \) by Stern et al., 1993, C. Lehner
- \( w; \text{cycA}^{\text{CELA}^2}/\text{TM3, Sb Ubx-lacZ} \) by Sigrist and Lehner, 1997, C. Lehner
- \( w; \text{cycA}^{\text{CELA}^2}/\text{TM3, Sb} \) by Lehner and O'Farrell, 1989, C. Lehner
- \( \text{cycB}^3/\text{CyO, ftz-lacZ} \) by Jacobs et al., 1998, C. Lehner
- \( \text{cycB}^3/\text{CyO, ftz-lacZ} \) by Jacobs et al., 1998, C. Lehner
- \( \text{cycB}^3/\text{TM3, Ubx-lacZ} \) by Jacobs et al., 1998, C. Lehner
- \( w; b \text{cyE}^{2877} \text{ pr cn bw}/\text{CyO, ftz-lacW} \) by Knoblich et al., 1994, C. Lehner
- \( w; \text{cycE}^{\text{CELA}^2}/\text{CyO, wg-lacZ} \) by Duronio et al., 1998, C. Lehner
- \( \text{dap}^{454}/\text{CyO, wg-lacZ} \) by de Nooij et al., 1996, J. de Nooij
- \( w; \text{Dweel}^\text{E}^2\text{cn}/\text{CyO, cn ftz-lacZ} \) by Price et al., 2000, S. Campbell
- \( w; \text{Dweel}^\text{E}^2\text{cn}/\text{CyO, cn ftz-lacZ} \) by Price et al., 2000, S. Campbell
- \( \text{E2f}^{\text{M770}} \) by Duronio et al., 1995, L. Johnston
- \( \text{E2f}^{\text{M770}}/\text{TM3, Sb ftz-lacZ} \) by Duronio et al., 1995, L. Johnston
- \( \text{fsc}^3 \text{ cn bw}/\text{CyO} \) by Dawson et al., 1993, C. Lehner
- \( \text{fsc}^3 \text{ b pr cn wx}^{2877} \text{ bw}/\text{CyO} \) by Dawson et al., 1993, C. Lehner
- \( \text{Df}(1)\text{bi-D3, w}^+\text{FM7c (uncovers fizzy-related)} \) by Banga et al., 1986, BL-3204
- \( \text{Df}(3L)pbl-X1/\text{TM6B, Tb}^1 (\text{uncovers pebble}) \) by Hime and Saint, 1992, BL-1420
- \( \text{Rbf}^3/\text{FM7, ftz-lacZ} \) by Du and Dyson, 1999, Wei Du
- \( \text{Rbf}^3/\text{FM7, ftz-lacZ} \) by Du and Dyson, 1999, Wei Du
- \( \text{P[ry}^{+7.2}=\text{PZ})\text{Rca1}^{02300} \text{ cn}/\text{CyO: ry}^{006} \) by dong et al., 1997, BL-P1294
- \( \text{Rca1}^{TS}\text{K2}^{S2.30.1} \text{ cn bw}/\text{SM6a} \) by Gift from B. Thomas, B. Thomas
- \( \text{rux}^2 \) by Lindsley and Grell, 1968, BL-1506
- \( \text{rux}^2 \) by Lewis, 1945, BL-95
- \( \text{rux}^2 \text{ y' cho}^2/\text{FM7c} \) by Thomas et al., 1994, B. Thomas
- \( \text{rux}^2 \text{ h th' st' cu' sr' e' stg}^7\text{ ca'/TM3, Sb}^1 \text{ hb-lacZ or/TM6, Tb} \) by Tearle and Nusslein-Volhard, 1987, UM-Z328
- \( \text{rux}^2 \text{ h th' st' cu' sr' e' stg}^7\text{ ca'/TM3, Sb}^1 \text{ hb-lacZ or/TM6, Tb} \) by Tearle and Nusslein-Volhard, 1987, UM-Z328
- \( \text{rux}^2 \text{ h th' st' cu' sr' e' stg}^7\text{ ca'/TM3, Sb}^1 \text{ hb-lacZ or/TM6, Tb} \) by Tearle and Nusslein-Volhard, 1987, UM-Z328
- \( \text{rux}^2 \text{ h th' st' cu' sr' e' stg}^7\text{ ca'/TM3, Sb}^1 \text{ hb-lacZ or/TM6, Tb} \) by Tearle and Nusslein-Volhard, 1987, UM-Z328
- \( \text{rux}^2 \text{ h th' st' cu' sr' e' stg}^7\text{ ca'/TM3, Sb}^1 \text{ hb-lacZ or/TM6, Tb} \) by Tearle and Nusslein-Volhard, 1987, UM-Z328
- \( \text{str}^{454}/\text{TM3, Sb hb-lacZ or/TM6, Tb} \) by Edgar et al., 1994, L. Johnston
- \( w^{118}; \text{Df}(3R)3450/\text{TM6B, Tb}^1 (\text{uncovers string}) \) by BDGP Project Members, BL-430
- \( \text{Df}(3R)p{1215}/\text{TM3, Sb}^1 (\text{uncovers string}) \) by BDGP Project Members, BL-5424
- \( \text{Df}(3L)\text{ri-79c}/\text{TM3, Sb}^1 (\text{uncovers tribbles}) \) by Jurgens et al., 1984, BL-3127
- \( \text{Df}(3L)\text{rdg-co2, th' st' in1 kni}^{01} \text{ p's}/\text{TM6C, cu' Sb}^1 \text{ ca' (uncovers tribbles)} \) by Steele and O'Tousa, 1990, BL-2052
2.2 Fly Manipulations

2.2.1 Fly crosses and stock maintenance
Stocks were maintained and crosses were carried out at 25°C in plastic vials containing standard organic media supplemented with dry yeast, except where stated otherwise. Approximately ten females and five males were used in most crosses. Where necessary, the required progeny of a cross were identified by selection against dominant markers on balancer chromosomes. Interaction test crosses were carried out in a single tray kept at a constant place within the incubator so as to minimize environmental effects. Oregon R was used as the wild type strain.

2.2.2 \(l(3)neo40\) germline clone generation
Virgin females of the genotype \(yw hs-flp; FRT-82B \ l(3)neo40'/ TM3, Sb\) were crossed with males of the genotype \(w; FRT-82B ovo^D'/ TM3, Sb\) and left for 24 hours to allow females to clear unfertilized eggs. Adults were then transferred to fresh vials on each of three consecutive days. These vials were heat shocked at 37°C in a water bath for 4 hours on the third, forth and fifth day after egg laying (that is, during the 2\textsuperscript{nd} and 3\textsuperscript{rd} larval instar stage) in order to induce recombination between FRT sites. \(Sb\) (i.e. \(FRT-82B ovo^D'/ FRT-82B \ l(3)neo40'\)) virgin females were collected after ~10 days and mated to \(yw\) males for 2 days so as to stimulate oogenesis. \(ovo^D\) germ lines that do not undergo recombination arrest oogenesis at stage 3-4. Only those germlines in which recombination at FRT sites occurred eliminate the \(ovo^D\) mutation (and will be concomitantly homozygous for \(l(3)neo40'\)) and have ovarioles containing later stage egg chambers.

2.2.3 P element excision
The mating scheme used is detailed in Figure 3.4.

2.2.4 Heat shock induction of String
\(Ex>Arm^{6}/CyO\) females were crossed to \(w\) or \(hs-String/TM3\) males in vials and a 24 hour embryo collection was made. Progeny were raised normally at 25 °C for 5 days, and then vials were subjected to a 1 hour heat shock at 37 °C by inserting them into preheated metal blocks in a 37 °C oven. Larvae were then allowed to recover for 1 hour at 25 °C before late third instars were dissected. \(hs-String\) wing discs were distinguished from \(TM3\) discs by the presence of increased mitotic figures in the former.

2.3 Wing mounting and Screening Procedure
One to two day-old adult flies of the correct genotype were collected at random and stored in isopropanol in air-tight tubes. Wings from males and females were mounted and analysed separately as wing phenotypes.
were sexually dimorphic. Only one wing from each fly was taken so as to optimize the validity of the sample collection. (The two wings of an individual fly were usually very similar while wing phenotypes between different flies showed greater variation.) Wings were dissected in isopropanol, mounted in Euparal (Agar Scientific) and baked at 65°C overnight. Adult wings were analysed and photographed on a Zeiss Axioplan 2 microscope using 64T Ektachrome film (Kodak). Appropriate control crosses were performed at the same time and in the same conditions as the 'test' crosses.

For the quantitative analysis of En>Arm and C96>Arm wings, ectopic margin bristles were counted. Ventral and dorsal surfaces were counted separately so as to minimize counting errors. 'Ectopic bristles' were classed as those bristles that were clearly not incorporated into the wing margin such that supernumerary bristles within the margin were not counted. (For C96>Arm wings, only those ectopic bristles that were in the region between veins L2-L4 were counted as the majority were found in this definable region.) Typically 12 female (En>Arm) or 24 female and male (C96>Arm) wings were analysed for each genotype.

For the quantitative analysis of En>CADi^ wings, the number of endogenous margin bristles between veins L4-L5 was determined. Bristle number in this defined region proved to be a reliable read-out of either enhancement or suppression of the phenotype. Typically 24 wings of each sex were analysed for each genotype.

C96>CADi^ interactions were assessed by counting the number of notches in the wing margin. Though crude, this measure was the only easily quantifiable aspect of the phenotype. Typically 24 wings of each sex and genotype were analysed.

The raw data of all these quantitative analyses are available upon request.

2.4 Dissection, staining and analyses of Drosophila tissues

2.4.1 Ovary dissection and mounting
Virgin females of the correct genotype were kept in fresh yeasted vials with wild type males (so as to stimulate oogenesis in the females) for three to four days. Females were then CO2-anaesthetized and their ovaries dissected out in Drosophila glutamate saline solution in a solid watchglass. Dissected ovaries were transferred to a second solid watchglass containing 4% paraformaldehyde in 0.1% PTw and, after separating out the ovarioles using needles, were fixed for 20 minutes at room temperature. Fixed ovaries were transferred to a 1.5ml microcentrifuge tube containing 0.1% PTw and washed for ten minutes, rocking at room temperature. The PTw was then removed and Fluoromount-G (Southern Biotechnology Associates, Inc.) added. Ovaries were allowed to equilibrate with the mountant for at least 4 hours at room temperature. Needles were used to dissect out individual ovarioles or egg chambers in Fluoromount-G on a cavity slide before being mounted in Fluoromount-G on a fresh slide.

2.4.2 Immunolabelling of imaginal wing discs
Approximately twenty females and ten males of appropriate genotypes were added to a fresh vial that had been kept at 25°C. Overnight collections of embryos were made and allowed to age for five to six days within the same vial. Wandering third instar larvae were then collected from the side of the vials. Where appropriate, larvae of the desired genotype were chosen by selecting against the dominant Tubby mutation on the TM6 balancer chromosome.

Fixation was performed essentially as described by White (1998) but with the following differences. Wandering third instar larvae were collected and washed in solid watch glasses. Dissected larval heads were kept on ice until fixation. Amassed larval heads were transferred to 3.8% formaldehyde (from a 38% stock solution, Merck) in glass scintillation vials (Camlab) and fixed for 20 minutes, rolling at room temperature.
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Heads were then rinsed three times in PBS, washed for 20 mins in PBS and then washed for 20 minutes in PTx, each time rolling at room temperature.

Blocking and antibody incubation occurred exactly as detailed by White (1998). Incubations in the secondary antibody(ies) occurred for 1.5 hours. Imaginal discs were dissected out in solid watch glasses using forceps and mounted individually in Vectashield with or without DAPI (Vector Laboratories).

Primary antibodies used: mouse anti-Armadillo (1:100, Developmental Studies Hybridoma Bank), rabbit anti-βgalactosidase (1:1200, Cappell), rabbit anti-phosphohistone H3 (1:1000, Upstate Biotechnology), mouse anti-Myc (1:200, Santa Cruz Biotechnology), mouse anti-HA (1:4000, BabCo). Secondary antibodies used: Alexa 488 goat anti-mouse, Alexa 594 goat anti-rabbit, Alexa 488 goat anti-rabbit, Alexa 594 goat anti-mouse (each diluted at 1:300, Molecular Probes).

Immunofluorescent imaginal discs were analysed initially on a Zeiss Axioplan 2 microscope. Where necessary, appropriate genotypes were selected by discriminating against marked balancer chromosomes. Typically 15-25 discs of each genotype were examined for each experiment and 5 representative examples were selected for confocal imaging using a Leica TCS SP laser scanning confocal microscope and Leica TCS NT software. Images were collected using a 40X or 63X objective lens. 16 slices in the Z axis were taken through the entire disc and projections of 2-3 apical slices (~4 μm thick) just below the apical cell surface are presented in this thesis. Images were processed using NIH Image software and assembled in Adobe Photoshop.

2.4.3 GFP-Armadillo analysis in imaginal wing discs

Five homozygous GFP-Armadillo wing discs were fixed as above and mounted in Vectashield containing DAPI. Discs were imaged using a DeltaVision cooled CCD system, visualized on an O2 Silicon Graphics Workstation and analysed using the Data Inspector function of the SoftWorX package. 10 slices in the Z axis were taken through a 2 μm thick apical section of the disc, just below the peripodial membrane: projections of slices 2-5 were used in this analysis. GFP-Armadillo cytoplasmic fluorescence was estimated in high magnification projections by calculating total pixel values within a defined circular area of constant size in selected cells of each disc. The circle size used was determined to include the maximum amount of exclusively cytoplasmic fluorescence in the smallest interphase cell examined, and total pixel values were then calculated within this same area for the centre of each mitotic cell and for the neighbouring interphase cells. The mean pixel value within this circle was calculated (Figure 5.22). As cells change their shape but not their absolute size during mitosis, this approach allows a valid comparison of GFP-Armadillo fluorescence in a defined volume of cytoplasm between interphase and mitotic cells.

2.5 Molecular biology

2.5.1 General techniques

Bacterial transformation, plasmid DNA preparation and restriction digests were performed according to standard protocols (Sambrook and Russell, 2000).

2.5.2 PCR assays

PCRs were performed using template DNA from either single flies or plasmid rescued clones. PCRs from single flies were performed according to a protocol obtained from J. Wasserman (LMB, Cambridge). Briefly, single flies were first frozen in a microfuge tube at -20 °C. They were subsequently ‘squished’ using a pipette tip for about a minute in 50 μl ‘squishing’ buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl) plus
0.01 volumes of proteinase K and 1 µl RNase A (10 µg/µl). Tubes were then incubated at 37 °C for 30
minutes, then at 90 °C for 2 minutes to inactivate the proteinase K. Protein was pelleted by centrifugation for
2 minutes and the 1 µl of supernatent was used in a standard 20 µl PCR (Sambrook and Russell, 2000).

Primers were designed to amplify either 5’ pUC sequence (157 bp) or 3’ neo sequence (986 bp) in
the P[hsneo] transposon (Figure 3.6). pUC forward: 5’-TTCGCCATTCAGGCTACGCA-3’; pUC reverse:
5’-GCACTGCGCGTCGTTTTACA-3’; neo forward: 5’-GAGACAGGATGAGGATCGT-3’; neo reverse:
5’-AGAACTCCAGCATGATCC-3’. The following thermal cycling profile was used: 2.5 minutes at 94
°C, followed by 35 cycles of [35 seconds at 94 °C, 35 seconds at 52 °C, and 50 seconds at 72 °C], and a final
step of 5 minutes at 72 °C.

2.5.3 Plasmid rescue and sequencing
Plasmid rescue was performed according to the protocol of Rubin and Spradling (1982). Restriction enzymes
used for rescuing 5’ flanking DNA were Xho I and Nar I, and Sal I and Eco RI were used to rescue 3’
flanking DNA (Figure 3.6). 3 independent Xho I clones, 3 independent Nar I clones, and 3 independent Sal I
clones were thus obtained.

DNA products for sequencing were prepared according to the manufacturer’s instructions (Perkin
Elmer). The following primers were used: SJM2 (5’ flank): 5’- TAAGCTTGGATGCTTCTTGCCG-3’; SJM3 (3’
flank): 5’-TAAGCTTGGATGCTTCTTGCCG-3’. Sequencing was performed ‘in house’ using ABI Prism 377
machines.
"l(3) neo40' was discarded from the P element collection some time ago because our own analysis determined that the mutant phenotype was not caused by the P insertion."

Allan Spradling (E-mail communication, 1998)
3.1 Introduction

In the original screen conducted by Greaves, the interaction of ten chromosomal regions could not be ascribed to a single cloned gene (Greaves, 1999; Greaves et al., 1999). One such region, termed ‘3R2’, comprised the 86C-88A cytological interval on the third chromosome, and was defined by five non-overlapping deficiencies that suppressed the $En>CADi$ wing phenotype and/or enhanced the $En>Arm$ phenotype. Eight P insertions within this region were subsequently tested for genetic interaction with Armadillo. Only one of these, an insertion at 87E11-12 called $l(3)neo40^I$, clearly suppressed $En>CADi$ and concomitantly enhanced $En>Arm$, and was thus selected for further analysis (Greaves, 1999; Greaves et al., 1999). Armadillo-sensitized phenotypes were also generated in the Drosophila eye using the GMR-GAL4 driver, and heterozygosity for $l(3)neo40^I$ modified these phenotypes in a similar manner to those in the wing (Greaves, 1999; Greaves et al., 1999). This demonstrated that the genetic interactions were specific neither to a particular organ nor GAL4 driver. In summary, $l(3)neo40^I$ is a dosage-dependent modifier of Armadillo-sensitized phenotypes, whose wild type function might compromise the role of Armadillo in Wingless signalling (see section 1.5.1).

At the time of investigation, the $l(3)neo40^I$ gene and the protein it encoded were completely uncharacterized. $l(3)neo40^I$ was the only known mutant allele, generated in a P[hsneo] mutagenesis screen conducted in the Spradling laboratory, and recessive lethality was the only reported phenotype (Cooley et al., 1988). Therefore, several phenotypic and molecular analyses of the $l(3)neo40^I$ mutation were undertaken by Greaves (Greaves, 1999).

Mutations in genes encoding members of the Wingless pathway usually cause embryonic lethality and display strong segment polarity phenotypes in their cuticles (Perrimon, 1994). However, homozygosity for $l(3)neo40^I$ resulted in late embryonic death
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with no overt cuticle defects (Greaves, 1999). The Wingless pathway also has a key role in wing development (see section 1.2.1) and wing clones that transduce the Wingless signal aberrantly show distinct morphological defects (Couso et al., 1994; Zecca et al., 1996). However, clones of cells homozygous for l(3)neo40 in the adult wing were found to be phenotypically wild type (Greaves, 1999). These results indicated that l(3)neo40 does not encode an integral member of the Wingless signalling pathway.

There are at least two drawbacks to the phenotypic analyses described above. First, as the somatic clones were unmarked, subtle defects may have been missed or gone undetected because of the elimination of l(3)neo40 mutant cells during development. Secondly, homozygous l(3)neo40 embryos may retain some wild type l(3)neo40 courtesy of their heterozygous mothers who deposit RNA and protein in the oocyte during oogenesis. This 'maternal contribution' of wild type l(3)neo40 may have masked any loss-of-function phenotype in the initial analysis. To address this latter point, clones of l(3)neo40 cells were generated in the maternal germline so as to eliminate all wild type l(3)neo40 function in the homozygous mutant embryos derived from matings with l(3)neo40 males (Engstrom et al., 1997). However, embryos were not obtained from such germline clones because oogenesis was compromised in the mothers (Greaves, 1999). This observation was particularly interesting as armadillo or shotgun (which encodes Drosophila E-cadherin) germline clones also cause defects in oogenesis as a result of defective cell adhesion (Peifer et al., 1993; White et al., 1998). Thus l(3)neo40 might encode a protein that modulates Armadillo function at adherens junctions. However, egg chambers derived from l(3)neo40 germ line clones did not show defects in their actin cytoskeletons (Greaves, 1999).

It was thought that the analysis of l(3)neo40 function would benefit greatly from having additional mutant alleles. To this end, the l(3)neo40 line was re-exposed to a source of transposase enzyme to generate imprecise excision events in which both the P and
flanking DNA were removed to create a small genomic deletion (Spradling et al., 1995). Two ‘excision alleles’ were thus obtained that were lethal when in trans to the original l(3)neo40\textsuperscript{i} mutation, and dominantly modified the Armadillo-sensitized wing phenotypes in a manner similar to l(3)neo40\textsuperscript{i} (Greaves, 1999). Strangely, the excision alleles showed weaker genetic interactions than l(3)neo40\textsuperscript{i} and they also complemented deficiency chromosomes spanning the 87E region (Greaves, 1999). These latter observations serve to cast doubt on their being true imprecise excisions of the l(3)neo40\textsuperscript{i} P insertion.

Exposure of l(3)neo40\textsuperscript{i} flies to transposase also generated homozygous viable revertants in which the P element was presumably excised cleanly (Greaves, 1999). This result demonstrated that the P insertion itself, rather than secondary mutations in the stock, was the cause of lethality. Furthermore, such revertants did not dominantly modify the Armadillo-sensitized wing phenotypes, indicating that the P insertion was specifically responsible for the observed genetic interactions (Greaves, 1999).

DNA flanking the insertion site of the P element was cloned by the plasmid rescue technique and sequenced (Rubin and Spradling, 1982; Greaves, 1999; Greaves et al., 1999). Homology between the l(3)neo40 sequence to previously cloned and characterized genes might have facilitated analysis of the functional relationship between Armadillo and the protein defined by l(3)neo40. However, sequence alignments using the BLAST server failed to show any homology to known genes.

In summary, l(3)neo40 identifies an uncloned gene in the 87E11-12 chromosomal region that is essential for embryogenesis (Cooley et al., 1988). It also appears to have a critical function during oogenesis, and genetic interactions indicate that it antagonizes Armadillo function (Greaves, 1999; Greaves et al., 1999). Based on this background, my initial project had three broad aims: to characterize the l(3)neo40\textsuperscript{i} phenotypes more thoroughly; to confirm that the P insertion itself was responsible for these phenotypes; and finally to clone the l(3)neo40\textsuperscript{i} gene and thus investigate its functional relationship with
Armadillo. My data confirm that \( l(3)\text{neo40}^l \) disrupts oogenesis and dominantly enhances \( En>Arm \). However, while the \( P \) insertion at 87E appears to be responsible for this latter phenotype, it does not seem to cause the recessive lethality that is associated with the \( l(3)\text{neo40}^l \) chromosome.

### 3.2 \( l(3)\text{neo40} \) may be required for oogenesis

#### 3.2.1 Introduction

*Drosophila* females possess two ovaries, each consisting of 15-20 ovarioles, and each of these comprising a series of eggs of progressively more developed stages (see Spradling, 1993 for a review). The earliest stages are located in an anterior germarium, in which a germline-derived progenitor cystoblast undergoes four successive divisions and becomes surrounded by somatic follicle cells. When this egg chamber buds from the posterior of the germarium into the vitellarium, the posterior-most cystocyte is fated to be the oocyte, with the other fifteen cystocytes being 'nurse cells' that are connected to the oocyte via cytoplasmic bridges. The egg chamber then develops through 14 continuous stages in the vitellarium (King, 1970). Stages 1-7 are previtellogenic, during which time the germline cells grow and the nurse cells undergo polyploidization. During stages 8-9 the oocyte starts to accumulate yolk, almost all of the follicle cells migrate over the surface of the oocyte, and the vitelline membrane begins to be secreted by these follicle cells. At stage 10, the oocyte comprises 50% of the egg chamber and the nurse cells begin 'dumping' their cytoplasmic contents into the oocyte. By stage 12, the nurse cells have degenerated and the oocyte occupies most of the egg chamber. Finally, the eggshell layers are laid down over the vitelline membrane and specialized eggshell features are formed.
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Initial experiments indicated that wild type l(3)neo40 function was required for oogenesis, as ovaries derived from l(3)neo40^l germ line clones did not contain egg chambers later than stage 10 of development (Greaves, 1999). Arrest at this stage might be significant as it correlates with nurse cell dumping, a process that requires modification of the actin cytoskeleton and thus perhaps Armadillo function at adherens junctions. As these initial studies were preliminary I sought to confirm the oogenesis phenotype and stage of arrest by repeating the experiment.

3.2.2 Ovaries derived from l(3)neo40^l germline clones are blocked in mid-oogenesis

l(3)neo40^l/l(3)neo40^l germlines were generated using the autosomal FLP-DFS technique (Engstrom et al., 1997; see Chapter 2.2.2). Briefly, the technique uses the FLP-recombinase to cause site-specific recombination between FLP-recombinase target (FRT) sites in order to generate female germline cells that are both homozygous for the lethal of choice and negative for the dominant female sterile (DFS) mutation, ovo<sup>DI</sup>.

Ovaries derived from l(3)neo40^l mutant ovaries were dissected out and observed under DIC optics (Figure 3.1). FRT-mediated recombination was clearly effective as putative l(3)neo40^l ovaries contained egg chambers at later stages than ovo<sup>DI</sup> controls, in which oogenesis is arrested at stage 3-4 (Perrimon and Gans, 1983; compare Figure 3.1C to 3.1A). However, homozygous l(3)neo40^l ovaries were severely compromised in their oogenesis compared to wild type ovaries (compare Figures 3.1B and C). Closer inspection of individual ovarioles revealed previtellogenic egg chambers up to and including stage 7-8, after which time they appeared to shrink and disintegrate (compare Figures 3.1E to 3.1D).

The cause of ovariole atrophy at this developmental time is not clear, although morphological events that occur at stage 7-8 include yolk accumulation by the oocyte and microtubular changes within the oocyte (Spradling, 1993; Knowles and Cooley, 1994).
Figure 3.1  Ovaries derived from homozygous \( l(3)\text{neo}40^I \) germline clones show a development block at stage 7-8 of oogenesis

A, \( ovo^{D1} \) ovaries. B, wild type (WT) ovarioles. C, \( l(3)\text{neo}40^I \) ovaries. A-C are shown at the same magnification. D, E, Higher power views of a WT ovariole and of an ovariole derived from a \( l(3)\text{neo}40^I \) germline clone respectively. Arrows indicate a stage 7-8 egg chamber: \( l(3)\text{neo}40^I \) germline clones do not develop further than this stage.
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The maternal effect of the zygotic lethal \( l(3)neo40^{EI} \) and \( l(3)neo40^{E2} \) mutations was not assessed.

3.2.3 Summary and discussion

Wild type \( l(3)neo40 \) function may be required for normal oogenesis. Removal of the maternal contribution of \( l(3)neo40 \) results in atrophying ovarioles and consequent female sterility. However, I find that the oogenesis block occurs earlier than that reported by Greaves (1999), at stage 7-8 rather than stage 10. The reason for this discrepancy is not apparent, but perhaps Greaves observed rare ovarioles that contained later stage egg chambers that I missed. Nonetheless, the observation that most \( l(3)neo40^{E1}/l(3)neo40^{E1} \) egg chambers do not progress to stage 10 negates the possibility that \( l(3)neo40 \) identifies a factor with a specific function in nurse cell dumping that starts at this time. Assigning a definite oogenic role to the \( l(3)neo40 \)-associated factor would require further analyses of the germline clone phenotype.

There is at least one caveat to both Greaves' and my data: in neither case was the chromosome bearing the \( l(3)neo40^{l} \) mutation 'cleaned'. That is, secondary mutations in addition to \( l(3)neo40^{l} \) may have been generated during the original mutagenesis and these might have interfered with normal oogenesis. In this scenario, such a mutation would be on the 3R arm of the third chromosome because FRT-mediated recombination was induced on this arm to generate the germline clones.
3.3 \( l(3)\text{neo}40^l \) dominantly enhances the \( En>CADi \) and \( En>Arm \) wing phenotypes

3.3.1 Introduction

The original study reported that \( l(3)\text{neo}40^l \) dominantly suppressed the \( En>CADi^{10} \) wing phenotype to wild type, suppressed the more severe \( En>CADi^{5} \) phenotype towards wild type, and significantly enhanced the \( En>Arm^{2} \) wing phenotype (Greaves, 1999; Greaves et al., 1999). Interactions with the excision alleles \( l(3)\text{neo}40^{EI} \) and \( l(3)\text{neo}40^{E2} \) were similar but weaker (Greaves, 1999). Several of these interaction tests were repeated to assess the reproducibility of these reports.

3.3.2 \( l(3)\text{neo}40^l \) dominantly enhances both \( En>CADi \) and \( En>Arm \)

In stark contrast to the findings reported by Greaves (Greaves et al., 1999; Greaves, 1999), I found that heterozygosity for \( l(3)\text{neo}40^l \) clearly enhanced both the \( En>CADi^{10} \) and \( En>CADi^{5} \) wing phenotypes (Figures 3.2 and 3.3, upper chart), and that the excision allele, \( l(3)\text{neo}40^{EI} \), also dominantly enhanced \( En>CADi^{5} \) (Figure 3.3, upper chart). However, Greaves and I are in agreement with respect to the dominant enhancement of the \( En>Arm \) phenotype by \( l(3)\text{neo}40^l \) (Figures 3.2 and 3.3, lower chart). Therefore, heterozygosity for \( l(3)\text{neo}40^l \) results in the same type of phenotypic modification in both an Armadillo loss-of-function and gain-of-function background.

The excision alleles \( l(3)\text{neo}40^{EI} \) and \( l(3)\text{neo}40^{E2} \) were not tested for their ability to dominantly modify the \( En>CADi^{10} \) or \( En>Arm^{10} \) wing phenotype.

3.3.3 Summary and discussion

I was unable to reproduce the observations reported by Greaves regarding the dominant suppression of \( En>CADi \) wing phenotypes by \( l(3)\text{neo}40^l \) (Greaves, 1999; Greaves et al., 1999). Indeed, I found the direction of the interaction to be opposite. The root cause of
Figure 3.2  Heterozygosity for $l(3)\text{neo40}^i$ enhances both the $En>\text{CADi}$ and $En>\text{Arm}$ wing phenotypes

A, $En>\text{CADi}^+/+$ control. B, Heterozygosity for $l(3)\text{neo40}^i$ weakly enhances the $En>\text{CADi}^i$ (compare with A). C, $En>\text{CADi}^{10}/+$ control. These wings exhibit a weaker phenotype compared to $En>\text{CADi}^i$ wings as they contain more blade material and margin bristles. D, Heterozygosity for $l(3)\text{neo40}^i$ enhances the $En>\text{CADi}^{10}$ phenotype (compare with C). E, $En>\text{Arm}^{16}/+$ control. F, Heterozygosity for $l(3)\text{neo40}^i$ also enhances the $En>\text{Arm}^{16}$ phenotype; notice the extra ectopic bristles compared to E. Wings heterozygous for $l(3)\text{neo40}^i$ alone have wild type morphology (not shown). Female wings that are representative of the range produced by each genotype are shown.
A: $\text{En} \times \text{CADi}[5]/+$

B: $\text{En} \times \text{CADi}[5]/+; l(3)\text{neo40}[1]/+$

C: $\text{En} \times \text{CADi}[10]/+$

D: $\text{En} \times \text{CADi}[10]/+; l(3)\text{neo40}[1]/+$

E: $\text{En} \times \text{Arm}/+$

F: $\text{En} \times \text{Arm}/+; l(3)\text{neo40}[1]/+$
Figure 3.3  Quantitative analysis of the enhancement of Armadillo mis-expression wing phenotypes by heterozygosity for *l(3)neo40* mutations

**Upper chart** *En>CADi* interactions. The mean number of posterior wing margin bristles remaining between longitudinal veins 4-5 (L4-L5) is shown. Lower counts represent greater degrees of enhancement. **Lower chart** *En>Arm*\(^{16}\) interactions. The mean number of ectopic bristles in the posterior blade in shown. Here, higher counts represent enhancement. Analyses are of wings heterozygous for *En>CADi* or *En>Arm*\(^{16}\) and for the mutation indicated. *l(3)neo40*\(^{E1}\) and *l(3)neo40*\(^{E2}\) are imprecise P excision alleles whereas the P element was removed cleanly in *l(3)neo40*\(^{*}\) (see text). Wings heterozygous for the mutation alone have wild type morphology (not shown). Error bars are 95% confidence intervals on the mean. At least 10 female *En>CADi*\(^{s}\) and 6 female *En>Arm*\(^{16}\) wings of each genotype were analyzed.
Mean no. of ectopic bristles

Mean no. of margin bristles (L4-L5)

**Genotype**

**EN-ARM x (3)neo40 mutants**

**EN-CAD x (3)neo40 mutants**
this is uncertain but there are at least two possibilities. First, it is far from clear how many wings of each genotype and sex were examined in Greaves' study and thus it is impossible to judge the variability and reproducibility of the original data. Second, Greaves routinely used sibling genotypes for control phenotypes (S. Greaves, personal communication), while I crossed the Armadillo-sensitized lines to wild type flies to produce a reference phenotype. Greaves' method suffers from the fact that balancer chromosomes contain mutations that enhance En>CADi (for example, TM3, Serrate and CyO; not shown) such that aberrant interactions could have been scored. In conclusion, I prefer to believe my own data where it contradicts that of Greaves as I viewed several wings of each genotype and judged phenotypic modification against a more appropriate control.

Uniquely, Greaves and I were concordant regarding the dominant enhancement of the En>Arm wing phenotype by l(3)neo40l. This interaction appears to be reproducible, although the finding that heterozygosity for l(3)neo40l also enhances En>CADi appears to cast doubt on its significance. However, as discussed later (Chapter 4.5), it is not necessarily wise to assume that bona fide interators ought to modify the En>Arm and En>CADi phenotypes in a complementary manner.

Finally, it is possible that secondary chromosomal mutations, rather than the l(3)neo40l mutation itself, were responsible for the phenotypic modifications described here.

3.4 The l(3)neo40l P insertion does not cause lethality

3.4.1 Introduction

Are the phenotypes and genetic interactions described for the l(3)neo40 mutant chromosomes caused by the associated P insertion? In theory, one, some, or all of these
effects could actually be caused by secondary mutations on the \( l(3)\text{neo}40^i \) chromosome that were induced by the original mutagenesis (Spradling \textit{et al.}, 1995). It was critical to confidently discount this possibility before committing to a concentrated analysis of the wild type function of \( l(3)\text{neo}40 \) and of the Armadillo-\( l(3)\text{neo}40 \) interaction.

Two approaches were taken to this end. First, I excised the P from the \( l(3)\text{neo}40^i \) stock and tested the resulting flies for homozygous lethality and retention of the genetic interaction with Armadillo. Second, I performed a lethal complementation analysis by crossing the \( l(3)\text{neo}40^i \) stock to flies bearing genomic deletions of the 87E region. In conjunction with this I cloned and sequenced DNA flanking the \( l(3)\text{neo}40^i \) insertion in order to confirm that the P insertion was indeed within the 87E chromosomal interval. Although each of these analyses had been undertaken by Greaves (see section 3.1), I nevertheless wished to perform them myself.

3.4.2 Excision of \( l(3)\text{neo}40^i \) reverts lethality and the interaction with En>Arm

Reversion tests are thought to be the most direct way of correlating a P insertion with mutant phenotypes. Thus \( l(3)\text{neo}40^i \) flies were crossed to another transgenic strain that expressed the transposase enzyme, known as \( \Delta 2-3 \), and reversion of the lethality was detected by the appearance of homozygous viable adults in a subsequent generation (Figure 3.4, scheme 1).

The full \( l(3)\text{neo}40^i \) third chromosome genotype is \( l(3)\text{neo}40^i \ red\ e/\ TM3, Sb^i \). \textit{red Malpighian tubules} (red) and \textit{ebony} (e) are recessive markers on the mutant chromosome: \textit{red/red} produces rusty red-coloured Malpighian tubules and brown adult eyes, while \textit{e/e} causes dark to black pigmentation of the adult body; \textit{Stubble} (Sb) is a dominant adult marker on the \textit{TM3} balancer chromosome: \textit{Sb/+} results in abnormally short notal bristles. After exposure to transposase, potential revertants were crossed back to this parental strain (Figure 3.4, scheme 1). This cross yielded a single \textit{red e/ red e} fly, representing a viable
Figure 3.4  Mating scheme for reversion of the $l(3)neo40^i$ mutation by remobilization of the P element

**SCHEME 1**  The $l(3)neo40^i$ chromosome was exposed to transposase ($\Delta2\cdot3$) by mating the $l(3)neo40^i$ stock to flies transgenic for $\Delta2\cdot3$ and selecting against the *Stubble* (*Sb*) and *Tubby* (*Tb*) dominant markers in the progeny. Revertants were recovered by back-crossing these progeny to the parental genotype and then screening for $Dr^+ Sb^+ red^e$ flies in the F2 generation. A single male was recovered. See text for details.

**SCHEME 2**  The revertant chromosome was propagated by first crossing the single revertant male to the *TM3* balancer. 24 male *TM3* progeny were then mated individually to *CxD/TM3* females, selecting against the dominant *CxD* mutation in the F2 generation. These F2 flies were then selfed so as establish 24 sublines: half of these were predicted to carry the $l(3)neo40^i*$ revertant chromosome and the other half to bear the original $l(3)neo40^i$ chromosome. See text for details.
SCHEME 1:

\[
\begin{align*}
\text{TM3, Sb} & \quad \text{TM6, Tb} \\
\text{TM3, Sb} & \quad \text{TM3, Sb}
\end{align*}
\]

SCHEME 2:

\[
\begin{align*}
\text{TM3, Sb} & \quad \text{TM3, Sb} \\
\text{TM3, Sb} & \quad \text{TM3, Sb}
\end{align*}
\]

24 sublines: 50% carry revertant chromosome
Chapter 3: Analysis of the genetic interaction between Armadillo and l(3)neo40

revertant, compared to 267 red e/TM3, Sb siblings. This reversion frequency is similar to those reported previously (Cooley et al., 1988).

In order to recover and propagate the revertant chromosome, the single revertant male was crossed to the TM3 balancer and 24 sublines were started from individual male progeny (Figure 3.4, scheme 2). Of these, half were expected to contain the excised locus (l(3)neo40^*). Genomic DNA from a fly of each subline was extracted and analysed for the presence of P[hsneo] transposon sequences by PCR, and each subline was also backcrossed to the original l(3)neo40^ stock (Figure 3.5). Phenotypic reversion was always correlated with a loss of P[hsneo] sequences (10/19 sublines; Figure 3.5), thus proving that a viable revertant stock had been successfully established.

The revertant stock was then used to test for genetic interaction with Armadillo. Consistent with the reversion of lethality, heterozygosity for l(3)neo40^* no longer enhanced the En>Arm wing phenotype (Figure 3.3, lower chart). However, the En>CADI^ phenotype was dominantly enhanced by l(3)neo40^* to a similar extent as by the original l(3)neo40^ mutation (Figure 3.3, upper chart).

Taken together, these analyses appear to confirm that the lethality and dominant modification of En>Arm associated with the l(3)neo40^ stock are both caused by the P insertion. The dominant enhancement of En>CADI^ wings by the l(3)neo40^ chromosome is apparently not caused by the P. From this standpoint, the finding that l(3)neo40^ dominantly enhances both the En>CADI and En>Arm wing phenotypes (section 3.3) is not significant as the two interactions stem from different causes: the former is not associated with the P insertion while the latter is. Thus, the ability of l(3)neo40^ to dominantly enhance En>Arm should be seen as a bona fide interaction that is not belittled by the En>CADI result.

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Figure 3.5  Phenotypic reversion correlates with loss of the P insertion in l(3)neo40^1* flies

19 sublines survived of the 24 initially generated. Each was balanced over TM3 and carries either the l(3)neo40^1* revertant chromosome or the l(3)neo40^1 mutan chromosome (as described in Figure 3.4). Nine sublines retained the P[hsneo] transposon (‘+’ in the table), as judged from a PCR that amplified 5' pUC sequence from within the transposon and generated a ~150bp product. All nine of these also retained a third chromosome lethal, as judged by the absence of non-TM3 progeny in a back-cross to the original l(3)neo40^1/TM3 stock (‘-’ in the table). The other ten sublines harboured the revertant chromosome as they lacked P[hsneo] sequences and were viable when trans-heterozygous with l(3)neo40^1. The gel shows PCR products from 12 representative sublines, the Df(3R)ry85 stock as a negative control (-ve), and the progenitor pP[hsneo] plasmid (gift from V. Pirotta) as a positive (+ve) control. The band from subline #1 is weak, but present.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PUC sequence present?</th>
<th>Back-cross result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>+</td>
<td>n/a</td>
</tr>
<tr>
<td>$pP[hsneo]$/plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>(Df(3R)ry85/MKRS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subline #1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #4</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #8</td>
<td>Died</td>
<td>Died</td>
</tr>
<tr>
<td>Subline #9</td>
<td>Died</td>
<td>Died</td>
</tr>
<tr>
<td>Subline #10</td>
<td>Died</td>
<td>Died</td>
</tr>
<tr>
<td>Subline #11</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #12</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #13</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #14</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #15</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #16</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #17</td>
<td>Died</td>
<td>Died</td>
</tr>
<tr>
<td>Subline #18</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #19</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Subline #20</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #21</td>
<td>Died</td>
<td>Died</td>
</tr>
<tr>
<td>Subline #22</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #23</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Subline #24</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
3.4.3 Deficiency mapping reveals the absence of a lethal within 87E in the l(3)neo40I stock

Several complementation tests between l(3)neo40I and Deficiencies or other lethals in the 87E region had been conducted before I started this project (Table 3.1). These tests produced conflicting results: Greaves reported three cases of non-complementation with l(3)neo40I, whereas other researchers had found that these same trans-heterozygous combinations were viable (Cooley et al., 1988; BDGP; Table 3.1).

In order to confirm one report or the other, I repeated these complementation tests and also conducted several others using l(3)neo40I, l(3)neo40EI and l(3)neo40E2, and appropriate Deficiencies and lethals (Tables 3.2, 3.3, 3.4 and 3.5). All Deficiencies tested that uncover the 87E region complemented all three l(3)neo40 mutations (Tables 3.2, 3.4 and 3.5). Similarly, all lethals in the 87E interval that were tested were found to complement l(3)neo40 mutant alleles (Table 3.3). Non-complementation was only observed for l(3)neo40I/l(3)neo40EI, l(3)neo40EI/l(3)neo40E2 and l(3)neo40I/l(3)neo40E2 (Tables 3.4 and 3.5).

These data validate the reports of the Berkeley Drosophila Genome Project and Cooley and colleagues (BDGP; Cooley et al., 1988) and clearly demonstrate that there is no recessive lethal mutation in the entire 86C-87F region of the l(3)neo40I third chromosome. Therefore, assuming that the P insertion in this line is mapped correctly at 87E11-12 (see section 3.4.4 below), the most parsimonious explanation of these data is that the homozygous lethality of the l(3)neo40I stock is not associated with the P, but rather is caused by a secondary lethal elsewhere on the third chromosome (as the stock is kept stably over a third chromosome balancer). The fact that l(3)neo40I, l(3)neo40EI and l(3)neo40E2 are all lethal when trans-heterozygous with each other is consistent with there being a common background lethal mutation in each of these stocks. However, these conclusions are clearly contradictory to the reversion data described above, an issue which is addressed
Table 3.1  Previous complementation analysis of \( l(3) neo40 \)

All instances of non-complementation are reported by Greaves alone (S. Greaves, personal communication). Where tested, these data are contested by independent sources. Deficiencies/mutations that uncover the 87E11-12 region (the reported insertion site of \( l(3) neo40 \)) are shown in **bold**—notice that Greaves reported non-complementation between \( l(3) neo40 \) and three deficiencies/mutations that map outside of this region. BDGP, Berkeley *Drosophila* Genome Project.

Table 3.2  Deficiency mapping of the \( l(3) neo40 \) insertion site

\( l(3) neo40 \) is complemented by all of the Deficiencies tested. Notice that the two non-complementing deficiencies of Greaves (\( Df(3R)M-Kx1 \) and \( Df(3R)ry615 \)) complement \( l(3) neo40 \) in my hands, the latter being in agreement with previous independent reports. (Table 3.1.) ‘Transheterozygous progeny’ is the number of Deficiency/\( l(3) neo40 \) progeny (i.e. instances of complementation) given as a percentage of the total number of progeny collected after crossing the Deficiency stock to the \( l(3) neo40 \) stock. Assuming that all viable genotypes are equally healthy, this figure is predicted to be \(~33\%\). Deficiencies that remove the 87E11-12 region are shown in **bold**.
### Table 3.1

<table>
<thead>
<tr>
<th>Deficiency/mutation</th>
<th>Breakpoints/insertion site</th>
<th>Complements?</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3R)M-Kxl</td>
<td>86C1; 87B1-5</td>
<td>NO</td>
<td>S. Greaves</td>
</tr>
<tr>
<td>Df(3R)T-32</td>
<td>86E2-4; 87C6-7</td>
<td>YES</td>
<td>S. Greaves</td>
</tr>
<tr>
<td>Df(3R)kar-D1</td>
<td>87A7; 87D1-2</td>
<td>YES</td>
<td>BDGP</td>
</tr>
<tr>
<td>Df(3R)ry615</td>
<td>87B11-13; 87E8-11</td>
<td>YES/NO</td>
<td>Cooley et al., 1988/ S. Greaves</td>
</tr>
<tr>
<td>l(3)0346303463</td>
<td>87D7-9</td>
<td>YES/NO</td>
<td>BDGP/ S. Greaves</td>
</tr>
<tr>
<td>P[hsneo]132 l(3)'</td>
<td>87E</td>
<td>YES</td>
<td>S. Greaves</td>
</tr>
<tr>
<td>l(3)0513705137</td>
<td>87E7-8</td>
<td>YES/NO</td>
<td>BDGP/ S. Greaves</td>
</tr>
</tbody>
</table>

### Table 3.2

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Breakpoints</th>
<th>Transheterozygous progeny</th>
<th>Complements?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3R)M-Kxl</td>
<td>86C1; 87B1-5</td>
<td>37/97 =38%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)T-32</td>
<td>86E2-4; 87C6-7</td>
<td>25/59 =42%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)E229</td>
<td>86F6-7; 87B1-2</td>
<td>86/152 =57%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry615</td>
<td>87B11-13; 87E8-11</td>
<td>72/226 =32%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry85</td>
<td>87B15-C1; 87F15-88A1</td>
<td>37/103 =36%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)kar-Sz11</td>
<td>87C7-8; 87E5-6</td>
<td>44/109 =40%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)kar-Sz28</td>
<td>87C7-8; 87E9-10</td>
<td>66/134 =49%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry27</td>
<td>87D1-2; 87F1-2</td>
<td>75/247 =30%</td>
<td>YES</td>
</tr>
<tr>
<td>Tp(3;Y)ry506-85C</td>
<td>87D1-2; 88E5-6</td>
<td>23/64 =36%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry619</td>
<td>87D7-9; 87E12-F1</td>
<td>79/227 =35%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)126c</td>
<td>87D14-E1; 87F11-12</td>
<td>49/175 =28%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)IC4a</td>
<td>87E5-7; 87E11-F1</td>
<td>88/253 =35%</td>
<td>YES</td>
</tr>
</tbody>
</table>
Table 3.3  Complementation tests between \( l(3)neo40^i \) and other homozygous lethal mutations in the 87E region

\( l(3)neo40^i \) is complemented by all of the mutations tested. Notice that the two non-complementing mutations of Greaves \((l(3)03463^{03463} \text{ and } l(3)05137^{05137})\) complement \( l(3)neo40^i \) in my hands, in agreement with previous independent reports. (Table 3.1.) Lethals that have been mapped to the 87E11-12 region are shown in bold. See legend to Table 3.2.

Table 3.4  Complementation analysis of \( l(3)neo40^{EI} \)

Non-complementation of \( l(3)neo40^{EI} \) was observed with \( l(3)neo40^i \) and \( l(3)neo40^{E2} \) only. Deficiencies/mutations that affect the 87E11-12 region are shown in bold. See legend to Table 3.2.

Table 3.5  Complementation analysis of \( l(3)neo40^{E2} \)

Non-complementation of \( l(3)neo40^{E2} \) was observed with \( l(3)neo40^i \) and \( l(3)neo40^{EI} \) only. Deficiencies/mutations that affect the 87E11-12 region are shown in bold. See legend to Table 3.2.
Table 3.3

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Insertion site</th>
<th>Transheterozygous progeny</th>
<th>Complements?</th>
</tr>
</thead>
<tbody>
<tr>
<td>l(3)neo39^</td>
<td>87D1-E12</td>
<td>26/83 =31%</td>
<td>YES</td>
</tr>
<tr>
<td>l(3)03463^03463</td>
<td>87D7-9</td>
<td>33/90 =37%</td>
<td>YES</td>
</tr>
<tr>
<td>P[hsneo]132, l(3)^*</td>
<td>87E</td>
<td>38/100 =38%</td>
<td>YES</td>
</tr>
<tr>
<td>l(3)05137^05137</td>
<td>87E7-8</td>
<td>35/103 =34%</td>
<td>YES</td>
</tr>
<tr>
<td>l(3)87Ek^2</td>
<td>87E10-11</td>
<td>30/74 =41%</td>
<td>YES</td>
</tr>
<tr>
<td>l(3)87Ek^2</td>
<td>87E11-12</td>
<td>66/222 =30%</td>
<td>YES</td>
</tr>
</tbody>
</table>

Table 3.4

<table>
<thead>
<tr>
<th>Mutation/Deficiency</th>
<th>Insertion site /breakpoints</th>
<th>Transheterozygous progeny</th>
<th>Complements?</th>
</tr>
</thead>
<tbody>
<tr>
<td>l(3)neo40^</td>
<td>87E11-12</td>
<td>0/71 =0%</td>
<td>NO</td>
</tr>
<tr>
<td>l(3)neo40^E2</td>
<td>87E11-12</td>
<td>0/99 =0%</td>
<td>NO</td>
</tr>
<tr>
<td>Df(3R)M-Kxl</td>
<td>86C1; 87B1-5</td>
<td>41/115 =36%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry85</td>
<td>87B15-C1; 87F15-88A1</td>
<td>6/16 =38%</td>
<td>YES</td>
</tr>
<tr>
<td>Tp(3;Y)ry506-85C</td>
<td>87D1-2; 88E5-6</td>
<td>13/45 =29%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry619</td>
<td>87D7-9; 87E12-F1</td>
<td>27/83 =33%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)126c</td>
<td>87D14-E1; 87F11-12</td>
<td>63/179 =35%</td>
<td>YES</td>
</tr>
<tr>
<td>l(3)87Ek^2</td>
<td>87E11-12</td>
<td>41/95 =43%</td>
<td>YES</td>
</tr>
</tbody>
</table>

Table 3.5

<table>
<thead>
<tr>
<th>Mutation/Deficiency</th>
<th>Insertion site /breakpoints</th>
<th>Transheterozygous progeny</th>
<th>Complements?</th>
</tr>
</thead>
<tbody>
<tr>
<td>l(3)neo40^</td>
<td>87E11-12</td>
<td>0/20 =0%</td>
<td>NO</td>
</tr>
<tr>
<td>l(3)neo40^E2</td>
<td>87E11-12</td>
<td>0/99 =0%</td>
<td>NO</td>
</tr>
<tr>
<td>Df(3R)M-Kxl</td>
<td>86C1; 87B1-5</td>
<td>52/157 =33%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry85</td>
<td>87B15-C1; 87F15-88A1</td>
<td>19/43 =44%</td>
<td>YES</td>
</tr>
<tr>
<td>Tp(3;Y)ry506-85C</td>
<td>87D1-2; 88E5-6</td>
<td>21/52 =40%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)ry619</td>
<td>87D7-9; 87E12-F1</td>
<td>51/154 =33%</td>
<td>YES</td>
</tr>
<tr>
<td>Df(3R)126c</td>
<td>87D14-E1; 87F11-12</td>
<td>60/152 =39%</td>
<td>YES</td>
</tr>
<tr>
<td>l(3)87Ek^2</td>
<td>87E11-12</td>
<td>33/96 =34%</td>
<td>YES</td>
</tr>
</tbody>
</table>
in section 3.4.5.

The complementation data for \( l(3)\text{neo}40^I \) presented here differ from those of Greaves (Table 3.1). An explanation for this is elusive. Intriguingly, Greaves did find that the two excision alleles complemented Deficiency chromosomes spanning the 87E region (Greaves, 1999), results consistent with my data.

3.4.4 Sequencing of DNA flanking the P insertion confirms that \( l(3)\text{neo}40^I \) is located at 87E

in situ hybridization to polytene chromosomes had been used originally to map the \( l(3)\text{neo}40^I \) P insertion to the 87E region (BDGP; Cooley et al., 1988). If this mapping were incorrect, then the inconsistencies between the Deficiency mapping and the reversion data with respect to the homozygous lethality of the stock would be explained. That is, \( l(3)\text{neo}40^I \) homozygous lethality could be caused by a P insertion outside of the 87E region.

In order to test this remote possibility, I took advantage of the ‘plasmid rescue’ technique to clone genomic DNA flanking the P insertion (Rubin and Spradling, 1982); see Chapter 2.5.3. Briefly, the \( P[\text{hsneo}] \) transposon includes a bacterial origin of replication, an Ampicillin resistance gene and unique restriction enzyme sites, features which allow plasmid rescue (Figure 3.6A). Genomic DNA from \( l(3)\text{neo}40^I \) flies was extracted and digested with an enzyme that cuts the vector on only one side of the origin of replication and resistance sequences within the transposon sequence and at an unspecified site in the flanking genomic DNA (Figure 3.6A). Digested DNA was then allowed to ligate at high dilution to promote intramolecular ligation and the formation of circular plasmids. These plasmids were then transformed into high efficiency bacteria, the bacterial cells plated on Ampicillin, and surviving transformants amplified. Finally, \( l(3)\text{neo}40^I \) genomic flanking DNA was sequenced using primers designed to the \( P[\text{hsneo}] \) termini (Figure 3.6A).
Figure 3.6  Quality analysis of plasmid rescued genomic DNA flanking the l(3)neo40' P insertion

A, Schematic of the P[hsneo] transposon inserted into genomic DNA. The scale of base pairs (bp) is given along the top. The next line down shows the sites of the 5' pUC and 3' neo PCR products, and the sites used for the sequencing primers, SJM2 (for 5' flanking DNA) and SJM3 (for 3' flanking DNA). The unique restriction sites used for plasmid rescue are given below the transposon cartoon, as are the regions encoding the bacterial origin of replication (ori) and the Ampicillin resistance gene (ampR). Thus, Xho 1 or Nar 1 restriction digests can yield plasmids containing the ori, ampR and 5' flanking genomic DNA, while a Sal 1 digest can produce plasmids containing the ori, ampR and 3' flanking DNA. P5' and P3' are the 5' and 3' P element sequences respectively. B, PCR assays to check the quality of plasmid rescues. The 5' pUC PCR product should be present only in the 5' Xho 1 and Nar 1 rescues, while the 3' neo PCR product should be present only in the 3' Sal 1 rescues. However, only the Xho 1-30, Xho 1-11, Nar 1-26 and Nar 1-8.2 clones show the predicted pattern of PCR products. Two of these, Xho 1-30 and Nar 1-8.2, and also the 3' Sal 1-8.1 clone were sequenced. pP[hsneo] is the progenitor plasmid for the P[hsneo] transposon and is thus a positive control.
A

$P[hsneo]$ (5075bp)

```
0  1000  2000  3000  4000  5000
```

SJM2 (5' flank) 5' PCR 3' PCR SJM3 (3' flank)

5' genomic DNA

\[
P'\text{poly} & \text{linker} & \text{pUC8} & \text{Hsp70Bb} & \text{neoR} & P'3\text{'}
\]

SalI[718]

ori

ampR

neoR[Hsp70Bb]

B

Predicted PCR result?

| pUC (5') | + | + | + | - | - | + | + | + |
| neo (3') | - | + | - | + | + | - | + | + |

Predicted PCR result?

| pUC (5') | + | + | + | - | - | + | + | + |
| neo (3') | - | + | - | + | + | - | + | + |
Chapter 3: Analysis of the genetic interaction between Armadillo and l(3)neo40

Xho 1 and Nar 1 restriction enzymes were used to obtain 5' flanking DNA, and SalI to rescue 3' flanking DNA (Figure 3.6A). Three independent transformants were obtained using each enzyme (making nine in total) and plasmid DNA was checked for quality by using PCR to detect either 5' pUC sequences or 3' neo sequences that are present in the P[hsneo] transposon (Figures 3.6B): only rescues of 5' DNA (Xho 1 and Nar 1 digests) should have 5' pUC sequences, while only 3' rescues should retain 3' neo sequences (Sal I digests). Four clones, Xho 1-30, Xho 1-11, Nar 1-8.1, Nar 1-8.2, all of which included 5' flanking DNA, showed the expected pattern of PCR products (Figures 3.6B).

Two of the validated clones, Xho 1-30 and Nar 1-8.2, were sequenced in duplicate. The first round of sequencing was of poor quality, yet BLAST analysis identified only one hit that was shared between the two sequences amongst high scoring segment pairs: a Drosophila P1 clone named DS04219 (BDGP; GenBank accession no. AC003923; not shown). This P1 maps to the Actin87E contig that is centred on the cytological region 87E12-F1. In the second round of sequencing, the Xho 1-30 clone did not yield good sequence. However, the Nar 1-8.2 clone was 98% identical to sequences within the same P1 clone as was identified before (not shown).

Despite its aberrant pattern of PCR products (Figures 3.6B), the Sal 1-8.1 clone, which represented 3' flanking DNA, was also sequenced. BLAST analysis indicated that the 3' sequence was 100% identical to DNA flanking the P insertion named l(3)05137 which itself maps to cytological region 87E7-E8 (not shown).

Taken together, sequencing of DNA flanking the P insertion associated with l(3)neo40 confirmed that the P maps to cytological region 87E and that previous analyses had not mis-mapped the insertion. Hence, the complementation analysis using Deficiencies that uncover the 87E region yielded valid and informative data.
3.4.5 Summary and discussion

The homozygous lethality of the l(3)neo40\textsuperscript{l} mutant chromosome is associated with a single P insertion (Cooley et al., 1988). This P was originally mapped to the 87E cytological region by in situ hybridization to polytene chromosomes, but was later refined to 87E11-12 based on further in situ analysis and its non-inclusion within Df(3R)ry615 (which uncovers 87B11-13; 87E8-11; BDGP). However, I find that the lethality associated with l(3)neo40\textsuperscript{l} is not uncovered by any Deficiency chromosome that removes part or all of the 87E region. This has at least two implications. First, the non-inclusion of a lethal within Df(3R)ry615 is not a valid criterion for refining the 'left limit' of the P insertion from 87E to 87E11 (BDGP). The second and more important conclusion is that a recessive lethal loss-of-function mutation does not reside in the 87E region of the l(3)neo40\textsuperscript{l} chromosome.

Sequencing of plasmid rescued DNA flanking the P insertion confirmed its insertion within the 87E region. Taking this result and the complementation analyses together, it seemed clear that the lethality of the l(3)neo40\textsuperscript{l} stock was not caused by the P insertion. Indeed, around the time of these experiments I received a belated reply to an enquiry made to Allan Spradling regarding the l(3)neo40\textsuperscript{l} mutation. He informed me that "l(3)neo40\textsuperscript{l} was discarded from the P element collection some time ago because our own analysis determined that the mutant phenotype was not caused by the P insertion." (Unfortunately, this information had not been imparted to the relevant Drosophila databases at the time.)

Arguing against the evidence that the P insertion does not cause lethality, I found that the phenotype could be reverted by excising the P transposon, as had Greaves (Greaves, 1999). This result directly implicated the P in being the causing of recessive lethality, but is obviously at odds with the Deficiency mapping data. The discrepancy may be resolved in one of two ways. First, it is possible that the sole revertant was generated by a freak recombination event that eliminated a secondary mutation in addition to the P at 87E, rather than a specific and clean excision of the P alone. If this were the case then
there may have been several P excision events that went undetected as the chromosome still harboured a lethal, although the reversion frequency seen here was similar to previous reports. Second, one can posit that \( l(3)\text{neo}40^l \) produces a protein with dominant-active effects, such that homozygosity is deleterious and lethal but heterozygosity or hemizygosity are each tolerated and viable as they are functionally equivalent with respect to hypermorphic alleles. Hence, if ‘++’ is the gain-of-function effect of \( l(3)\text{neo}40^l \), then +++/+ (homozygosity) is lethal, while +++/+TM3 (heterozygosity) and +++/0 (hemizygosity) are viable, as was observed. However, this model relies upon the unlikely scenario of all three \( l(3)\text{neo}40 \) mutations \( (l(3)\text{neo}40^f \text{ and } l(3)\text{neo}40^*) \) being hypermorphic as they all behaved identically in the complementation analyses (Tables 3.2-3.5). Furthermore, the wild type product identified by \( l(3)\text{neo}40^l \) would have to be dispensable as hemizygotes lack all wild type function and are viable; that is, a null mutation of \( l(3)\text{neo}40 \) would be predicted to be homozygous viable in this scenario.

In the absence of a convincing explanation for these discrepancies, the true relationship between the \( l(3)\text{neo}40^l \) P insertion and the cause of lethality is not absolutely clear. However, in considering the single instance of reversion against the body of evidence from my complementation analyses and the work referred to by Spradling, I prefer to consider that the \( l(3)\text{neo}40^l \) P insertion at 87E is non-lethal.

In addition to zygotic viability, the \( l(3)\text{neo}40^l \) chromosome is also required maternally for normal oogenesis and it interacts genetically with Armadillo. If the excision data is to be believed, then the associated P insertion caused the dominant enhancement of the \( En>\text{Arm} \) wing phenotype but not the dominant enhancement of \( En>CADi \). Thus regardless of whether the P is responsible for the lethality of the \( l(3)\text{neo}40^l \) stock, it remains a specific modifier of \( En>\text{Arm} \), which was the original reason for its analysis (Greaves, 1999; Greaves et al., 1999).Whether the P is the specific cause of the oogenesis defects resulting from \( l(3)\text{neo}40^l \) germline clones was not investigated.
3.5 Summary and discussion

- \( l(3)\text{neo}40^l \) was initially defined as a recessive lethal mutation associated with a \( P[\text{hsneo}] \) insertion at 87E
- Further analyses of \( l(3)\text{neo}40^l \) by Greaves found that it interacted genetically with Armadillo, that homozygotes died in late embryogenesis, and that a maternal contribution was required for oogenesis
- \( l(3)\text{neo}40^l \) germline clones arrest oogenesis at stage 7/8, although the cause of this is unclear
- \( l(3)\text{neo}40^l \) dominantly enhanced both the \( \text{En>CADi} \) and \( \text{En>Arm} \) wing phenotypes
- Sequencing of DNA flanking the \( P \) confirmed its insertion within 87E
- Deficiency mapping indicated the absence of a lethal mutation in the 87E region, while excision of the \( P \) caused reversion of lethality
- The \( l(3)\text{neo}40^l \) \( P \) insertion appears to be responsible for the dominant modification of \( \text{En>Arm} \), but not \( \text{En>CADi} \)
- Thus it is not fully apparent whether the \( P \) is truly responsible for the recessive lethality of the \( l(3)\text{neo}40^l \) chromosome, nor whether the different phenotypes (lethality, genetic interaction with Armadillo, and defective oogenesis) are each caused by a single mutation

Despite my finding that the \( P \) insertion caused a bona fide modification of \( \text{En>Arm} \), it was decided that the analysis of \( l(3)\text{neo}40^l \) and its relationship to Armadillo function should be terminated. This decision was taken primarily because of the opposing results from the two independent tests of whether the \( P \) insertion caused lethality: it was deemed that analysing
the developmental functions of a likely non-essential gene could prove to be both difficult and unproductive. A second reason for aborting this project was that the sole datum linking \( l(3)\text{neo}40^I \) to Armadillo function was its dominant modification of \( En>Arm \): the modification of \( En>CADi \) was not caused by the P insertion, while neither the \( l(3)\text{neo}40^I \) zygotic phenotype, \( l(3)\text{neo}40^I \) somatic clones, nor \( l(3)\text{neo}40^I \) germline clones generated a phenotype characteristic of deregulated Armadillo activity.

Discrepancies between Greaves' data and my own were numerous in this project. An explanation for these is not apparent. All I can say is that I believe that I carried out the experiments described in this Chapter in a well-controlled and careful manner. On this note it is worth pointing out that I obtained the \( l(3)\text{neo}40^I \) stock from two independent sources: one via Greaves and the other from the Bloomington stock centre. Both generated identical results in genetic interaction and complementation tests.
ADDENDUM TO CHAPTER 3

Fly BLAST (BDGP) and GadFly (FlyBase) were used to align and position genomic DNA flanking the l(3)neo40 insertion site within the *Drosophila* genome sequence (Section 3.4.4; Figure 3.7). The l(3)neo40 insertion is within cytogenetic region 87E8 and is very close to that of two other P elements, l(3)05137 and EP(3)3703 (FlyBase; BDGP; Figure 3.7A). All three P insertions are located in the first (and only) intron of the *diminished discs* (*did*) gene and thus might disrupt its function (Figure 3.7B). Although several *did* mutations exist (FlyBase), *did* (also known as l(3)IX-11) is the only characterized allele: *did* homozygotes are larval/prepupal lethal and larvae have smaller imaginal discs than normal (Shearn et al., 1978, *Genetics* 89, 355; Gatti and Baker 1989, *Genes Dev.* 3, 438). This phenotype may be explained by the observation that *did* larval ganglia (and thus probably other imaginal tissues) exhibit a very low mitotic index, indicating a defect in cell cycle progression (Gatti and Baker, 1989). These data are intriguing in light of the cell cycle-Armadillo interactions reported in Chapters 4 and 5. Alternatively, P insertions within the *did* intronic sequence may cause aberrant transcriptional regulation of either adjacent genes, such as BcDNA:GH02431, which encodes a mitochondrial dicarboxylic acid transporter (Figure 3.7B; FlyBase), or more distant genes. A third possibility is that P insertions within this small genomic region have no significant mutational affect. In this respect, two different 'recessive lethal' l(3)05137 mutations exist, but have not been validated, while no phenotypic data is available for the EP(3)3703 insertion (FlyBase).

**Figure 3.7** l(3)neo40 is inserted in the intron of the *diminished discs* gene

A, Genomic DNA sequence surrounding the l(3)neo40 insertion site. Black and boxed: scaffold DNA sequence (+ strand of scaffold segment AE003699; 5' is to the left); green: 5' of l(3)05137 insertion (BDGP); red: 5' and 3' of EP(3)3703 insertion (BDGP); blue: 3' of l(3)neo40 insertion (my SalI-8.1 rescue); magenta: 5' of l(3)neo40 insertion (my XhoI-30 rescue). Non-identical bases are underlined. The insertion sites of l(3)05137 and EP(3)3703 are indicated by the **bold** typeface in the relevant sequences. The site of insertion of l(3)neo40 is indicated by the dashed line. Flanking genomic DNA sequences are referred to as 5' or 3' relative to each particular P insertion: note that the orientation of the l(3)neo40 insertion is 3'-5' while that of both l(3)05137 and EP(3)3703 is 5'-3' with respect to the scaffold sequence.

B, Genetic map of the region surrounding the l(3)neo40 insertion site (FlyBase: GadFly). l(3)neo40, l(3)05137 and EP(3)3703 (turquoise arrow) are all inserted within the sole intron of the *diminished discs* (*did*) gene (which is transcribed from the + strand). The BcDNA:GH02431 gene, from which two alternative transcripts are made from the + strand, is located nearby and the first exon of the longer transcript lies within the *did* intronic sequence. Intron-exon structures of *did* and BcDNA:GH02431 are confirmed by cDNAs (FlyBase GeneSeen; not shown). The region shown lies within region 87E8 (BDGP). Divisions are 1000bp. Bases 9115000-9125000 from chromosome arm 3R are shown.
Chapter 4: Characterization of genetic interactions between Armadillo and cell cycle regulators

CHAPTER 4: CHARACTERIZATION OF GENETIC INTERACTIONS
BETWEEN ARMA DIILO AND CELL CYCLE REGULATORS

"interact verb. to act on or in close relation with each other"


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Chapter 4: Characterization of genetic interactions between Armadillo and cell cycle regulators

4.1 Introduction

In addition to uncloned genes, the screen by Greaves et al. (1999) also identified known factors whose genetic interaction with Armadillo was unexpected. The case in point is a group of five Drosophila cell cycle regulators: Cdk1, Cyclin A, String, Twins and Pebble. While it has been known for many years that Wnt/Wingless signalling contributes to the regulation of cell proliferation, direct links between signalling components and discrete cell cycle regulators have only been characterized during the last two to three years (see Chapter 1.4). Thus at the time that this investigation started, the possibility of a clear and functional link between cell cycle regulation and Wingless-Armadillo signalling was both relatively novel and highly interesting.

This chapter describes my investigation and characterization of genetic interactions between Armadillo and several cell cycle regulators using Armadillo-sensitized wing phenotypes in Drosophila. I find that Cyclin E, Dacapo, Cdk1, Cyclin A, Cyclin B, String and Twins show reliable genetic interactions with the En>CADi ('Armadillo loss-of-function') wing phenotype, while the En>Arm ('Armadillo gain-of-function') wing phenotype is very sensitive to levels of String.

4.2 Preliminary tests identify putative genetic interactions between cell cycle regulators and Armadillo

4.2.1 Introduction

Greaves reported that five different cell cycle regulators were dosage-sensitive for Armadillo-sensitized phenotypes in the wing (Greaves et al., 1999). Remarkably, all five are positive regulators of mitotic entry or progression (see Chapter 1.3.2). Briefly, Cdk1
(encoded by the cdc2 gene) is the conserved Cyclin-dependent protein kinase that induces a cascade of protein phosphorylations which in turn cause the morphological changes of mitosis (Stern et al., 1993). *Drosophila* Cyclin A is one of three mitotic Cyclins that bind to Cdk1 and is necessary for its mitotic activation (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990). String is the Cdc25 phosphatase that activates the Cdk1-Cyclin complex and is rate limiting for mitotic entry in *Drosophila* (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990; Neufeld et al., 1998). Twins is a regulatory subunit of protein phosphatase 2A that is needed for the normal separation of sister chromatids and appears to dephosphorylate substrates of Cdk1 (Gomes et al., 1993; Mayer-Jaekel et al., 1994). Lastly, Pebble is a GTP exchange factor for the Rho family of small GTPases and is required for cytokinesis ((Hime and Saint, 1992; Lehner, 1992).

As shown in Table 4.1, heterozygosity for a loss-of-function mutation in each of these genes was reported to enhance to the *En>Arm* wing phenotype and, with the exception of *cyclin A*, concomitantly suppress the *En>CADi* wing phenotype (Greaves et al., 1999). These genetic interactions are consistent with each factor having a negative effect on the signalling function of Armadillo in wild type cells. My aims were to confirm these findings and to eventually characterize functional interactions between the regulation of Armadillo and the cell cycle.

### 4.2.2 Cdk1, Cyclin A, String and Twins interact genetically with Armadillo

I wished to reproduce the putative genetic interactions described above before undertaking any further analyses. Furthermore, I considered it important to confirm the specificity of the interactions by testing at least two different mutant alleles of each gene, where possible. In this way, I hoped to minimize the possibility that a secondary mutation in any one particular mutant stock was contributing to or was the actual cause of a phenotypic modification.
Table 4.1  Summary of genetic interactions between Armadillo and cell cycle regulators described by Greaves *et al.* (1999)

'Modification' is a qualitative measure of the effect of heterozygosity for a mutation upon the *En>CADi* or *En>Arm* wing phenotype as compared to control phenotypes. -/-/-, weak/strong suppression, i.e. the wings have a more wild type morphology than the controls; +/-++, weak/strong enhancement; i.e. the wing phenotype is exaggerated over that of controls; 0, no effect. The allele class listed is as described on Flybase.

Table 4.2  Re-tests of the genetic interactions described in Table 4.1

In addition to the *En>CADi*<sup>10</sup> line used by Greaves, I used an *En>CADi*<sup>5</sup> line that shows a slightly more severe wing phenotype- see Figure 3.2. As the *En>Arm*<sup>2</sup> line used in the original screen had died, the *En>Arm*<sup>16</sup> line, which exhibits a stronger wing phenotype, was used. The allele class listed is as described on Flybase. Approximately 20 wings of each genotype and sex were compared to the same number of *En>CADi/+* or *En>Arm/+* control wings of each sex. Notation as in Table 4.1. Note the numerous discrepencies between this data and that of Greaves *et al.* (1999; Table 4.1).
Table 4.1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele tested (class)</th>
<th>Modification of En&gt;CAD&lt;sup&gt;10&lt;/sup&gt;</th>
<th>Modification of En&gt;Arm&lt;sup&gt;2&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>cdc2</td>
<td>Dmd&lt;sup&gt;cdc2&lt;sup&gt;2847&lt;/sup&gt; (amorph)</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>cdc&lt;sup&gt;2EI-24&lt;/sup&gt; (hypomorph)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>cyclin A</td>
<td>cycA&lt;sup&gt;183&lt;/sup&gt; (amorph)</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>string</td>
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<td>-</td>
<td>++</td>
</tr>
<tr>
<td>twins</td>
<td>tws&lt;sup&gt;01436&lt;/sup&gt; (?)</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>pebble</td>
<td>Df(3L)pbl-X1 ('amorph')</td>
<td>--</td>
<td>++</td>
</tr>
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</table>

Table 4.2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alleles tested (class)</th>
<th>Modification of En&gt;CAD&lt;sup&gt;10&lt;/sup&gt;</th>
<th>Modification of En&gt;CAD&lt;sup&gt;15&lt;/sup&gt;</th>
<th>Modification of En&gt;Arm&lt;sup&gt;16&lt;/sup&gt;</th>
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<td>+</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cdc&lt;sup&gt;2EI-24&lt;/sup&gt; (hypomorph)</td>
<td>++</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>cyclin A</td>
<td>cycA&lt;sup&gt;ChLR1&lt;/sup&gt; (amorph)</td>
<td>++</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>cycA&lt;sup&gt;neo114&lt;/sup&gt; (?)</td>
<td>+</td>
<td>++</td>
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</tr>
<tr>
<td>string</td>
<td>stg&lt;sup&gt;7B&lt;/sup&gt; (strong)</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>stg&lt;sup&gt;7M&lt;/sup&gt; (strong)</td>
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<td>0</td>
<td>++</td>
</tr>
<tr>
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<td>twins</td>
<td>tws&lt;sup&gt;01436&lt;/sup&gt; (?)</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>tws&lt;sup&gt;02414&lt;/sup&gt; (?)</td>
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<tr>
<td>pebble</td>
<td>Df(3L)pbl-X1 ('amorph')</td>
<td>0</td>
<td>n.t.</td>
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</tr>
</tbody>
</table>
As shown in Table 4.2, I was unable to corroborate the majority of Greaves' observations (compare to Table 4.1). Indeed, I found that heterozygosity for mutation of cdc2, cyclin A, string or twins enhanced (rather than suppressed) En>CADi phenotypes, while the reported dominant modification of the En>Arm phenotype by mutation of cdc2, cyclin A or pebble was not apparent. Only the interactions between string or twins and En>Arm were consistent with Greaves' original report (Greaves et al., 1999): dominant enhancement of the En>Arm phenotype was confirmed in each case.

### 4.2.3 Summary and discussion

Mutations in four genes encoding regulators of mitotic induction or progression each dominantly enhanced En>CADi, indicating that their wild type role may be to upregulate Armadillo function in some way and that passage through mitosis could have a positive effect on Armadillo activity. However, heterozygosity for mutation of either string or twins also appeared to enhance the En>Arm phenotype, leading to the opposite conclusions (discussed further in section 4.5).

The discrepancies between my findings and those reported by Greaves et al. (1999) may be explained in two ways. Firstly, the fact that I used an En>Arm line that exhibited a stronger phenotype than that used by Greaves and colleagues (as the original stock had been lost) may have rendered my En>Arm interaction tests less sensitive to modification. Secondly, while I crossed the Armadillo-sensitized lines to wild type flies to give a reference phenotype, Greaves routinely used wings from sibling flies in each cross as a control phenotype which suffers from there being modifying mutations on the balancer chromosomes (as discussed in Chapter 3.3.3). For this reason, and because I used two different mutations to confirm the specificity of most interactions, my own data is likely to be the more reliable (as was confirmed by further tests- see section 4.3).
Chapter 4: Characterization of genetic interactions between Armadillo and cell cycle regulators

The finding that certain promoters of the G2-M transition showed genetic interactions with Armadillo suggested two immediate questions. Firstly, would additional regulators of mitosis show similar interactions? Secondly, would regulators of other phases of cell cycle progression interact genetically with Armadillo, and if so, how? Such additional cell cycle factors might have gone undetected in the original modifier screen as relevant interactors can often be overlooked when screening with Deficiencies and the screen was not performed to saturation (see Chapter 1.5.2). To these ends, I tested mutations in genes encoding several other cell cycle regulators for their ability to dominantly modify CADi and Armadillo over-expression phenotypes.

4.3 Several mitotic regulators, Cyclin E, and Dacapo show genetic interaction with CADi over-expression phenotypes

4.3.1 Introduction
Preliminary tests indicated that mutations in genes encoding three additional promoters of mitotic progression dominantly enhanced \( En>CADi \) (not shown). These were the Cyclins B and B3 that bind to Cdk1 and co-ordinate its mitotic functions (Jacobs et al., 1998; Lehner and O'Farrell, 1990), and Fizzy, an activating subunit of the anaphase promoting complex/cyclosome (Dawson et al., 1993; Sigrist et al., 1995). The G1-S regulators, Cyclin E and Dacapo, also enhanced \( En>CADi \) in preliminary tests (not shown). Cyclin E binds to and activates Cdk2 and is thought to be the limiting factor for entry into S phase (Knoblich et al., 1994; Neufeld et al., 1998), while Dacapo is a specific inhibitor of this Cdk2-Cyclin E complex and thus inhibits the G1-S transition (de Nooij et al., 1996; Lane et al., 1996).
Chapter 4: Characterization of genetic interactions between Armadillo and cell cycle regulators

Three approaches were taken in order to assess the reproducibility, significance, and specificity of all candidate modifiers of the \textit{En}>CADi} phenotype that have been described thus far. First, the interaction tests between \textit{En}>CADi} and eight potential interactors (Dacapo, Cyclin E, Cdk1, Cyclin A, Cyclin B, Cyclin B3, String and Twins) were repeated. These were done simultaneously in conjunction with a suitable control so as to allow fair scoring of any phenotypic modification and valid comparison between interactors. Secondly, mutations were tested for their ability to dominantly modify a second wing phenotype in which the \textit{UAS-CADi} transgene was expressed from a different GALA driver. This was to ensure that the \textit{En}>CADi} interactions were not due to indirect perturbations of \textit{En-GALA} expression. Finally, some cell cycle regulators were over-expressed in the context of \textit{En}>CADi}, the prediction being that opposite interactions to those seen by decreasing gene dosage might be observed.

\subsection{4.3.2 Mutation of \textit{dacapo}, \textit{cyclin E}, \textit{cdc2}, \textit{cyclin A}, \textit{cyclin B}, \textit{cyclin B3}, \textit{string} or \textit{twins} dominantly enhances \textit{En}>CADi}}

Two different null or hypomorphic mutations of each gene were tested for interaction with \textit{En}>CADi} so as to confirm their specificity. Phenotypic modification was quantified by counting the number of bristles remaining on the posterior margin of the wing. The \textit{En}>CADi}^5 line, rather than the weaker \textit{En}>CADi}^{10} line, was used in these experiments as the former showed clearer interactions in the preliminary tests (Table 4.2 and not shown).

The results are displayed in Figures 4.1 and 4.3 (upper chart). Each of the mutations tested gives clear dominant enhancement of the \textit{En}>CADi} phenotype. The relative interaction strengths may be divided into two classes: mutation of \textit{dacapo}, \textit{cdc2} or \textit{cyclin B} gives strong dominant enhancement with both alleles tested, while mutations of \textit{cyclin E}, \textit{cyclin A}, \textit{cyclin B3} or \textit{twins} results in weaker dominant enhancement (Figure 4.3).
Figure 4.1  Mutation of genes encoding cell cycle regulators dominantly enhances the 
$En\textgreater CADi$ wing phenotype

A, $En\textgreater CADi^{+/+}$ control. Arrows indicate the few bristles that typically remain on the posterior margin. B-I, Wings heterozygous for $En\textgreater CADi^{3}$ and the mutation indicated Each shows a more severe phenotype than that in A. $dacapo^{4}$ and $string^{AB2}$ are amorphs; cyclin $E^{po28}$ is a strong hypomorph; cyclin $B^{1}$ and cyclin $B^{3}$ are defined as loss-of-function alleles (Flybase). See Table 4.1 for the class of the other mutations listed here. Wings heterozygous for the mutation alone have wild type morphology (not shown). Female wings that are representative of the range produced by each genotype are shown.
**Figure 4.2** Mutation of genes encoding cell cycle regulators dominantly enhances the C96>CADi wing phenotype

**A**, UAS-CADi°/+; C96-GAL4/+ control. Arrow indicates a notch in the posterior margin. **B-I**, Wings heterozygous for C96>CADi° and the mutation indicated. Each shows a more severe phenotype than that in **A**. *cyclin B2* and *cyclin B3*- are defined as loss-of-function mutations (Flybase). See Tables 4.1 and legend to Figure 4.1 for the class of the other mutations. Wings heterozygous for the mutation alone have wild type morphology (not shown). Female wings that are representative of the range produced by each genotype are shown.
Figure 4.3  Quantitative analysis of the dominant enhancement of CADi over-expression wing phenotypes by mutations in genes encoding cell cycle regulators

Upper chart  En>CADi interactions. The mean number of posterior wing margin bristles remaining between longitudinal veins 4-5 (L4-L5) is shown and gives a quantitative indication of the extent of phenotypic modification. Lower counts represent greater degrees of enhancement. Lower chart  C96>CADiΔ interactions. The mean number of notches in the wing margin is shown. This measure is a crude but effective indication of the extent of modification of the phenotype. Higher counts represent greater degrees of enhancement. Wings analyzed were heterozygous for En>CADiΔ or C96>CADiΔ and the mutation indicated. dacapo4414 is a hypomorph, cyclin E4895 is an amorph (Flybase). See Tables 4.1 and legends to Figures 4.1 and 4.2 for the class of the other mutations. Wings heterozygous for the mutation alone have wild type morphology (not shown). Error bars are 95% confidence intervals on the mean. Approximately 20 wings of each genotype and sex were analyzed from flies reared simultaneously in identical conditions.
**En>CADI** x cell cycle mutants

![Graph showing mean number of margin bristles (L4-L5) for different genotypes and sexes.](image)

**C96>CADI** x cell cycle mutants

![Graph showing mean number of notches for different genotypes and sexes.](image)
4.3.3 Mutation of dacapo, cyclin E, cdc2, cyclin A, cyclin B, string or twins also dominantly enhances C96>CADi

In order to discount the possibility that some, or all, of the genetic interactions described above might be caused by Engrailed-dependent effects on the expression of GAL4, I repeated the interaction tests using a similar wing phenotype generated using the C96-GAL4 driver. C96-GAL4 is expressed during the latter half of larval development in a band of cells that straddles the prospective wing margin and so can be used to generate phenotypes in the anterior and posterior of the wing margin in the adult fly (Gustafson and Boulianne, 1996; Johnston and Edgar, 1998). Again, all of these interaction tests were performed simultaneously in identical conditions, while the number of notches in the wing margin was used to measure the degree of phenotypic enhancement.

C96>CADi/+ wings display a weakly penetrant notching phenotype (Figures 4.2 and 4.3, lower chart). Mutation of seven of the eight candidate genes under investigation resulted in clear dominant enhancement of this phenotype, the exception being cyclin B3, which did not interact significantly in this system (Figure 4.3, lower chart). Consistent with their modification of En>CADi, mutation of dacapo or cdc2 gave a very strong dominant enhancement of the C96>CADi phenotype. In contrast, the enhancement of C96>CADi by cyclin B mutations was not as clear as with En>CADi, while mutation of string showed a more marked enhancement than seen with En>CADi.

Based on this secondary interaction test, I decided to remove Cyclin B3 from my list of cell cycle regulators that interact with CADi over-expression phenotypes. However, the other seven regulators show consistent genetic interactions with both En>CADi and C96>CADi and so are confirmed as robust interactors.
4.3.4 Over-expression of Dacapo or String suppresses En>CADi

As reducing the gene dosage of certain cell cycle regulators could reliably enhance CADi-associated phenotypes, I hypothesized that over-expression of these same regulators via UAS transgenes might cause phenotypic suppression. Such observations would support the thesis that these phenotypes are truly dosage-sensitive to specific factors.

For these experiments, I used En>CADi rather than C96>CADi as the latter phenotype is not sufficiently penetrant to detect suppression reliably. I tested four out of the seven candidate interactors for their ability to suppress En>CADi when over-expressed. Two different lines of each UAS construct were tested so as to confirm the significance of any interaction. The results are shown in Figures 4.4 and 4.5 (upper chart). Co-expression of either Dacapo or String dramatically suppressed the En>CADi phenotype compared to control wings. (This is consistent with my earlier observations that reducing the gene dosage of dacapo or string enhanced En>CADi.) These effects are not due to dilution of GAL4 as co-expression of either LacZ or GFP did not suppress En>CADi (Figures 4.4A and 4.5, upper chart). Co-expression of either Cyclin E or Cdk1 had no effect. As Cyclin E is thought to be the limiting factor for G1-S progression (Knoblich et al., 1994; Neufeld et al., 1998) increasing the amount of cyclin E transcripts is expected to augment its function. As mutation of cyclin E dominantly enhances En>CADi, the lack of phenotypic suppression upon over-expression of Cyclin E is therefore somewhat surprising. Contrariwise, the absence of effect upon co-expression of Cdk1 is not unexpected as Cdk1 protein is thought to be in excess for its cell cycle function in Drosophila cells (Edgar and O'Farrell, 1990; Edgar et al., 1994b; Neufeld et al., 1998).

In interpreting these data, it is crucial to consider the effect of En-GAL4-mediated expression of these cell cycle regulators alone (Figure 4.5, lower chart). Consistent with its inhibitory role at the G1-S transition, Engrailed-GAL4-driven expression of Dacapo results
Figure 4.4  Co-expression of Dacapo or String suppresses the En>CADi wing phenotype, while co-expression of Cyclin E or Cdc2 has no effect

A, En>CADi^+/UAS-GFP^{null} control.  B, E, En>CADi^+/UAS-Dap^{II,2} and En>CADi^+/UAS-Stg^{III,1} respectively. The phenotypes are suppressed compared to A.  C, D, En>CADi^+/UAS-CycE^{III,3} and En>CADi^+/UAS-Cdc2^{III,1} respectively. Wings are similar to that in A. Over-expression of Dacapo also results in fewer, larger cells in the posterior half of the wing (B), while over-expression of Cyclin E produces more, smaller cells within the posterior compartment (C). Female wings that are representative of the range produced by each genotype are shown.
Figure 4.5  Quantitative analysis of the effect of over-expressing cell cycle regulators on En>CADi and wild type wings

Upper chart  Co-expression of either Dacapo or String clearly suppresses the En>CADi^ wing phenotype. Higher counts represent phenotypic suppression. Analyzed wings were heterozygous for En>CADi^ and for the transgene indicated. Lower chart Over-expression of String increases, while over-expression of either Dacapo or Cyclin E decreases the number of posterior margin bristles. Analyzed wings were heterozygous for En-GAL4 and for the transgene indicated. The mean number of posterior wing margin bristles between longitudinal veins 4-5 (L4-L5) is shown. Error bars are 95% confidence intervals on the mean. 10 wings of each genotype and sex were analysed from flies reared simultaneously in identical conditions.
En-GAL4 x UAS-[cell cycle regulator]

Mean no. of margin bristles (L4-L5)

- UAS-GFP[nls]
- UAS-Dap[II.2]
- UAS-Dap[III.1]
- UAS-CycE[II.1]
- UAS-CycE[III.3]
- UAS-Cdc2[II.1]
- UAS-Cdc2[III.1]
- UAS-Stg[II.7]
- UAS-Stg[III.1]

Females □ Males

Mean no. of margin bristles (L4-L5)

- WT
- UAS-LacZ[3a]
- UAS-GFP[nls]
- UAS-Dap[II.2]
- UAS-Dap[III.1]
- UAS-CycE[II.1]
- UAS-CycE[III.3]
- UAS-Cdc2[II.1]
- UAS-Cdc2[III.1]
- UAS-Stg[II.7]
- UAS-Stg[III.1]

Females □ Males
in fewer, larger cells, and consequently fewer margin bristles, throughout the posterior wing (not shown and Figure 4.5, lower chart). This cell cycle defect is retained when Dacapo is co-expressed in En>CADi wings and makes the suppression of this ‘tissue loss’ phenotype even more remarkable (Figure 4.4B). In contrast En>Cyclin E wings have more, smaller cells in the posterior compartment of the wing (not shown). This demonstrates that over-expressed Cyclin E failed to suppress the En>CADi phenotype despite a marked augmentation in its cell cycle function. However, a small degree of phenotypic suppression may have been masked as En>Cyclin E wings exhibit a loss of margin bristles (Figure 4.4, lower chart), maybe because their specification or differentiation is inhibited by the forced induction of extra cell cycles.

Consistent with its being rate-limiting for mitotic induction, over-expression of String produces more marginal bristles and, by extension, increases the number of cells in the posterior wing (Figure 4.5, lower chart). Previous studies found that En-GAL4-driven expression of String did not increase cell number by the end of larval development (Neufeld et al., 1998), and so these extra cell divisions presumably occur during pupal stages.

As Cdk1 is present in cellular excess, it is not surprising that En>Cdc2 wings do not have a dominant phenotype.

In summary, these over-expression studies clearly establish that the cellular levels of Dacapo and String are critical in determining the response to CADi over-expression.

4.3.5 Summary and discussion

Mutation of dacapo, cyclin E, cdc2, cyclin A, cyclin B, string or twins dominantly enhanced both the En>CADi and C96>CADi wing phenotypes in a robust and reproducible manner. Of these, mutation of dacapo or cdc2 exhibited the strongest interactions. Significantly, co-expression of either Dacapo or String suppressed En>CADi wings to near wild type
morphology. Taken together, these data implicate Dacapo and String/Cdk1 as potent modulators of the consequences of CADi over-expression, such that Wingless-Armadillo signalling is potentiated by their activity.

The results reported in this section support the earlier proposition that passage through mitosis in general, or activation of the Cdk1 kinase in particular, might promote the signalling function of Armadillo in some fashion. The observations that mutation of cdc2, cyclin A, cyclin B or string dominantly enhances En>CADi and C96>CADi, and that over-expression of String almost rescues the En>CADi phenotype to wild type are particularly persuasive.

It is striking that Dacapo, an inhibitor of proliferation and cell cycle progression, should interact with En>CADi in the manner described. The observation that over-expression of Dacapo suppresses the En>CADi 'tissue loss' phenotype in such an effective manner is particularly remarkable. However, the exact significance of this robust interaction is unclear as heterozygosity for mutation of either cyclin E or dacapo was found to enhance both the En>CADi and C96>CADi phenotypes. The fact that both the major inducer of S phase (Cyclin E) and its cognate inhibitor (Dacapo) interact similarly may indicate that these genetic interactions do not reflect wild type functions of these proteins. Alternatively, this inconsistency could be explained if Dacapo had a role in promoting G1 progression at low concentrations, as has been described for its mammalian counterparts (Sherr and Roberts, 1999), although such a function has not yet been reported in Drosophila. Dacapo may also have additional functions at a later stage in the cell cycle as it is expressed in cells that arrest in G2 (de Nooij et al., 1996). This latter finding suggests the possibility that the interaction between En>CADi and Dacapo/Cyclin E might even be connected with the several interactions observed with the known regulators of G2-M.

Taken at face value, the finding that mutation of seven different cell cycle regulators clearly enhanced the En>CADi phenotype in a dominant fashion is rather surprising, as the
wild type functions of these regulators vary considerably with regard to mechanism and time of action within the cell cycle. Moreover, of some fifteen cell cycle regulators tested in these studies, no mutations that reliably suppressed En>CADi were identified. It may be that the wing phenotypes used here are simply more amenable to enhancement than suppression, or that there are few/no cell cycle regulators that negatively affect Armadillo function. However, a more worrisome possibility is that CADi-associated phenotypes are very sensitive to non-specific modification, as may be demonstrated by the interaction of Cyclin E and Dacapo discussed above.

4.4 String shows robust genetic interactions with Armadillo over-expression wing phenotypes

4.4.1 Introduction
Preliminary studies indicated that three different cell cycle regulators might interact genetically with the En>Arm phenotype: Dacapo, Cyclin E, and String (not shown and Table 4.2). In order to assess the significance of these observations, I used tests similar to those employed for confirming genetic interaction with En>CADi (section 4.3).

4.4.2 cyclin E and dacapo show inconsistent interactions with En>Arm
In my preliminary tests, heterozygosity for either of two dacapo mutations (including the amorphic dacapo allele) appeared to enhance the En>Arm phenotype, while heterozygosity for the amorphic cyclin E allele seemed to suppress this phenotype (not shown). These opposing genetic interactions are consistent with Dacapo and Cyclin E having opposing functions in the G1-S transition in the cell cycle. I therefore hypothesized that control of cell cycle entry at G1-S might have functional consequences on Armadillo
activity. To this end, I repeated the original interaction tests between En>Arm and mutations in dacapo or cyclin E. I also studied the effect of over-expressing these factors in the context of En>Arm, using two different UAS transgenes in each case. These tests were performed simultaneously in identical conditions.

As shown in Figure 4.6, these retests did not confirm dacapo mutations as dominant enhancers of En>Arm, but rather found that they had no significant modifying effect. Likewise, co-expression of Dacapo using the UAS-Dap\textsuperscript{II,2} construct had no effect (Figure 4.6). Strangely, co-expression of Dacapo via the weaker UAS-Dap\textsuperscript{III,1} transgene enhanced the phenotype compared to control wings (Figure 4.6).

Heterozygosity for either of two amorphic cyclin E mutations suppressed the En>Arm wing phenotype. However, co-expression of Cyclin E also suppressed En>Arm. This latter finding is inconsistent with a simple model in which the level or function of Cyclin E modulates Armadillo function, although the dominant effects of over-expressed Cyclin E on margin bristles (section 4.3.4) could feasibly have masked subtle positive effects on Armadillo activity.

In conclusion, Dacapo does not interact simply or reproducibly with En>Arm, while Cyclin E does not interact robustly with En>Arm. In light of these ambiguous interactions, Dacapo and Cyclin E were not studied further in this context.

### 4.4.3 String gives robust genetic interactions with En>Arm

The strongly hypomorphic string\textsuperscript{78} allele was identified as a dominant enhancer of the En>Arm phenotype in the original screen by Greaves et al. (1999; Table 4.1). Consistent with this, heterozygosity for each of three different string mutant alleles clearly enhanced En>Arm in my preliminary experiments (Table 4.2). Despite these consistencies, it was still important to demonstrate formally that String interacted genetically with En>Arm in a reproducible and robust manner before undertaking further analyses.
The mean number of posterior wing margin bristles remaining between longitudinal veins 4-5 (L4-L5) is shown. Lower counts represent suppression of the phenotype; higher counts represent enhancement. Wings analyzed were heterozygous for En>Arm\textsuperscript{hs} and for the mutation or transgene indicated. \textit{dap\textsuperscript{a}} and \textit{cycE\textsuperscript{Arg}} are amorphs; \textit{dap\textsuperscript{hs}} and \textit{cycE\textsuperscript{28}} are hypomorphs. Wings heterozygous for the mutation alone have wild type morphology (not shown). Error bars are 95% confidence intervals on the mean. 12 female wings of each genotype were analyzed from flies reared simultaneously in identical conditions.
Seven different *string* mutant alleles were tested in parallel for their ability to dominantly modify the *En>Arm* wing phenotype. Six of these, including the amorphic *string*\textsuperscript{AR2} allele, and also two genomic deletions that remove the *string* locus, each strongly enhanced *En>Arm* when heterozygous (Figures 4.7 and 4.9). The amorph and Deficiencies were among the strongest interacting alleles, as would be expected if String function is an important determinant of Armadillo activity. Exceptionally, the, *string*\textsuperscript{6A} mutation dominantly suppressed the *En>Arm* phenotype (Figure 4.9). The reason for this is not apparent (especially as this allele acts as a dominant enhancer of another Armadillo over-expression wing phenotype- see next section), though it could feasibly be related to the temperature-sensitivity of this allele (Neufeld et al., 1998).

As before, I reasoned that if the *En>Arm* phenotype was specifically sensitive to the levels of String, then increasing String levels via an *UAS-String* transgene might suppress *En>Arm*. As shown in Figures 4.7 and 4.9, this is indeed the case for each of two different *UAS-String* lines. *En>String* control wings are phenotypically wild type (Figure 4.7E), although they do possess extra bristles within their endogenous posterior margin (Figure 4.5, lower chart). This latter point suggests that the reduction of ectopic bristle number upon co-expression of String in *En>Arm* wings is not an artefact of over-expression.

Taken together, these data indicate that the *En>Arm* phenotype is specifically and acutely sensitive to String levels.

**4.4.4 String also gives robust genetic interactions with *C96>Arm***

In order to demonstrate that the strong genetic interaction between String and *En>Arm* were not due simply to modification of *Engrailed*-mediated GAL4 expression, I tested whether a *C96>Arm* phenotype was sensitive to changes in the levels of String.

*C96>Arm* wings have ectopic bristles in their wing blades near to the margin (Figure 4.8A). Significantly more ectopic bristles are produced when such wings are also
Figure 4.7  Changing the dosage of string modifies the En>Arm wing phenotype

A, B, En>Arm^{16}/+ and En>Arm^{16}/+; UAS-LacZ^{68}/+ controls respectively. Arrows in A indicate ectopic bristles in the posterior wing blade. C, En>Arm^{16}/+; stg^{AB2}/+. Notice the increase in the number of ectopic bristles compared to A. stg^{AB2} is an amorph; wings heterozygous for stg^{AB2} alone have wild type morphology. D En>Arm^{16}/UAS-Stg^{II}.7. Notice the clear suppression of the phenotype shown in B. E, En-GAL4/+; UAS-Stg^{III}/+. En-GAL4-mediated expression of String alone produces a morphologically wild type wing. Female wings that are representative of the range produced by each genotype are shown.
Figure 4.8 Changing the dosage of string modifies the C96>Arm wing phenotype

A, C96>Arm^{23}/+ control. Arrow indicates an ectopic bristle. B, C96>Arm^{23}/UAS-LacZ^{64} control. These wings typically have more ectopic bristles than C96>Arm^{23}/+. C, C96>Arm^{23}/stg^{AR2}. Notice the large increase in the number of ectopic bristles compared to A. stg^{AR2} is an amorph; wings heterozygous for stg^{AR2} alone have wild type morphology. D, UAS-Stg^{II.7}/+; C96>Arm^{23}/+. Notice the slight suppression of the phenotype shown in B. E, UAS-Stg^{II.7}/+; C96-GAL4/+. C96-GAL4-mediated expression of String alone produces a morphologically wild type wing. Female wings that are representative of the range produced by each genotype are shown.
Figure 4.9  Quantitative analysis of the modification of Armadillo over-expression wing phenotypes by changing the dosage of string

Both wing phenotypes are enhanced by heterozygosity for string and suppressed by co-expression of String. **Upper chart** The mean number of ectopic bristles in the posterior wing is shown. **Lower chart** The mean number of ectopic bristles between longitudinal veins 2 and 4 (L2-L4) is shown. Higher counts represent phenotypic enhancement; lower counts represent suppression. Wings analyzed were heterozygous for En>Arm\textsuperscript{16} or C96>Arm\textsuperscript{23} and the deficiency, mutation or transgene indicated. Df(3R)3450 and Df(3R)01215 are genomic deletions that uncover the string locus. stg\textsuperscript{Ar2} is an amorph, stg\textsuperscript{78} and stg\textsuperscript{8A} are strong hypomorphs, and stg\textsuperscript{7M}, stg\textsuperscript{9K} and stg\textsuperscript{13D} are weak hypomorphs (classifications according to Flybase). The reason why co-expression of LacZ or GFP enhances the C96>Arm phenotype is not known. Wings heterozygous for the deficiency or mutation alone have wild type morphology (not shown). Error bars are 95% confidence intervals on the mean. 12 female (En>Arm) or 24 female and male (C96>Arm) wings of each genotype were analyzed from flies reared simultaneously in identical conditions.
Effect of changing *string* dosage on *En>Arm*

Effect of changing *string* dosage on *C96>Arm*
made heterozygous for any one of seven *string* alleles or each of two genomic deletions that uncover the *string* locus (Figures 4.8 and 4.9). In contrast, co-expression of String gives a weak, but clear, suppression of the *C96>Arm* phenotype compared to control wings (Figures 4.8 and 4.9). These interactions are very similar to those seen with *En>Arm* except that the temperature sensitive *string<sup>^n</sup>* allele now behaves in a manner similar to the other *string* mutations and dominantly enhances the *C96>Arm* phenotype.

Taken together, these data indicate that String is a *bona fide* dosage sensitive modifier of the *C96>Arm* phenotype.

### 4.4.5 Genetic interactions of negative regulators of String function with *En>Arm* and *C96>Arm*

The *tribbles* and *Dweel* loci each encode a negative regulator of String function (see Chapter 1.3.2). Tribbles is thought to act by specifically targeting String for proteolysis (Mata *et al*., 2000), while Dweel is a kinase that phosphorylates and inhibits the String target, Cdk1 (Campbell *et al*., 1995). As *string* mutations dominantly enhance *En>Arm* wings, mutation of either *tribbles* or *Dweel* might be expected to suppress this phenotype. Such observations would add significant support to the functional significance of the String-Armadillo interaction.

These predictions were not upheld. Instead, dominant enhancement of *En>Arm* was observed upon heterozygosity for the *Dweel<sup>ES2</sup>* allele or for either of two Deficiency chromosomes that remove *tribbles* (Figure 4.10). Moreover, co-expression of either *tribbles* or *Dweel* in the context of *En>Arm*, which might be expected to enhance the phenotype, actually resulted in phenotypic suppression (Figure 4.10).

I sought to confirm these rather surprising data by testing for the ability of mutation of either *tribbles* or *Dweel* to dominantly modify the *C96>Arm* phenotype (Figure 4.11). As was found for *En>Arm*, heterozygosity for either of two genomic deletions that remove
the *tribbles* locus enhanced the $C96>Arm$ phenotype. However, co-expression of Tribbles had no effect on $C96>Arm$. Both of the $Dweel$ mutant alleles tested were dominant enhancers of $C96>Arm$, although the effect was weak and was only seen in females. Consistent with this and the interactions with $En>Arm$, co-expression of $Dweel$ suppressed the phenotype.

Although these results at first appear to cast doubt on the functional relevance of the String-Armadillo interaction, they are subject to certain caveats. Firstly, the phenotypic modifications caused by different alleles/Deficiencies were not 'internally consistent'. For example, heterozygosity for $Dweel^{ES2}$ but not $Dweel^{ES1}$ enhanced $En>Arm$, and the dominant enhancement of $C96>Arm$ caused by heterozygosity for for $Df(3L)ri-79c$ was much stronger than for $Df(3L)rdg-co2$. As each $Dweel$ allele is a null mutation (Price et al., 2000), and as both Deficiency chromosomes completely remove the *tribbles* locus, the extent of interaction ought to have been similar in each case if the interaction was caused specifically by a reduction in the dosage of these genes. In this respect it is pertinent that both Deficiency chromosomes used to uncover *tribbles* also remove the *Drosophila-pesenilin* locus (Cox et al., 2000). As D-presenilin normally functions to lower Armadillo levels itself (Cox et al., 2000; Noll et al., 2000), the inclusion of its genetic locus in these deletions makes them an ineffective tool for assessing the specific effect of halving *tribbles* dosage on $En>Arm$. A second caveat is that $En-GAL4-driwen$ expression of Tribbles or $Dweel$ alone reduces the number of endogenous margin bristles (not shown). These dominant phenotypic effects may obscure 'true' genetic interactions in the $En>Arm$ wings. This hypothesis is supported somewhat in the case of *tribbles* as $C96-GAL4$-mediated expression of Tribbles does not result in a dominant phenotype (not shown) and co-expression of Tribbles in the $C96>Arm$ background had no modification effect. However, co-expression of $Dweel$ in the context of $C96>Arm$ suppressed the phenotype despite the lack of any dominant effect of $C96-GAL4$-driven expression of $Dweel$ alone (not shown).
Effect of changing *tribbles* or *Dweel* dosage on *En>Arm*

**Figure 4.10** Quantitative analysis of the modification of the *En>Arm* wing phenotype by changing the gene dosage of regulators of String function.

The mean number of ectopic bristles in the posterior wing is shown. Higher counts represent phenotypic enhancement; lower counts represent suppression. Wings analyzed were heterozygous for *En>Arm* and the deficiency, mutation or transgene indicated. *Df(3L)ri-79c* and *Df(3L)rdg-co2* uncover *tribbles*; *Dweel* and *Dweel* are amorphs. Wings heterozygous for the mutations or deficiencies alone have wild type morphology (not shown). Error bars are 95% confidence intervals on the mean. At least 10 female wings of each genotype were analyzed and compared to controls from flies reared simultaneously in identical conditions.
**Figure 4.11** Quantitative analysis of the modification of the C96>Arm wing phenotype by changing the dosage of tribbles or Dweel.

The mean number of ectopic bristles between longitudinal veins 2 and 4 (L2-L4) is shown. Higher counts represent phenotypic enhancement; lower counts represent suppression. Wings analyzed were heterozygous for C96>Arm and the deficiency, mutation or transgene indicated. DweelES1 and DweelES2 are amorphs; Df(3L)ri-79c and Df(3L)rdg-co2 uncover tribbles. Wings heterozygous for the mutation or deficiency alone have wild type morphology (not shown). Error bars are 95% confidence intervals on the mean. 24 wings of each sex and genotype were analyzed from flies reared simultaneously in identical conditions.
Finally, Dweel function has been found to be largely dispensable for *Drosophila* development (Price et al., 2000), suggesting that inhibitory phosphorylation of Cdk1 by Dweel is not significant, or that there is functional redundancy. Furthermore, it is formally possible that the effect of String on Armadillo function does not operate via Cdk1 at all. If this were so, it would render the effects of manipulating Dweel levels on the Armadillo-sensitized phenotypes irrelevant or indirect.

In conclusion, genetic interactions between Dweel or Tribbles and Armadillo are similar, albeit weaker, to those observed between String and Armadillo. This is seemingly incompatible with any of these cell cycle regulators being relevant to Armadillo function *in vivo* as they have opposing effects on String/Cdk1 activity and thus induction of mitosis. However, there are several important caveats to the significance of the Tribbles and Dweel interactions with Armadillo that may well render them irrelevant or indirect.

### 4.4.6 Summary and discussion

Mutation of *dacapo* appeared to dominantly enhance, while mutation of *cyclin E* seemed to dominantly suppress the *En>Arm* phenotype in preliminary tests, suggesting a possible connection between passage from G1 phase to S phase and Armadillo function. However, I could not substantiate these postulates by more thorough analyses conducted under well-controlled conditions.

Heterozygosity for several different *string* mutations clearly enhanced both *En>Arm* and *C96>Arm* phenotypes, while co-expression of String in either background suppressed these phenotypes. These results establish String as a specific, dosage-dependent inhibitor of Armadillo function in Wingless signalling, and therefore associates induction or passage through mitosis with low Armadillo activity.

Bizarrely, genetic interactions between Armadillo and Dweel or Tribbles were found to be similar to those of Armadillo and String. However, the latter interaction is stronger,
more robust in several different tests, and is subject to fewer caveats. By these criteria, I considered that the interaction between String and Armadillo was still worthy of further analysis (see Chapter 5).

It is notable that the \textit{En>Arm} phenotype was not obviously enhanced by mutation of \textit{cdc2}, \textit{cyclin A} or \textit{cyclin B} (Tables 4.2 and 4.3) considering the very strong interactions shown between String and \textit{En>Arm}. However, these cell cycle regulators are thought to be present in excess for their role at G2-M, whereas String is limiting for this phase transition and its activity is primarily regulated through a burst of transcription shortly before mitosis (Edgar and O'Farrell, 1990; Neufeld \textit{et al.}, 1998). Therefore, one might expect that G2-M ought to be very sensitive to \textit{string} dosage, but relatively insensitive to the dosage of \textit{cdc2} and the mitotic Cyclins.

\section*{4.5 Summary and discussion}

\begin{itemize}
  \item \textit{cyclin E, dacapo, cdc2, cyclin A, cyclin B, string} and \textit{twins} each show dosage-dependent interactions with CADi over-expression wing phenotypes
  \item These genetic interactions are consistent with their wild type protein products having a positive effect on Armadillo function in Wingless signalling
  \item Dacapo, Cdk1 and String show the most robust interactions with CADi based on both loss- and gain-of-function data and on the strength of their interactions with two different CADi-associated phenotypes
  \item The finding that several promoters of mitosis interact similarly with CADi indicates that a mitotic-specific activity may up-regulate Armadillo function
\end{itemize}
Chapter 4: Characterization of genetic interactions between Armadillo and cell cycle regulators

- String is the only cell cycle regulator tested that shows robust genetic interactions with Armadillo over-expression wing phenotypes.
- In contrast to the conclusions above, this finding suggests that there is a mitotic-specific activity that down-regulates Armadillo function (see Chapter 5).

A summary of all the genetic interactions uncovered between cell cycle regulators and Armadillo is presented in Table 4.3. These are also shown in the context of their cell cycle role in Figure 4.12. Other regulators of S phase entry (Rbf, E2f), of G1 progression (Roughex, Fizzy-related) and G2 progression (Regulator of Cyclin A1) were also tested, but failed to give consistent or significant interactions (not shown). However, the lack of genetic interaction in these tests does not necessarily imply absence of a relevant functional relationship in wild type cells as some factors will be functionally redundant or present in excess such that they go undetected in these tests. Several other important regulators of the Drosophila cell cycle were not tested for genetic interaction with Armadillo in this system because of time constraints or the unavailability of mutations.

Does each cell cycle regulator interact independently with Armadillo?

It is important to remember that all of the cell cycle regulators identified as interactors really do form part of a regulatory cycle. As such, perturbation in the expression of a single regulator is likely to have many secondary effects on the expression and function of the others. From this standpoint, it is difficult to judge the independence of the different interactions reported here. In an extreme model, there might be only one cell cycle regulator that interacts directly with En>CADi, with all other interactions being indirect and acting through their effects on this single ‘true’ interactor. There is no simple way of assessing the likelihood of this scenario.
Table 4.3  Summary of genetic interactions between Armadillo and cell cycle regulators

This table attempts to synthesize all the data presented in this chapter. However, for clarity, I have listed only the phenotypic modification observed upon halving gene dosage in the $En>CADi$ and $En>Arm$ backgrounds. +/++/+++ weak/strong/very strong enhancement; 0 no interaction; ? interaction is ambiguous; n.t. not tested. fizzy could be added to this table based on preliminary data, but its interaction was not checked thoroughly in controlled tests.

Figure 4.12  Armadillo-cell cycle interactions in context

The major factors known to regulate the Drosophila cell division cycle are shown. Green arrow: activation; red line: inhibition; no line shown: promotes general cell cycle progression. Black text: not (directly) tested; orange text: interacts with Armadillo in this study; purple text: does not interact (robustly) with Armadillo in this study. See legend to Figure 1.4.
Table 4.3

<table>
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<th>Cell cycle regulator</th>
<th>Modification of En&gt;CADi</th>
<th>Modification of En&gt;Arm</th>
<th>Implied effect on Arm function in WT</th>
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</thead>
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<td>dacapo&lt;sup&gt;++&lt;/sup&gt;</td>
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<td>0</td>
<td>Positive</td>
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Figure 4.12

![Cell cycle diagram](image)
Whether independent or interrelated, there is no a priori reason that these interactions represent a common cell cyclical phenomenon. For example, some interactions may be caused by cell cycle factors being targets of Wingless signalling, other cell cycle regulators may interact because of non-cell cycle regulatory roles (Gao and Zelenka, 1997), and some might be the result of there being shared regulatory components (see below). Likewise, these genetic data do not necessary implicate Armadillo itself as the primary target of these interacting factors: for example, a change in the activity or localization of Shaggy, Dishevelled or *Drosophila* E-cadherin would also be expected to modify the sensitized wing phenotypes through their effects on Armadillo (see Figure 1.6).

*How does changing the effective gene dosage of a cell cycle regulator affect its function?*

Heterozygosity for a loss-of-function mutation in a gene presumably reduces the rate of production or the absolute number of transcripts at the time(s) when that gene is normally expressed. For a cell cycle regulator whose levels are regulated primarily at the transcriptional level and/or which is limiting for a particular function (for example, String), reducing its gene dosage is likely to impair its normal function. Thus a particular phase transition may be delayed or Cdk substrates might be phosphorylated to suboptimal levels. However, reducing the dosage of some genes may not affect the function of the protein they encode if that protein is functionally redundant or is not limiting for the biological process in which it takes part. Thus the lack of phenotypic modification upon reducing the dosage of a gene cannot be taken to indicate that it has no relationship to the process under investigation.

Over-expression of cell cycle regulators is a far less subtle method of manipulating effective gene dosage. *GAL4-UAS*-based over-expression presumably results in transcripts and protein of the regulator being present at high levels through the cell cycle. This can cause aberrant cell cycle phasing, anomalous cell deaths and dominant phenotypes (e.g.
Figure 4.5, lower chart; Neufeld et al., 1998). It is also likely to result in gain-of-function effects and abnormal cross-talk between regulators that would not normally be co-expressed. Furthermore, over-expression may result in over-loading of transcriptional, translational or proteolytic machinery, and produce genetic interactions unrelated to any cell cycle regulatory role. However, the advantage of over-expression is that it can reveal relevant interactions when reducing gene dosage does not (see above).

Throughout these interaction studies, I used heterozygosity as an initial, relatively subtle method to perturb cell cycle regulator function and thus identify factors that might have relevant effects on Armadillo function in vivo. Only then was GAL4-UAS-based over-expression used to prove the dosage-dependence and specificity of a particular interactor.

Can genetic interactions with En>CADi and En>Arm be compared directly?

At one level, the En>CADi and En>Arm wing phenotypes can be regarded as ‘opposites’ with respect to Armadillo function in Wingless signalling and thus serve as a useful filter for selecting robust interactors (Greaves et al., 1999; see Chapter 1.5). In this view, a bona fide interactor should dominantly enhance one phenotype and concomitantly suppress the other. How reasonable is this rationale? The extent to which over-expressed Armadillo protein (as in En>Arm wings, for example) accumulates is presumably dependent on a cell’s proximity to a source of Wingless (see Chapter 1.5.1); only cells that receive high levels of Wingless can stabilize this extra Armadillo to sufficient levels to hyper-activate target genes. Over-expression of Armadillo in the developing wing therefore results in many supernumerary bristles near to adult wing margin but fewer in the blade, consistent with the expression of Wingless at the prospective margin in the imaginal wing disc (see Chapter 1.2.1).

Over-expression of CADi results in cytoplasmic Armadillo accumulating at cell junctions in stable complexes with CADi molecules. Assuming CADi is expressed in large
excess, significant amounts of cytoplasmic Armadillo are sequestered at the cell membrane regardless of the cell’s proximity to the source of Wingless. In the wing, these effects result in loss of the wing margin and adjacent blade material.

Therefore, while the cellular effects of over-expressing either wild type Armadillo or CADi may be considered as opposite at the level of Wingless signalling, they are not so with respect to how they modulate Armadillo localization, stability and protein levels. This is a crucial point, especially since the mechanisms that regulate the exchange between cadherin-bound and cytoplasmic Armadillo are not understood (see Chapter 1.2.5). Furthermore, the associated wing phenotypes must develop through very different means: over-expression of Armadillo causes discrete cell fate changes, while over-expression of CADi results in gross tissue loss or under-proliferation of cells. Thus, depending on the exact nature of a factor’s molecular-cellular interaction with Armadillo, it may not be reasonable to expect it to cause complementary dominant modifications of the En>CADi and En>Arm phenotypes. Indeed, of all the cell cycle regulator interactions tested in this study, only mutations in cyclin E behaved in such a fashion, while most enhanced the En>CADi phenotype and had no detectable effect on En>Arm. Moreover, String showed similar genetic interactions with both En>CADi and En>Arm, apparently indicating that execution of mitosis can both promote and inhibit the function of Armadillo in signalling when both clearly cannot be true (see Chapter 5).

Can functional relationships be deduced from genetic interaction data?

It is important to remember that the genetic interactions identified here represent only potential functional interactions during wild type development. Does Wingless-Armadillo signalling really link to cell cycle regulation at the molecular-cellular level? How might this occur and how direct is the connection? Several possibilities exist and are presented in Figure 4.13.
Chapter 4: Characterization of genetic interactions between Armadillo and cell cycle regulators

It is especially necessary to be cautious of the wild type significance of the interactions identified here for at least three reasons. First, several of the interactions were identified by a candidate gene approach, rather than in an unbiased screen. Second, the sensitized phenotypes used were generated by over-expression and in one case utilized a recombinant protein. Third, genetic interaction was scored in the adult wing and therefore actually represents potentially several relevant interactions that occurred during development. It is therefore necessary to investigate candidate interactions at the cellular-molecular levels at earlier stages of development in order to assess their functional significance. This was done for the String-Armadillo interaction and is described in the next Chapter.
Figure 4.13 Models for how Armadillo may interact with the cell cycle during *Drosophila* wing development.

**A, B, 'Direct' mechanisms.** A, Cell cycle regulators are downstream targets of Wingless signalling. B, Aspects of the cell cycle or individual regulators affect Wingless signal transduction directly.

**C, D, 'Indirect' mechanisms.** C, Wingless signalling and cell cycle regulation have independent roles during normal wing development that converge downstream. D, The Wingless pathway and cell cycle regulators have completely independent functions but genetic interactions are uncovered as a result of shared regulatory mechanisms that become limiting in Armadillo-senstized backgrounds. Wg, Wingless; Arm, Armadillo.
## Chapter 5: Analysis of the genetic interaction between Armadillo and String in wing imaginal discs

"Say what you see"

Roy Walker, Catchphrase (television quiz show)

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Chapter 5: Analysis of the genetic interaction between Armadillo and String in wing imaginal discs

5.1 Introduction

What is the cellular or developmental basis for the robust genetic interaction between String and Armadillo in the *Drosophila* wing? Is Wingless-Armadillo signalling regulated by String/mitosis or does String act downstream of or in a parallel pathway to Wingless-Armadillo to affect bristle number in the adult wing (Figure 4.13)? If the former is true, on what component of the Wingless pathway does String act? Or is junctional Armadillo the primary target of String? Furthermore, when in development does the relevant interaction occur? It is possible that the interaction revealed in adult wings is the additive effect of perturbing String and Armadillo at different developmental times, rather than reflecting a functional relationship.

This chapter describes my attempts to answer these questions in *En>*Arm and *C96>*Arm imaginal wing discs, the larval primordia of the adult wings (Figure 5.1). I find a strong negative correlation between String activity and cytoplasmic Armadillo levels. It appears that this effect occurs through promotion of Armadillo proteolysis as non-degradable Armadillo is not sensitive to *string* dosage. Consistent with the cell cycle regulatory role of String, mitotic cells have relatively low cytoplasmic Armadillo in *En>*Arm wing discs, while heat-shock induction of mitoses reduces Armadillo-positive cell number. Finally, analysis of a GFP-Armadillo protein in wild type discs reveals that both cytoplasmic and junctional Armadillo is degraded at mitosis. The cell biological consequences and reasons for this phenomenon are discussed.
**Figure 5.1**  Adult wings develop from larval imaginal wing discs

The upper image shows a late third instar imaginal wing disc and the lower image depicts the adult wing that it produces after metamorphosis (see Blair, 1995 and references therein). The ‘wing pouch’ (marked as the ‘wing blade’ in the upper image) develops into the wing proper while the remainder of the disc gives rise to the body wall of the mesothorax (not shown). The D-V boundary of the wing pouch is the prospective wing margin and is indicated by the dotted line. Cells at the D-V boundary secrete Wingless, which acts to pattern the margin and stimulate disc growth. Brown marks the posterior (P) compartment, as defined by the posterior-specific expression of *engrailed*, and yellow marks the anterior (A). The blue line shows the boundary of the A-P lineage compartments. However, in late discs and pupal wings, *engrailed* expression spreads slightly into the ‘anterior’ compartment where it determines the point of transition between innervated and uninnervated bristles in the adult wing margin. ‘X’ marks the intersection of the D-V and A-P boundaries on both the disc and the wing. Image modified after Blair, 1995.
5.2 Changes in string dosage affect wild type Armadillo expression in sensitized wing discs

5.2.1 Introduction

A simple immunodetection approach was used as a starting point to study the cellular relationship underlying the genetic interaction between Armadillo and String in En>Arm and C96>Arm imaginal wing discs. Anti-Armadillo labelling was chosen as a read-out of Wingless/Armadillo signalling because its relative activity constitutes the pivotal step in Wingless transduction and this activity is primarily determined by stabilization of the cytoplasmic Armadillo protein. Thus, if String affects Wingless signalling via Armadillo itself or some upstream member of the pathway, then changes in Armadillo stabilization ought to be observed. This method should also address whether Armadillo and String functionally interact in the same spatio-temporal context.

To gauge whether this approach might be effective, it was first necessary to view the expression of Armadillo in the En>Arm and C96>Arm backgrounds (Figure 5.2). In En>Arm wing discs, Armadillo is over-expressed throughout the posterior of the wing disc, but is only maintained in the cytoplasm of a subset of these cells, particularly those close to the prospective wing margin where high levels of secreted Wingless stabilizes the Armadillo protein (Figure 5.2B). In C96>Arm wing discs, Armadillo is over-expressed in a broad band of cells centred on the prospective wing margin. Cytoplasmic protein is maintained in the middle of this band, in those cells receiving the highest levels of Wingless (Figure 5.2C).
Figure 5.2  Armadillo expression in wild type, En>Arm, and C96>Arm imaginal wing discs

A, wild type. Armadillo localizes to the membrane of all wing disc cells. However, it is highly expressed and stabilized in the cytoplasm of cells that border the source of Wingless at the prospective margin (arrowheads).  B, En>Arm\textsuperscript{16}/+. Expression of Armadillo throughout the posterior of the disc results in many more cells with high levels of cytoplasmic Armadillo (arrows), particularly those adjacent to the source of Wingless.  C, C96>Arm\textsuperscript{23}/+. Expression of Armadillo in a band of cells straddling the prospective wing margin also increases the number of cells with high cytoplasmic Armadillo levels (arrows). These and most subsequent immunofluorescence images are projections of two confocal sections of the apical surface of the wing disc epithelium- see Chapter 2.4.2. Anterior is to the left and dorsal is up in these and all subsequent images of wing discs. All wing discs shown in this thesis were dissected from ‘wandering’ late third instar larvae.
5.2.2 String has dosage-dependent effects on cellular Armadillo levels in imaginal discs

The amorphic string^AR2 allele was used to study the effects of reducing string dosage on the En>Arm and C96>Arm disc phenotypes. Halving the gene dosage of string in this way resulted in a modest but reproducible increase in Armadillo expression levels across the posterior of En>Arm discs, as revealed by the increased number of cells that maintain high levels of cytoplasmic Armadillo (Figure 5.3D). In contrast, co-expression of String dramatically reduced cytoplasmic Armadillo levels in En>Arm discs, as reflected by the marked decrease in Armadillo-positive cells (Figure 5.3E). Very similar effects were observed in C96>Arm discs in which string dosage was manipulated (Figure 5.4). These findings are entirely consistent with the genetic interactions observed in adult wings and clearly demonstrate that changes in string dosage affect the expression or stabilization of the Armadillo protein. Moreover, they indicate that String acts as a negative regulator of Armadillo protein levels.

5.2.3 Summary and discussion

The results described in this section establish that the Armadillo-String interaction initially identified in adult wings is also apparent in larval imaginal wing discs. They also demonstrate that the interaction in adult wings is not due to anachronistic, additive effects during development, but rather that it could reflect functional consequences at the cellular level. Furthermore, these data show that String antagonizes Wingless signalling upstream or at the level of Armadillo itself such that cytoplasmic Armadillo levels are lowered.

The negative effect of String on Armadillo is likely a result of its cell cycle regulatory function. Changing the dosage of string would be expected to modify this function. Therefore the results described here support a model in which mitotic induction
Figure 5.3  Armadillo levels are sensitive to string dosage in En>Arm imaginal wing discs

A, En>Arm^{16}/UAS-GFP^{pos}.  En-GAL4 is expressed throughout the posterior of the wing disc. The anterior compartment acts as a positive control. B, C, En>Arm^{16}/+ and En>Arm^{16}/UAS-GFP^{pos} controls respectively. Armadillo protein is increased in the cytoplasm of only a few posterior cells (arrows in B), primarily those close to the source of Wingless at the D-V boundary. (C and A are the same disc.) (En>Arm^{16}/UAS-GFP^{pos} discs consistently showed a greater number of Armadillo-positive cells compared to En>Arm^{16}/+. The reason for this is unknown and was not reflected in adult wing phenotypes (Figure 4.9).) D, En>Arm^{16}/+; stg^{Ar^2}/+. More posterior cells maintain high Armadillo levels compared to B. E, En>Arm^{16}/UAS-Stg^{II}. Very few posterior cells have high levels of Armadillo compared to C. Engrailed-GAL4-mediated expression of String alone has no detectable effect on Armadillo expression in imaginal wing discs (see Figure 5.15). 23-37 discs of each genotype were examined and representative examples are shown.
Figure 5.4  Armadillo levels in C96>Arm imaginal wing discs are sensitive to string dosage

A, UAS-GFP\textsuperscript{mrt}/+; C96>Arm\textsuperscript{21}/+. C96-GAL4 is expressed in a broad band of cells straddling the D-V boundary of the wing pouch. B, C, C96>Arm\textsuperscript{21}/+ and UAS-GFP\textsuperscript{mrt}/+; C96>Arm\textsuperscript{21}/+ controls respectively. Cytoplasmic Armadillo levels are increased in a subset of cells (arrows in B) within the C96-GAL4 domain, primarily those close to the source of Wingless at the prospective margin. (C and A are the same disc.) D, C96>Arm\textsuperscript{21}/stg\textsuperscript{Ar2}. High Armadillo expression is maintained in a greater number of cells compared to B. E, UAS-Stg\textsuperscript{mrt}/+; C96>Arm\textsuperscript{21}/+. Very few cells maintain high levels of Armadillo compared to C. 25-31 discs of each genotype were examined and representative examples are shown.
or passage through mitosis lowers cytoplasmic Armadillo levels and thus impairs Wingless signalling.

5.3 String affects the proteolysis of Armadillo

5.3.1 Introduction
String appears to modulate Wingless signalling by affecting cytoplasmic Armadillo, the levels of which are determined primarily by regulated proteolysis (reviewed by Dale, 1998; see Chapter 1.2.2). It is therefore likely that the String-dependent effect modulates Armadillo degradation. Alternatively, String could alter the subcellular localization of Armadillo protein or the translational efficiency of \textit{armadillo} mRNA to affect its cytoplasmic expression. In order to address these issues, I sought to test whether the activity or expression of non-degradable forms of Armadillo were sensitive to \textit{string} dosage.

Figure 5.5 depicts two non-degradable forms of Armadillo (referred to generically as 'Armadillo*') that have been cloned downstream of \textit{UAS} sequences. Both mutant proteins lack the consensus phosphorylation sites for the Shaggy kinase that normally targets Armadillo for proteolysis. Thus Armadillo* signalling activity is constitutive and independent of Wingless. If String modulates the protein stability of wild type Armadillo through the canonical pathway, expression of these stabilized forms and phenotypes arising from their ectopic expression ought to be relatively insensitive to changes in \textit{string} dosage.

5.3.2 Over-expression of non-degradable Armadillo generates wing phenotypes that are sensitive to \textit{string} dosage
\textit{C96>GAL4}-mediated expression of Armadillo* produces viable and fertile adults. Wings from these flies have many supernumerary bristles within and adjacent to the wing
Figure 5.5  Non-degradable Armadillo proteins

A, Wild type Armadillo. Armadillo consists of N and C-terminal functional domains separated by several 'Armadillo repeat' motifs (see Figure 1.2). The activity of Armadillo in Wingless signalling is normally kept low as a result of its proteolysis by the Slimb/proteosome pathway after phosphorylations by the Shaggy kinase at the site shown. The role of Armadillo at adherens junctions depends on the ability of α-catenin to bind at the site indicated. B, Armadillo^Δ510 (Pai et al., 1997). A 54 amino acid N-terminal deletion removes the Shaggy phosphorylation site but keeps intact the α-catenin binding domain. This protein can thus presumably support cell-cell adhesion in addition to being constitutively active in Wingless signalling. It carries a Myc tag in its C terminus that does not interfere with the function of the C-terminal transcriptional activation domain. C, Armadillo^ΔFlu (Zecca et al., 1996). A larger section of the N terminus (154 amino acids) is deleted that removes both the Shaggy and the α-catenin sites. Therefore this protein is predicted to not make productive adherens junction complexes, but like Armadillo^Δ510, it has ligand-independent signalling activity. A Flu tag replaces the N-terminus of the native protein.
Figure 5.6 Changing string dosage modifies C96>Arm
and C96>Arm
wing phenotypes

A, E, UAS-Arm
/+; C96-GAL4/+ and C96>Arm
/+ controls respectively. Control wings have many extra bristles in both the margin and the blade (arrows in A). As the stability of these non-degradable Armadillo proteins is not dependent on Wingless, more ectopic bristles form and do so at a greater distance from the margin than in C96>Arm
wings (Figure 4.8A). B, F, UAS-Arm
/UAS-GFP
/+; C96-GAL4/+ and UAS-GFP
/+; C96>Arm
/+ respectively. These wings typically exhibit a slightly stronger phenotype than those in A and E. C, G, UAS-Arm
/+; C96-GAL4/stg
and C96>Arm
/stg
respectively. The phenotypes are enhanced compared to A and E. Wings heterozygous for string
alone have wild type morphology (not shown). D, H, UAS-Arm
/UAS-Stg
; C96-GAL4/+ and UAS-Stg
/+; C96>Arm
/+ respectively. Wings are either similar to the controls in B and F (as shown here) or the phenotypes are modestly enhanced compared to controls (not shown). C96-GAL4-mediated expression of String alone generates a morphologically wild type wing (not shown). Note that the C96>Arm
phenotypes are generally stronger than those of C96>Arm
. Female wings are shown.
margin in a manner exaggerated over that caused by C96-GAL4-driven expression of wild type Armadillo (Figure 5.6A and E; compare to Figure 4.8A). Surprisingly, heterozygosity for string\textsuperscript{AR2} caused enhancement of both the C96>Arm\textsuperscript{S10} and C96>Arm\textsuperscript{Flw-d} phenotypes, with more bristles forming over a wider area of the wing blade (Figures 5.6C and G). Although this is consistent with string mutations enhancing wing phenotypes resulting from over-expression of wild type Armadillo, it is seemingly inconsistent with the thesis that String affects Armadillo function via the modulation of protein stability. More surprising was the observation that co-expression of String occasionally enhanced C96>Arm\textsuperscript{S10} (not shown). This latter finding is inconsistent with both my previous data and the proposition that String promotes degradation of Armadillo.

As these interactions were rather unexpected, I analyzed them further at the time of bristle specification using neuralized expression as a marker of sensory organ precursors (SOPs) in late C96>Arm\textsuperscript{S10} imaginal wing discs (Figure 5.7). SOPs are the precursors to the large bristles found in the anterior margin of the adult wing, and neuralized is a Wingless target gene (Zecca \textit{et al.}, 1996). C96>Arm\textsuperscript{S10} discs show a slight increase in SOPs above wild type (compare Figures 5.7A and B). Consistent with its effect in adult wings, heterozygosity for string\textsuperscript{AR2} caused an increase in the number of SOPs and the area over which they were specified compared to control discs (Figure 5.7C). While this indicates that halving string dosage increases the number of ectopic bristles that are specified, it does not necessarily follow that the increase occurs through modulation of the expression/activity of non-degradable Armadillo\textsuperscript{S10} as this happens in the presence of wild type Armadillo. Thus the genetic interaction in this context may be a result of the effects of String on this endogenous wild type Armadillo (see section 5.3.3 below).

Co-expression of String could also cause an increase in SOP number over the control, but these SOPs were relatively small and formed organized lines (Figure 5.7D). This indicates that ectopic divisions of pre-existing SOPs, rather than extra Armadillo-
Figure 5.7  Increasing or decreasing string dosage enhances the C96>Arm<sup>510</sup> phenotype by different mechanisms

A, Wild type. *neuralized* (neu)-LacZ expression (red) specifically marks anterior sensory organ precursors (SOPs) in late third instar wing discs. The two rows of *neu*-LacZ cells that fall within the C96-GAL4 expression domain (green) generate the large bristles of the anterior wing margin. B, C96-GAL4-driven expression of Armadillo<sup>510</sup> causes a modest increase in the number of sensory organ precursors and disrupts their organization. C, Heterozygosity for *stg<sup>AR2</sup>* increases the number of C96>Arm<sup>510</sup>-generated SOPs over a wider area and in a pattern suggestive of *de novo* specification. D, Co-expression of String can also increase the number of C96>Arm<sup>510</sup>-generated SOPs, but this occurs within a confined area and their organization and size suggests that they arose by supernumerary divisions of the normal array of SOPS shown in A. 16-27 discs of each genotype were examined and representative examples are shown.
mediated specifications, may account for this phenotypic enhancement. This is not surprising as SOPs are arrested in G2 at the end of larval life and forced expression of String in this context is expected to induce mitosis and cell division (Usui and Kimura, 1992; Johnston and Edgar, 1998).

In conclusion, although observations in adult $C96^{>}Arm^*$ wings indicated that Armadillo$^*$ was sensitive to String levels, analyses in imaginal discs showed that this may not actually be the case. Hence these data do not detract from the thesis that String affects the proteolysis of Armadillo.

5.3.3 The expression of non-degradable Armadillo is unaffected by changes in string dosage

In order to test directly whether the expression of non-degradable forms of Armadillo is sensitive to String levels, I immunodetected the mutant proteins in $C96^{>}Arm^*$ imaginal wing discs after appropriate genetic manipulation of string dosage (Figures 5.8 and 5.9). This is possible as Armadillo$^{WT}$ is Myc-tagged and Armadillo$^{Fim-\Delta}$ is HA-tagged.

Unlike wild type Armadillo (Figure 5.4), over-expressed Armadillo$^*$ is maintained throughout the expression domain of $C96$-$GAL4$ (compare Figure 5.8A to 5.8C and Figure 5.9A to 5.9C). Co-expression of String in these contexts had no effect on either the level or domain of Armadillo$^*$ expression, consistent with its being unable to promote degradation of these stabilized forms of Armadillo (compare Figure 5.8E to 5.8C and Figure 5.9E to 5.9C). However, while heterozygosity for string$^{AR2}$ did not alter the levels of Armadillo$^*$ expression, it did modify its expression domain in two distinct ways. First, reducing string dosage resulted in more isolated Armadillo$^*$-positive cells being found outside of the main expression domain (compare Figure 5.8D to 5.8B and Figure 5.9D to 5.9B). The reason for this is not understood, but may be the result of changes in cell adhesion. Second, and more starkly, heterozygosity for string$^{AR2}$ appeared to enlarge the $C96$-$GAL4$ expression domain.
Figure 5.8 Altering string dosage has no effect on non-degradable Armadillo$^{S10}$ in C96>Arm$^{S10}$ wing discs

A, UAS-Arm$^{S10}$/UAS-GFP$^{pl}$; C96-GAL4/+ . The domain of C96-GAL4 expression is unaffected by co-expression of Armadillo$^{S10}$ (compare with Figure 5.4A). B, C, UAS-Arm$^{S10}$/+; C96-GAL4/+ and UAS-Arm$^{S10}$/UAS-GFP$^{pl}$; C96-GAL4/+ controls respectively. In contrast to wild type Armadillo (Figure 5.4B and C), Armadillo$^{S10}$ expression (detected via its Myc tag) is maintained throughout the C96-GAL4 domain. (C and A are the same disc.) D, UAS-Arm$^{S10}$/+; C96-GAL4/stg$^{AR2}$. The domain of C96-GAL4 expression is widened, maybe through positive feedback on C96-GAL4 (see text). Within its expression domain, the Armadillo$^{S10}$ staining is similar to B. However, there are more isolated Armadillo$^{S10}$-positive cells outside of the C96-GAL4 domain (arrows). E, UAS-Arm$^{S10}$/UAS-Stg$^{ll7}$; C96-GAL4/+ . Armadillo$^{S10}$ expression is similar to C. 21-32 discs of each genotype were examined and representative examples are shown.
Figure 5.9  Altering string dosage has no effect on non-degradable Armadillo\textsuperscript{Flu-Δ} in C96>Arm\textsuperscript{Flu-Δ} wing discs

A, UAS-GFP\textsuperscript{el+/+}; C96>Arm\textsuperscript{Flu-Δ}/+. Expression of Armadillo\textsuperscript{Flu-Δ} does not alter the domain of C96-GAL4 expression (compare with Figure 5.4A). B, C, C96>Arm\textsuperscript{Flu-Δ}/+ and UAS-GFP\textsuperscript{el+/+}; C96>Arm\textsuperscript{Flu-Δ}/+ controls respectively. Armadillo\textsuperscript{Flu-Δ} expression (detected via its HA tag) is maintained throughout the C96-GAL4 domain. (C and A are the same disc.) D, C96>Arm\textsuperscript{Flu-Δ}/stg\textsuperscript{AR2}/+. In contrast to C96>Arm\textsuperscript{s10} discs, the domain of C96-GAL4 expression is not greatly expanded by heterozygosity for stg\textsuperscript{AR2}. However, like C96>Arm\textsuperscript{s10} discs, Armadillo\textsuperscript{Flu-Δ} expression is unaffected within this domain and there are more isolated Armadillo\textsuperscript{Flu-Δ}-positive cells outside it (arrows). E, UAS-Stg\textsuperscript{II7}/+; C96>Arm\textsuperscript{Flu-Δ}/+. Armadillo\textsuperscript{Flu-Δ} expression is similar to C. 26-36 discs of each genotype were examined and representative examples are shown.
This effect was mainly seen in \textit{C96}\textgreater{}\textit{Arm}^{s10} discs (compare Figure 5.8D to 5.8B) and only occasionally in \textit{C96}\textgreater{}\textit{Arm}^{Fly-\Delta} discs (not shown). This observation may be explained by positive feedback on \textit{C96-GAL4} as the \textit{C96} promoter is positively regulated by Wingless signalling (L. Johnston, personal communication). That is, the reduction in \textit{string} levels in all disc cells might cause abnormal stabilization of wild type Armadillo and thus activate \textit{C96-GAL4} outside of its normal domain of expression. An alternative explanation is that reducing \textit{string} dosage caused increased cell proliferation within the normal \textit{C96} expression domain, which seems unlikely. Notwithstanding its explanation, the levels and pattern of Armadillo^{s10} expression within this expanded \textit{C96} domain are similar to those in control discs (compare Figure 5.8D to 5.8B).

As mentioned previously, over-expression of Armadillo* in \textit{C96}\textgreater{}\textit{Arm}^* discs occurs in the presence of wild type Armadillo, which itself is sensitive to \textit{string} dosage (Figures 5.3 and 5.4). I therefore examined the fate of this residual, endogenous Armadillo in the context of over-expressed Armadillo* and modified \textit{string} dosage (Figures 5.10 and 5.11). \textit{C96-GAL4}-mediated expression of Armadillo^{s10} appeared to displace endogenous wild type Armadillo away from cell membranes as only a weak cytoplasmic staining was detected (Figure 5.10B). Heterozygosity for \textit{string}^{Ar2} in this context resulted in a marked increase in this diffuse cytoplasmic staining within the \textit{C96} domain, although membrane staining is not restored (compare Figure 5.10D to 5.10B). In stark contrast, co-expression of String in \textit{C96}\textgreater{}\textit{Arm}^{s10} discs caused the \textit{C96} domain to be completely devoid of Armadillo staining (compare Figure 5.10E to C). Similar effects were observed in \textit{C96}\textgreater{}\textit{Arm}^{Fly-\Delta} discs (Figure 5.11). Effects on cytoplasmic Armadillo are less clear as over-expression of Armadillo^{Fly-\Delta} did not displace so much endogenous wild type protein from cell membranes (Figures 5.11B and C).

In conclusion, the expression level of non-degradable forms of Armadillo are insensitive to the gene dosage of \textit{string} while the cytoplasmic level of residual wild type
Figure 5.10  Residual wild type Armadillo is sensitive to the dosage of string in C96>Arm<sup>S10</sup> wing discs

A, UAS-Arm<sup>S10</sup>/UAS-GFP<sup>+</sup>; C96-GAL4/+. As shown in Figure 5.8, the domain of C96-GAL4 expression is unaffected by co-expression of Armadillo<sup>S10</sup> (compare with Figure 5.4A). B, C, UAS-Arm<sup>S10</sup>/+; C96-GAL4/+ and UAS-Arm<sup>S10</sup>/UAS-GFP<sup>+</sup>; C96-GAL4/+ controls respectively. Over-expression of Armadillo<sup>S10</sup> appears to displace endogenous wild type Armadillo from cell membranes, but weak cytoplasmic staining remains in the centre of the C96-GAL4 domain. (C and A are the same disc.) D, UAS-Arm<sup>S10</sup>/+; C96-GAL4/stg<sup>Ar2</sup>. Many more cells maintain high cytoplasmic Armadillo levels in the centre of the C96-GAL4 domain compared to B. E, UAS-Arm<sup>S10</sup>/UAS-Stg<sup>U7</sup>; C96-GAL4/+. The C96-GAL4 domain is almost devoid of Armadillo. Compare with C. N.B.: the monoclonal anti-Armadillo antibody used here does not detect the Armadillo<sup>S10</sup> protein as the N-terminal epitope is absent (E. Wieschaus, personal communication; compare this Figure to Figure 5.8). 9-10 discs of each genotype were examined and representative examples are shown.
Figure 5.11  Residual wild type Armadillo is sensitive to the dosage of string in 
$C96>Arm^{\text{flu-}\Delta}$ wing discs

A, UAS-GFP$^{n3}$/+; $C96>Arm^{\text{flu-}\Delta}$/+.  As shown in Figure 5.9A, the domain of $C96$-GAL4 expression is unaffected by co-expression of Armadillo$^{\text{flu-}\Delta}$.  B, C, $C96>Arm^{\text{flu-}\Delta}$/+ and 
UAS-GFP$^{n3}$/+; $C96>Arm^{\text{flu-}\Delta}$/+ controls respectively. Expression of Armadillo$^{\text{flu-}\Delta}$ appears to displace less wild type Armadillo from cell membranes but causes more cytoplasmic 
Armadillo to accumulate within the $C96$-GAL4 domain compared to when Armadillo$^{510}$ is 
expressed- see Figures 5.9B and C.  (C and A are the same disc.)  D, $C96>Arm^{\text{flu-}\Delta}/stg^{AR2}$. 
The area of high cytoplasmic Armadillo expression is widened compared to B.  E, UAS-
Stg$^{n7}$/+; $C96>Arm^{\text{flu-}\Delta}$/+.  The $C96$-GAL4 domain contains much less cytoplasmic 
Armadillo compared to C.  N.B.: the monoclonal anti-Armadillo antibody used here does not detect the Armadillo$^{\text{flu-}\Delta}$ protein as the N-terminal epitope is absent (E. Wieschaus, 
personal communication; compare this Figure to Figure 5.9).  25-35 discs of each genotype 
were examined and representative examples are shown.
Armadillo
UAS-GFP

GFP

Armadillo

UAS-GFP

B

C

+ UAS-GFP

D

E

stg[AR2] UAS-Stg
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Armadillo is dependent on string dosage. This is consistent with the view that String function impinges on Shaggy-mediated proteolysis of wild type Armadillo.

5.3.4 Summary and discussion

Changes in the dosage of string clearly affected a cell’s ability to stabilize wild type Armadillo in the cytoplasm. This is not the case for mutant forms of Armadillo that are resistant to Shaggy-mediated proteolysis. Taken together, these data support a model in which a String-dependent activity normally promotes the proteolysis of Armadillo through the canonical degradation pathway. It may activate or de-repress components of the destruction machinery, or inhibit more upstream transducers of Wingless, such as Frizzled or Dishevelled. Alternatively, there could be other functional elements that are disrupted in these Armadillo deletion mutants that mediate Shaggy-independent effects on protein stability or subcellular localization. The generation and subsequent testing of the String-sensitivity of an Armadillo mutant that is specifically mutated in the N-terminal serine/threonine residues that are targeted by Shaggy would be instrumental in addressing this issue. Similar testing of other ‘degradable’ Myc-tagged Armadillo proteins that are deleted for other functional domains might also be instructive (Orsulic and Peifer, 1996).

Over-expression of the two different Armadillo* forms gave qualitatively similar results with respect to the String-Armadillo interaction. However, differences were also observed. First, heterozygosity for string^AR2 resulted in significant broadening of the Armadillo^S10 expression domain but had only minimal effects on Armadillo^Flu-Δ expression. Second, expression of Armadillo^S10 clearly displaced endogenous wild type Armadillo from cell junctions, an effect not seen with Armadillo^Flu-Δ. Third, expression of Armadillo^Flu-Δ alone seemed to result in accumulation of cytoplasmic Armadillo in the C96 domain, while expression of Armadillo^S10 did not. The explanation for these differences is not clear, although may be associated with the different abilities of Armadillo^S10 and Armadillo^Flu-Δ to
participate in productive cell adhesion (Figure 5.5) or the different mechanisms by which they potentiate Wingless signalling (E. Wieschaus, personal communication).

When wild type Armadillo was over-expressed and string dosage was simultaneously altered, change in bristle number in adult wings proved a good correlate of changes in the cytoplasmic Armadillo level and thus the extent of Wingless-Armadillo signalling. This was not so for C96>Arm* wings and discs for the reasons discussed (section 5.3.2). This latter experience demonstrates the dangers of over-interpreting genetic interactions in adult structures and the need to investigate such potential relationships at the cellular level.

5.4 **String may affect the binding of Armadillo to E-cadherin**

5.4.1 **Introduction**

Thus far, the effects of String on Armadillo levels and activity have been discussed with respect only to its role in Wingless signalling. This is because String was identified as a dosage-dependent modifier of a ‘Wingless signalling’ phenotype in adult wings, and also because changes in string dosage clearly alter cytoplasmic Armadillo levels in imaginal wing disc cells. However, it is possible that the major effect of String is to modulate the association of Armadillo with E-cadherin at adherens junctions, and that the observed effects on cytoplasmic Armadillo levels and signalling are secondary to this. Changes in membrane-bound Armadillo might have been masked in the experiments thus far described by the more intense cytoplasmic staining.

I took two simple approaches to address this important issue. First, I re-examined the genetic interactions of String and the CADi protein in En>CADI imaginal wing discs as it was thought that any effect of String on the association of Armadillo with E-cadherin might be clearer in this context. Second, I examined the expression of the wild type E-
cadherin protein in En>Arm discs after manipulation of string dosage to assess whether the String affects the integrity of the adherens junction itself.

5.4.2 Over-expression of String alters Armadillo expression in En>CADi imaginal wing discs

En-GAL4-mediated expression of CADi caused almost all posterior cells to express Armadillo protein at high levels, regardless of their distance from the source of Wingless at the D-V boundary of the wing disc (Figure 5.12A). Armadillo accumulated at apical cell membranes in a punctate pattern that presumably correlates with sites of CADi localization. Surprisingly, cytoplasmic Armadillo levels also appear to be increased compared to anterior control cells. Heterozygosity for stringAR2 in this context had no obvious effect on the pattern or levels of Armadillo expression (compare Figure 5.12B to A), although the posterior of the disc is smaller than the control, consistent with the adult wing phenotype (Figure 4.1). When String was co-expressed however, the punctate membrane staining seen in control discs is almost absent (difficult to see on print, but compare Figure 5.12C to A). Unexpectedly, the size of the posterior compartment is often reduced compared to controls (compare Figure 5.12C to A). As adult wings of this genotype are nearly wild type in morphology, it is likely that phenotypic suppression depends on effects of String over-expression during the pupal period. In all genotypes, the expression of E-cadherin does not appear to be altered significantly by either the expression of CADi or by additional manipulation of string dosage (Figure 5.12).

In conclusion, an obvious functional reason for the strong genetic interaction between String and CADi seen in En>CADi wings is not revealed by immuno-detection of Armadillo in imaginal wing discs. Maybe String affects the En>CADi phenotype at a point downstream of Armadillo, or perhaps effects on Armadillo are too subtle to be detected by
Figure 5.12  Armadillo and Shotgun expression in En>CADi imaginal wing discs

Shotgun (Shg, Drosophila E-cadherin) localizes to apical cell membranes. A, En>CADi^+/+ control. Armadillo accumulates at apical cell membranes in a dotty, granular pattern that presumably correlates with sites of CADi localization. Surprisingly, cytoplasmic Armadillo levels also appear to be increased compared to anterior control cells. Notice also that the posterior compartment of the wing pouch (outlined), as defined by the area of high Armadillo expression, is smaller than normal. Armadillo expression in En>CA0i^+/UAS-LacZ^di control discs is similar to A (not shown). B, En>CADi^+/+; stg^AR2/+ . The pattern of Armadillo expression is similar to A. However, the posterior of the disc (outlined) is clearly smaller than in the control. C, En>CADi^+/+; UAS-Stg^m01/+ . The punctate membrane staining seen in control discs is almost absent. However, the size of the posterior compartment (outlined) is often reduced in size compared to controls. The significance or cause of the groove that routinely forms between the anterior and posterior compartments is not known. Shotgun levels are reduced slightly by the expression of CADi, (compare the anterior and posterior compartments in A) but this effect is not modulated by changes in string dosage- compare B and C to A. N.B.: the anti-Shotgun antibody does not detect the CADi protein. 5 discs of each genotype were examined and representative examples are shown.
Armadillo/Shotgun

A

A'

B

B'

C

C'

stg[AR2]

stg[AR2]

UAS-Stg

UAS-Stg

169
this method? On the other hand, over-expression of String did appear to reduce the punctate staining seen in control discs. If such staining represents Armadillo-CADi complexes at the plasma membrane, then String might feasibly release Armadillo from such complexes: this might lead to increased signalling and the genetic suppression of the $En>CADi$ phenotypes seen in adult wings (Figures 4.4 and 4.5).

5.4.3 E-cadherin expression is not altered by changes in string dosage in $En>Arm$ wing imaginal discs

It was thought that co-staining for Armadillo and E-cadherin might allow the membrane and cytoplasmic pools of Armadillo to be distinguished. Using this method, I assessed whether changing string dosage altered Armadillo levels in the cytoplasm or junctions or both of these locations in $En>Arm$ imaginal wing discs. In addition, this approach addresses whether the expression or localization of E-cadherin itself is modulated by changes in string dosage.

The merged images in Figures 5.13 show that, while cytoplasmic Armadillo is increased or reduced after altering string dosage, junctional Armadillo at cell membranes appears unaffected (compare the posterior to the anterior in each disc). As was found in $En>CADi$ discs, Shotgun ($Drosophila$ E-cadherin) staining in $En>Arm$ discs is unaffected by changes in string dosage, indicating that Shotgun expression is not sensitive to String levels. However, these images are not of sufficient magnification or resolution to be sure that there is no effect of String on junctional Armadillo or Shotgun.

5.4.4 Summary and discussion

Association of Armadillo with E-cadherin at adherens junctions appears to be unaffected by changes in string dosage. Thus it is likely that String primarily targets cytoplasmic Armadillo, as suggested by my previous experiments, and that E-cadherin-bound Armadillo
Cytoplasmic Armadillo is seen in green whereas membrane Armadillo appears yellow/orange in the merged images on the left. A, En>Arm^{16}/+. Over-expression of Armadillo does not significantly alter Shotgun (Drosophila E-cadherin) levels or localization- compare the posterior compartment to the anterior control. B, C, En>Arm^{16}/+; stg^{A02}/+ and En>Arm^{16}/UAS-Stg^{U7} respectively. Changes in string dosage in the context of over-expressed Armadillo does not affect Shotgun or membrane Armadillo expression (compare to A). However, cytoplasmic Armadillo is clearly sensitive to string dosage, as shown previously (Figure 5.3). 5-7 discs of each genotype were examined and representative examples are shown.
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is relatively insensitive to String-dependent effects. This is consistent with String modulating the stability of cytoplasmic Armadillo as junctional Armadillo is resistant to destruction (Dale, 1998; see Chapter 1.2.5). Nevertheless, it is still possible that E-cadherin-associated Armadillo is affected by String in a subtle way that is undetectable by the methods used here (see section 5.5.5). Analysis of α-catenin expression might be instructive in this regard as it forms a link between Armadillo and actin at adherens junctions (reviewed by Hynes, 1999; see Chapter 1.2.4): if String alters Armadillo-E-cadherin association to effect or contribute to the observed increased cytoplasmic Armadillo, then α-catenin is predicted to behave similarly.

It is notable that both the En>CADi and En>Arm phenotypes can be said to be somewhat similar at the cellular level, in that they each increase the amount of cellular Armadillo, albeit by different mechanisms and at different subcellular locations. Hence the identical genetic interactions between string and the two Armadillo-sensitized wing phenotypes (Chapter 4) might be a reflection of similar cell biological phenotypes (discussed further in section 5.6).

5.5 Mitotic cells have relatively low levels of Armadillo

5.5.1 Introduction

The only described function for the String Cdc25 phosphatase in Drosophila is in mitotic induction (see Chapter 1.3.2): string transcription anticipates mitosis by a few minutes (Edgar and O'Farrell, 1989); string mutant cells arrest in G2 (Edgar and O'Farrell, 1989); and ectopic expression of String causes G2 cells to enter M phase precociously (Edgar and O'Farrell, 1990; Neufeld et al., 1998). String removes inhibitory phosphate from Cdk1,
which in combination with an activatory Cyclin subunit, then initiates a phosphorylation cascade that co-ordinates the mechanics of mitosis and cell division (Alberts et al., 1994).

The negative effect of String on cytoplasmic Armadillo identified in this study is presumably associated with this mitotic inducing activity. The first approach I took to test this hypothesis was to simply correlate the level of cytoplasmic Armadillo in mitotic versus interphase cells in \( En>Arm \) imaginal wing discs. Secondly, I examined the effect upon cytoplasmic Armadillo levels after heat-shock induction of mitoses in \( En>Arm \) discs. Finally, I examined the mitotic behaviour of a GFP-Armadillo fusion protein in otherwise wild type discs.

### 5.5.2 E-cadherin staining reveals that mitotic cells have relatively low cytoplasmic Armadillo in \( En>Arm \) imaginal wing discs

Immunodetection of Shotgun (\( Drosophila \) E-cadherin) in \( En>Arm \) imaginal wing discs outlined several large, ovoid cells (Figure 5.14). This cellular morphology is indicative of the later stages of mitosis (Alberts et al., 1994). Strikingly, such cells invariably have low cytoplasmic Armadillo in comparison to neighbouring interphase cells and, conversely, cells harbouring a high level of Armadillo in their cytoplasm are not mitotic (Figure 5.14). This is, of course, only a correlation. Moreover, the majority of the Armadillo-negative cells are not obviously mitotic and nor do all interphase cells have large amounts of cytoplasmic Armadillo. Nonetheless, the finding that mitotic cells contain relatively low cytoplasmic Armadillo is consistent with String inhibiting the cytoplasmic accumulation of Armadillo through induction of mitosis.

### 5.5.3 Immuno-detection of phosphohistone H3 confirms that mitotic cells have relatively low cytoplasmic Armadillo in \( En>Arm \) imaginal wing discs

Histone H3 is phosphorylated specifically during mitosis and is located on the DNA of condensed chromosomes (Hendzel et al., 1997). Immuno-detection of anti-phosphohistone
Figure 5.14  E-cadherin staining shows that mitotic cells have relatively low levels of cytoplasmic Armadillo in En>Arm wing discs.

An enlarged version of the En>Arm/+; stg^{AR}/+ wing disc from Figure 5.13B is shown. Anti-Shotgun staining reveals several large, mitotic cells (arrows in B) that are likely in late anaphase or early telophase. Such cells show relatively low staining for cytoplasmic Arm (arrows in A) compared to neighbouring interphase cells. Discs of the other genotypes in Figure 5.13 also exhibited this correlation, but this genotype was chosen for enlargement because of the greater number of Armadillo-positive cells (not shown).
Figure 5.15  Anti-phosphohistone H3 (PH3) is a specific marker for mitotic cells

A, En>LacZ/+ control. Mitoses occur over the wing pouch in an essentially random pattern with small groups of adjoining cells undergoing mitosis simultaneously (circles). At late third instar wing discs, more mitotic figures are often detected in the posterior (P) compartment compared to the anterior (A) (mitotic figures in P>A in 75% of discs; n=8). B, En-GAL4/UAS-Stg^+/. Note that Armadillo expression in the posterior half of the disc is similar to the anterior control compartment and is similar to A. Practically all En>Stg discs show a greater number of mitotic figures in their posterior half compared to the anterior control compartment (mitotic figures in P>A in 100% of discs; n=9). C, En>Arm^{+/+}. Over-expression of wild type Armadillo in the posterior of the disc results in a similar pattern of mitotic figures as shown in control discs (mitotic figures in P>A in 75% of discs; n=36). Co-expression of String enhances this differential to a level similar to that seen in En>Stg discs (mitotic figures in P>A in 96% of discs; n=23; not shown), while heterozygosity for string^{A87} in this context substantially decreases the differential (mitotic figures in P>A in 32% of discs; n=25; not shown). N.B.: The total number of mitotic cells detected by such staining in fixed discs is very variable and En>Arm wing discs do not consistently show more mitotic figures than control discs. Also note that the gain of the confocal laser was substantially higher in A and B compared to C.

The white dotted line indicates the approximate position of the A-P boundary.
H3 (PH3) in fixed wild type discs revealed several isolated cells and groups of cells undergoing mitosis across the wing pouch (Figure 5.15A), consistent with previous reports (Milan et al., 1996). Many more cells are detected with this antibody than were revealed by anti-E-cadherin staining (Figure 5.14) as anti-PH3 labels all stages of mitosis, though staining is most intense during metaphase and early anaphase (Giet and Glover, 2001).

Although the pattern and number of mitotic cells detected in this way is very variable, both wild type and En>Arm discs consistently showed a greater number of PH3-positive cells in the posterior compared to the anterior of the disc at the late third instar stage (Figure 5.15A' and C'). Interestingly, En-GAL4-mediated expression of String, either alone or in the context of En>Arm, enhances this differential (Figure 5.15B'), while heterozygosity for stringAB2 in En>Arm discs abrogates the differential (see legend to Figure 5.15). These observations indicate that altering string dosage in En>Arm discs leads to a change in the number of proliferating cells or an alteration of the time spent in the mitotic phase. Taken with the previous data on the effects of manipulating string dosage on Armadillo expression in En>Arm discs, these findings support the conjecture that passage through mitosis reduces cytoplasmic Armadillo levels.

Co-staining with anti-PH3 and anti-Armadillo in En>Arm discs largely confirms the correlation between low levels of cytoplasmic Armadillo and mitosis (Figure 5.16). Cells close to the source of Wingless at the prospective wing margin in En>Arm discs frequently stabilize their cytoplasmic Armadillo. The vast majority of these cells are in interphase, while almost all mitotic cells in this region have relatively low cytoplasmic Armadillo (Figure 5.16).

In order to better correlate mitosis with low cytoplasmic Armadillo, I repeated this experiment using the more strongly-expressing UAS-ArmAB2 construct. However, I first tested whether this highly expressed Armadillo was still sensitive to the levels of String. En>ArmAB2 adult wings show a severe phenotype: they are smaller than wild type or
Figure 5.16 Mitotic cells have relatively low cytoplasmic Armadillo in En>Arm wing discs

An enlarged version of the En>Arm/+ disc in Figure 5.15C is shown. Notice that mitotic cells adjacent to the dorsal-ventral boundary in the posterior compartment rarely harbour high levels of cytoplasmic Armadillo (white arrows), while those cells that do express Armadillo at high levels are in interphase. There is one exception (yellow arrow), but even this cell appears to have less intense cytoplasmic staining than its immediate neighbours. 5 discs were examined and a similar correlation to that shown here was observed in each case.
Figure 5.17  Co-expression of String suppresses the strong En>Arm\textsuperscript{23} phenotype in both the wing and the disc

A, En-GAL4/+; UAS-Arm\textsuperscript{23}/+. Notice the numerous ectopic bristles, disruption to wing venation, and blisters within the posterior compartment. In addition, the whole wing is much smaller than either wild type or En>Arm\textsuperscript{16}/+ wings. B, En-GAL4/+; UAS-Arm\textsuperscript{23} UAS-Stg\textsuperscript{III.1}/+. All aspects of the phenotype are dramatically suppressed. (The female wings in A and B are shown at the same magnification and are representative examples.)

C, En-GAL4/+; UAS-Arm\textsuperscript{23}/+. Almost all posterior cells express high levels of cytoplasmic Armadillo with cells adjacent to the D-V boundary expressing the highest levels (seen in 5/5 discs examined; the faint Armadillo staining in the anterior of the disc shown originates from the peripodial membrane.) The increased number of mitotic figures seen in the anterior compared to the posterior compartment was observed in 4/5 discs analysed (compare with Figure 5.15).

D, En-GAL4/+; UAS-Arm\textsuperscript{23} UAS-Stg\textsuperscript{III.1}/+. Only a few cells close to the D-V boundary retain high Armadillo levels in 5/5 discs analysed. The number of mitotic figures in the anterior and posterior halves of the disc was similar in 3/5 discs analysed and greater in the posterior in 2/5 discs.
En>Arm\textsuperscript{16} wings, show a fully-penetrant blistering phenotype, and have many ectopic bristles (Figure 5.17A). All aspects of the phenotype are effectively suppressed by co-expression of String (Figure 5.17B). Figure 5.17C shows the high level expression of Armadillo in En>Arm\textsuperscript{23} discs. Although Armadillo expression is highest near to the dorsal-ventral boundary, levels are also high across the entire posterior wing pouch, presumably as a result of over-loading of the Armadillo destruction machinery (Figure 5.17C; see Chapter 1.2.2). The suppression of this disc phenotype by co-expression of String is remarkable: only a few cells proximal to the dorsal-ventral boundary maintain high levels of Armadillo (Figure 5.17D).

Intriguingly, high level expression of Armadillo in this way appears to repress mitoses in the posterior disc compared to the anterior, an effect that is de-repressed upon co-expression of String (see legend to Figure 5.17). This suggests that Armadillo may inhibit mitotic induction such that Armadillo and String have antagonistic roles in promoting mitosis (see section 5.6).

Most, but not all, cells undergoing mitosis in En>Arm\textsuperscript{23} discs have low cytoplasmic Armadillo (Figure 5.18). Notably, those cells that express the highest levels of Armadillo close to the dorsal-ventral boundary are all in interphase. However, the correlation between mitosis and low cytoplasmic Armadillo is not absolute as there are plenty of interphase cells with relatively low Armadillo expression.

In conclusion, there is striking negative correlation between mitosis and levels of cytoplasmic Armadillo. That is, mitotic cells are frequently observed to have lower Armadillo levels than neighbouring interphase cells. This finding is in very good agreement with my previous data indicating that String is a negative regulator of Armadillo, and together these suggest that passage through mitosis has a significant effect on cytoplasmic Armadillo levels. The fact that the correlation between mitosis and low
Figure 5.18 Mitotic cells have relatively low cytoplasmic Armadillo in En>Arm$^{23}$ wing discs

An enlarged version of the En>Arm$^{23}$ disc in Figure 5.17C is shown. Most mitotic cells in the posterior of the disc have low Armadillo levels (white arrows). However, a few mitotic cells appear to retain a high amount of Armadillo (yellow arrows), while there are also examples of Armadillo-negative cells that are not in mitosis (light blue arrows). Similar observations were made in the other 4 En>Arm$^{23}$ discs examined. (The anti-Armadillo staining shown here is a projection of more basal sections than those used in Figure 5.17C.).
cytoplasmic Armadillo is not absolute suggests that additional regulators of Armadillo, such as the Wingless pathway, are important in determining the final outcome.

5.5.4 Heat shock induction of mitosis reduces the number of Armadillo-positive cells in En>Arm imaginal wing discs

I utilized a heat shock (hs)-String construct (Edgar and O'Farrell, 1990) to induce a discrete pulse of String expression and thus induce mitoses in En>Arm discs. This is a much better approach than simple GAL4-UAS-mediated over-expression of String, as this latter method causes constitutive and high level String expression through the cell cycle with somewhat unknown consequences on the timing of mitosis and activation of Cdk1 substrates.

Armadillo expression in En>Arm hs-String discs that did not receive heat shock is very similar to that in En>Arm/+ discs (Figure 5.19A). The number of Armadillo-positive cells increases greatly in control En>Arm discs that lack the hs-String construct but receive a heat shock (Figure 5.19B). This may simply be the consequence of the extra 'kick' given to the temperature sensitive GAL4-UAS system as a result of the heat shock, although it is striking that this correlates with a marked decrease in the mitotic index (Figure 5.19B'), an effect of heat shock that has been reported previously in Drosophila embryos (Maldonado-Codina et al., 1993). Heat shock induction of String results in many more cells simultaneously entering mitosis across the wing pouch and a concomitant decrease in the number of cells expressing Armadillo at high levels compared to control discs (compare Figure 5.19C to B). This result strongly implicates String-mediated induction of mitosis in the loss or decrease of cytoplasmic Armadillo.

Correlation analysis of Armadillo levels in mitotic versus interphase cells is facilitated by the many mitotic figures seen after heat shock induction of String. As shown in Figure 5.20, many PH3-positive cells have low Armadillo, though others retain higher levels. It is possible to distinguish metaphase from anaphase figures by anti-PH3 staining.
Figure 5.19  Heat shock induction of mitoses reduces the number of Armadillo-positive cells in $En>Arm$ wing discs

A, $En>Arm^{16}/+; hs-Stg/+$, no heat shock (h.s.). Anti-Armadillo and anti-PH3 staining is similar to that in $En>Arm^{16}/+$ discs shown previously. B, $En>Arm^{16}/+$ control, 1 hour heat shock at 37°C and 1 hour recovery. Almost all cells express Armadillo at high levels in the posterior of the disc while mitoses appear to be inhibited (compare B with A)—see text. C, $En>Arm^{16}/+; hs-Stg/+$, 1 hour heat shock at 37°C and 1 hour recovery. Many cells are induced to enter mitosis and there are far fewer Armadillo-positive cells (compare C with B). 5 discs of each genotype shown in A and B were examined and 11 discs of the genotype in C were examined with similar results to those shown here.
Figure 5.20  Armadillo may be degraded at the metaphase to anaphase transition

An enlarged version of the $En>Arm^{16}/+; hs-Stg/+$ disc in Figure 5.19C is shown. Anti-PH3 staining is most intense at metaphase and anaphase. Late anaphase figures are outlined in light blue: these cells appear to have no or very low levels of cytoplasmic Armadillo. Examples of cells that appear to be in metaphase are also indicated (arrows) and retain higher levels of Armadillo.
and it is notable that cells in late anaphase have very low cellular Armadillo compared to cells in earlier stages of mitosis (Figure 5.20). This observation fits well with the previous finding that the ovoid cells identified by E-cadherin staining invariably correlate with low cytoplasmic Armadillo expression (Figure 5.14), as mitotic cells only take on this appearance in late anaphase (Alberts \textit{et al.}, 1994). Therefore, Armadillo might be specifically degraded at the metaphase to anaphase transition.

5.5.5 Both cytoplasmic and membrane-bound GFP-Armadillo are degraded at mitosis

All analyses described thus far have addressed the relationship between String/mitosis and over-expressed wild type Armadillo. This is because the initial genetic interaction was identified in this system, but also because the level of cytoplasmic Armadillo is very low and thus hard to analyse in most wild type wing disc cells. Nonetheless, it was important to determine whether my findings bear relevance to the wild type situation. I therefore examined the expression of a GFP-Armadillo fusion protein by direct immunofluorescence at high resolution in otherwise wild type imaginal wing discs. This GFP-Armadillo protein has been shown to behave similarly to wild type Armadillo in \textit{Drosophila} embryos (C. Alexandre, personal communication).

GFP-Armadillo localizes to all cell membranes but is expressed highly at the membrane and in the cytoplasm of cells along the D-V boundary of the wing disc (Figure 5.21A). Mitotic cells, which can be identified by their ovoid appearance and condensed DNA, show lower levels of both membrane and cytoplasmic GFP-Armadillo compared to their interphase neighbours (Figure 5.21B). Five such discs were examined in this experiment and subjected to a quantitative analysis of cytoplasmic fluorescence intensities (Figure 5.22). Mitotic cells had significantly lower levels of cytoplasmic GFP-Armadillo than did interphase cells in each case.
Figure 5.21  Both cytoplasmic and junctional GFP-Armadillo is degraded at mitosis in wild type wing discs

A, Close-up view of the centre of a GFP-Armadillo disc showing direct GFP fluorescence. Most cells within and on either side of the D-V boundary (which runs left to right along the centre of the image) show high levels of both membrane and cytoplasmic fluorescence. However, mitotic cells (as judged by their shape and condensed DNA; arrows, and within the box) show lower cytoplasmic fluorescence compared to neighbouring interphase cells.

B, Higher power view of the boxed area in A. The two mitotic cells shown (arrowheads in B') have clearly reduced levels of both cytoplasmic and membrane Armadillo compared to neighbouring interphase cells. Interestingly, cells immediately adjacent to the dividing cells also appear to lose their junctional GFP- Armadillo.
Figure 5.22  Quantitative analysis of cytoplasmic fluorescence in GFP-Armadillo wing disc cells

Five GFP-Armadillo wing discs were analysed quantitively with similar results; three analyses are shown here. The average cytoplasmic GFP fluorescence intensity (pixel value) of 1-2 mitotic (M) and several neighbouring interphase (I) cells in each disc was calculated (see Chapter 2.4.3). Note that only cells within each disc can be compared directly and that the x-axis on each graph is set to a different scale. (‘Disc 1’ is the wing disc shown in Figure 5.21.) Error bars are 1 standard deviation.
In conclusion, these results suggest that wild type cytoplasmic Armadillo is subject to degradation at mitosis. Moreover, they show that the previous data implicating String as a negative regulator of Armadillo was not an artefact of over-expression. Finally, this more sensitive approach revealed that junctional Armadillo is also likely to be destroyed during mitosis in wild type cells. Possible implications for this finding are discussed in section 5.6. Due to time constraints, I was unable to determine the exact stage within mitosis when GFP-Armadillo was degraded.

5.5.6 Summary and discussion

Several data are consistent with cytoplasmic Armadillo being specifically degraded as a result of String-mediated mitosis in imaginal wing discs. First, co-staining En>Arm discs for Armadillo and E-cadherin demonstrated that cells in late stages of mitosis frequently contained less cytoplasmic Armadillo than their interphase neighbours, while cells expressing the highest levels of Armadillo were never seen to be undergoing mitosis. Second, an anti-PH3 antibody was used to specifically detect mitotic cells in both En>Arm and En>Arm discs. Although the correlation was not absolute, similar conclusions to those observed upon staining for E-cadherin were made. Third, a heat shock approach was taken to show that a single pulse of String expression was sufficient to both induce mitoses and substantially reduce the number of Armadillo-positive cells in En>Arm discs. Finally, the expression of a GFP-Armadillo protein was examined directly in a wild type wing disc. This last experiment demonstrated that the data presented in this Chapter may be relevant to normal cell biology and that both the junctional and cytoplasmic ‘pools’ of Armadillo are degraded as a cell progresses through mitosis.

Determination of the exact timing of Armadillo degradation within mitosis requires further investigation. Preliminary results involving co-staining for Armadillo and Shotgun or PH3 indicated that Armadillo might be degraded at the metaphase to anaphase transition.
This possibility could be confirmed by co-staining wing discs for Armadillo and discrete markers of mitotic subphases, or inducing the expression of indestructible Cyclin A, B or B3 to arrest mitosis at specific times in En>Arm imaginal wing discs and immunodetecting Armadillo (e.g. Parry and O'Farrell, 2001). Armadillo degradation at a specific mitotic sub-phase might account for the non-absolute correlation between the labelling of cells at all stages of mitosis (as detected by anti-PH3) and reduced cytoplasmic Armadillo levels. Alternatively, this observation may be explained simply by the String-dependent effect on Armadillo not being a dominant form of regulation.

The metaphase-anaphase transition is controlled by the activation of the anaphase promoting complex/cyclosome (APC/C^\text{Fizzy})\text{,} the multicomponent ubiquitin ligase that targets several cell cycle regulators for proteolysis (Zachariae and Nasmyth, 1999; see Chapter 1.3.2). Is it possible that APC/C^\text{Fizzy} also mediates the degradation of Armadillo during mitosis? Two observations appear to argue against this theory. First, Armadillo does not contain a 'destruction box' motif (not shown), a short destruction signal sequence that is traditionally found in all APC/C^\text{Fizzy} substrates (Jackson et al., 2000). Second, my earlier experiments using the non-degradable Armadillo proteins indicated that String-induced degradation of Armadillo occurs through the canonical Shaggy-SCF^\text{Slimb} pathway (section 5.3.3). However, both these points may be countered. In the first case, APC/C^\text{Fizzy}-mediated degradation of Drosophila Cyclin A occurs in the absence of canonical destruction box sequences, but rather depends on a longer and more complicated destruction signal. (Jacobs et al., 2001; Kaspar et al., 2001). It is therefore possible that Armadillo may be targeted for degradation by the APC/C^\text{Fizzy} in a manner similar to Cyclin A. In the second case, the non-destructible forms of Armadillo described earlier contain relatively large N-terminal deletions rather than being specifically mutant for the serine/threonine residues phosphorylated by the Shaggy kinase (Pai et al. 1997; Zecca et al. 1996; Figure
5.5). Hence these mutant proteins may well remove functional domains of Armadillo that are regulated independently of Shaggy, such as an N-terminal APC/C degradation signal.

Heat shock induction of String proved to be an effective method of demonstrating the discrete effect of mitosis on the level of cytoplasmic Armadillo. However, it would be even more informative if discs were fixed at several different times after heat shock and their levels of Armadillo examined on a Western blot or *in situ* using the GFP-Armadillo protein. This would provide a rough time-course for the dynamics of Armadillo degradation after mitotic induction and its (presumed) re-stabilization after entry into G1. Furthermore, it might also aid determination of precisely when Armadillo degradation occurs in mitosis as hs-String induction effectively synchronizes G2-M progression in receptive G2 cells (this study; Maldonado-Codina *et al.*, 1993).

The observation that junctional Armadillo disappears in mitotic cells is intriguing (and is discussed further in section 5.6 below). This phenomenon was not apparent in antibody-labelling experiments and was only discovered through the use of a GFP-Armadillo fusion protein. This is probably due to a combination of reasons, amongst which are that GFP-Armadillo provides a direct read-out of location and expression, that GFP-Armadillo was not over-expressed, and that these GFP-Armadillo imaginal discs were analysed at a much higher magnification than in previous experiments. In retrospect, the finding that over-expression of String in *En>CADi* discs appeared to reduce the number of CADi-Armadillo complexes at cell membranes (section 5.4.2) may be consistent with a 'mitotic activity' releasing Armadillo into the cytoplasm.
Summary and discussion

- Manipulating the dosage of string alters cytoplasmic Armadillo levels in En>Arm and C96>Arm imaginal wing disc cells in a manner consistent with genetic interactions in adult wings.
- These results suggest that String activity inhibits the cytoplasmic accumulation of wild type Armadillo.
- Expression of non-degradable Armadillo is not sensitive to string dosage, indicating that String reduces the levels of wild type Armadillo by promoting Shaggy-dependent proteolysis.
- Expression and localization of E-cadherin does not appear to be modulated by String.
- Consistent with the mitosis-inducing function of String, mitotic cells have relatively low cytoplasmic Armadillo compared to neighbouring interphase cells in En>Arm imaginal wing discs.
- Heat shock induction of String induces mitoses across the wing disc and reduces Armadillo levels.
- Both cytoplasmic and junctional GFP-Armadillo is down-regulated specifically in mitotic cells.
- Wingless/Armadillo signalling may inhibit entry into mitosis, indicating that an antagonistic relationship might exist between Wingless/Armadillo and String with respect to mitotic induction.

Based on the available literature (Chapter 1), it was somewhat unexpected that the original genetic interactions observed between Armadillo and String should eventually be explained.
Chapter 5: Analysis of the genetic interaction between Armadillo and String in wing imaginal discs

by String being an inhibitor of Wingless-Armadillo signalling. However, in retrospect, this situation is perhaps the clear implication from the interaction tests in adult wings. More surprisingly still was the finding that String should modify Armadillo function so directly. In a nutshell, the data described in this chapter indicate that passage through String-mediated mitosis results in increased Shaggy-mediated degradation of cellular Armadillo. However, several outstanding questions remain concerning the exact functional relationship between String and Armadillo, and the biological reason for a cell wanting to degrade its Armadillo at mitosis.

Why does String interact similarly with En>CADi and En>Arm?

As discussed previously, the ‘direction’ of the phenotypic modification caused by the manipulating string dosage in En>CADi and En>Arm adult wings is similar (Chapter 4.5). Taken at face value, opposing conclusions are drawn from each interaction: that mitosis promotes or inhibits Armadillo function respectively. Clearly these two cannot both be true. Indeed, the analyses described in this Chapter demonstrate that passage through String-mediated mitosis decreases Armadillo levels, thus validating the original interactions with En>Arm and questioning the relevance or interpretation of the those with En>CADi. It is therefore clear that genetic modification of CADi-derived phenotypes cannot be assumed to reflect functional modulation of Armadillo activity at the cellular level.

The question remains, however, as to how increasing String levels should suppress both En>CADi and En>Arm, while decreasing its levels enhances both phenotypes. One clue may come from the finding that expression of either wild type Armadillo or the CADi protein leads to increased cellular Armadillo, the former primarily in the cytoplasm and the latter at the cell membrane (Figures 5.2 and 5.12). Thus, if String acts to affect the interchange of Armadillo between junctions and cytoplasm, or the stability of Armadillo in one pool or the other, then similar genetic interactions with the two sensitized phenotypes
might result. Other clues might become evident if En>CADi discs were characterized for cell cycle parameters or co-stained for Armadillo and PH3 to detect mitotic cells.

Is the effect of String on Armadillo solely through its mitotic-promoting role?

Can dephosphorylation of Cdk1 and the consequent transition from G2-M account fully for the effect of String on Armadillo? In support that it can do so, there is a good correlation between mitosis and low cytoplasmic Armadillo. This is consistent with the reported cell cycle expression and function of String, and suggests that activation of Cdk1 results in Armadillo being targeted for degradation (Edgar and O’Farrell, 1989; Edgar and O’Farrell, 1990; Kumagai and Dunphy, 1991; Neufeld et al., 1998; Figure 5.23). Preliminary experiments using heat shock induction of Cdk1AF, an engineered protein that lacks the inhibitory phosphorylation sites (Sprenger et al., 1997) appeared to confirm this model, as they gave results similar to those described using the heat shock-String construct (not shown; Figure 5.19). A better experiment would be to activate String without inducing mitosis by over-expressing String in the presence of a mitosis-inhibiting drug, although such an experiment would be difficult to perform in imaginal discs.

Two observations appear to argue against the idea that all effects of String on Armadillo are mitosis-specific. First, the correlation between mitosis and low cytoplasmic Armadillo was never found to be absolute. Second, it is somewhat surprising that halving string dosage causes such robust phenotypic modifications if it only affects Armadillo levels once per cell cycle. These findings suggest the possibility that String has effects on Armadillo outside of its mitotic role. Indeed, of the three mammalian Cdc25 homologues, Cdc25A and Cdc25B are expressed throughout the cell cycle, with Cdc25A being required for G1-S progression (Murray and Hunt, 1993). However, the sole reported function for Drosophila String is in activation of Cdk1 at mitosis and so all of its affects on Armadillo are almost certainly to be mediated through Cdk1 (Figure 5.23). From this standpoint, the
Figure 5.23 Model for Armadillo regulation at mitosis

The established mechanisms for Wingless signalling and regulation of mitosis in *Drosophila* are summarized and boxed individually. The results described in this Chapter provide links between the two (shown in blue). The negative effect of String on Armadillo is likely to be via a substrate of a Cdk1-Cyclin complex that activates Shaggy-SCF\textsuperscript{Stimb} or APC\textsuperscript{Fizzy}-mediated proteolysis of Armadillo (see text). Wingless-Armadillo signalling may inhibit mitotic entry, although the relevant target is not known (see text). Thus, in this model, activation of String induces mitosis by stimulating Armadillo degradation (to relieve Wingless-dependent inhibition) in addition to directly promoting Cdk1 function.
observation of mitotic cells that retain high levels of Armadillo may be explained by Armadillo being degraded only at a certain subphase of mitosis, while the observation of interphase cells with low Armadillo may be simply the result of them responding to other dominant signals. Similarly, the strong genetic interactions between Armadillo and String might be explained by there being a heritable consequence to the manipulation of Armadillo degradation at mitosis, or that the cells expressing higher Armadillo levels in imaginal wing discs are in the String-sensitive G2 phase of the cycle.

**What is the String-dependent effector and does it target Armadillo?**

The negative effect of String on cytoplasmic Armadillo levels could be primarily mediated by one of three factors. First, a Cdk1-Cyclin complex. This is possible as components of the Wingless pathway that function ‘between’ the receptor and Armadillo are known to be serine/threonine phosphorylated (including Armadillo itself; Dale, 1998), although consenus phosphorylation sites for Cdk1 have not been found in such candidate targets (Moreno and Nurse, 1990; not shown). Second, the String phosphatase itself or a non-Cdk1 downstream target. This is unlikely as the only reported substrate for String is Cdk1 and heat shock induction of activated Cdk1 had similar effects to String on cytoplasmic Armadillo levels (not shown). Third, a substrate of a Cdk1-Cyclin complex. This is perhaps the most likely explanation. For example, one clear candidate is the APC/C that is known to target mitotic Cyclins for degradation at the metaphase-anaphase transition and could potentially target Armadillo for the same (Zachariae and Nasmyth, 1999; discussed in section 5.5.6). Alternatively, such a Cdk1 substrate could interact with a component of the Wingless pathway to affect Armadillo stability. Clearly, more work is required in order to assign an identity to the ‘String-dependent effector’ of Armadillo degradation at mitosis. This is not a trivial task.
As alluded to in the paragraph above, there are also three basic mechanisms through which the 'String-dependent effector' could act. First, mitotic-specific activation of the canonical Shaggy-SCF\textsuperscript{SIMb} pathway could destroy Armadillo. Candidate target proteins here include the Wingless receptor, Dishevelled, Shaggy, dAPC2, and Daxin, as modification of any of these factors could effect N-terminal phosphorylation and consequent proteolysis of Armadillo (Dale, 1998). Of these, Shaggy is perhaps the most likely as the activity of the vertebrate homologue, GSK-3\textbeta, has been found to be cell cycle regulated (Diehl \textit{et al.}, 1998; Fisher \textit{et al.}, 1999; Alt \textit{et al.}, 2000; Chapter 1.4.1). Second, activation of the APC/C in such a way that it tags Armadillo for degradation at the metaphase-anaphase transition. Third, direct targeting (e.g. phosphorylation) of Armadillo such that it becomes a better substrate for degradation via either an SCF- or APC/C-mediated degradation pathway at mitosis. At face value, each of these three possibilities appears to be equally possible, although elucidation of the true target would be made much easier if the identity of the primary effector molecule were known.

\textit{Why should Armadillo be destroyed at mitosis?}

The cell biological reason for mitotic proteolysis of Armadillo is presumably because its function in cell-cell adhesion or Wingless signalling has to be down-regulated for normal induction, progression or completion of mitosis. The reported rounding-up of dividing cells and the characteristic cell shape changes of late anaphase/cytokinesis suggest that adhesive contacts are modified at mitosis (Bauer \textit{et al.}, 1998b and references therein). Dissociation of Armadillo from E-cadherin and its subsequent cytoplasmic proteolysis might effect these necessary changes. Indeed, previous reports have implicated modulation of E-cadherin-catenin complexes in dividing vertebrate cells (Bauer \textit{et al.}, 1998b and references therein; see Chapter 1.4.3). The most thorough of these investigations found that a small proportion of β-catenin translocated from the membrane to the cytoplasm at
mitosis, a finding consistent with my GFP-Armadillo data (Bauer et al., 1998b). However, in contrast to my findings, cytoplasmic β-catenin actually increased at mitosis as it remained bound to E-cadherin and was thus protected from APC-mediated degradation. It would be informative to conduct a detailed analysis of adherens junction components and cytoplasmic Armadillo levels during the different stages of mitotic progression in Drosophila. These experiments could take advantage of both GFP-Armadillo and Drosophila α-catenin-GFP fusion proteins (C. Alexandre, unpublished; Oda and Tsukita, 1999).

An alternative explanation for the mitotic degradation of Armadillo is the intriguing possibility that Wingless signalling actively inhibits mitotic induction and that this has to be overcome for a cell to traverse G2-M (Figure 5.23). This was first suggested by the low mitotic index observed in the posterior of En>Arm23 wing discs in which Armadillo was expressed at high levels. Moreover, individual cells that expressed the highest levels of Armadillo were never found to be mitotic. (I note, however, that this scenario is incompatible with the thesis that Armadillo is not degraded until the metaphase-anaphase transition, as was proposed in section 5.5.6.) As far as I am aware, this possibility does not have a published precedent and the relevant target is not clear. (Candidates would include Tribbles, String and a Cdk1-Cyclin complex, but again, more work is required to elucidate the details.) However some unpublished data are in support of Wingless-Armadillo inhibiting G2-M (L. Johnston, personal communication): FACS analysis of wing discs containing clones that express non-degradable ArmadilloFluΔ are enriched for G2 phase cells, while discs containing clones expressing dominant-negative Pangolin have unusually few cells in G2. Unfortunately, these data suffer from the fact that ArmadilloFluΔ promotes the signalling activity of endogenous wild type Armadillo rather than having potent signalling potential itself (E. Wieschaus, personal communication). It would thus be advisable to repeat this experiment using Armadillo510.
A third possibility, and one that is compatible with metaphase-anaphase degradation of Armadillo, is that its proteolysis late in mitosis is required to establish desirable conditions in the G1 phases of the mitotic daughters. For example, if a mother cell was actively transducing the Wingless signal before cell division, then this signalling 'history' is effectively reset in daughter cells. This situation may be advantageous in proliferating tissues such that nascent cells do not necessarily adopt the same developmental fate as their parents. Such an effect would presumably occur even if one of the two possibilities discussed above were the primary reason for degrading Armadillo at this cell cycle time.

I cannot at present distinguish whether signalling or junctional Armadillo is the primary target for mitotic-specific degradation. On the one hand, it is more likely to be the junctional pool, with degradation of cytoplasmic Armadillo being an inevitable consequence, as targeted proteolysis of cytoplasmic Armadillo would not be expected to affect the stable association of Armadillo with E-cadherin. On the other hand, Wingless-mediated inhibition of G2-M is an attractive model that is consistent with other observations in Drosophila wing discs (this study; L.Johnston, personal communication; Figure 5.23A). Of course, it is also possible that both signalling and junctional Armadillo need to be degraded for a normal and timely mitosis to proceed. In order to address these issues, the precise effects of mitosis on Armadillo regulation, levels, and localization should be tested directly through biochemical approaches. For example, imaginal wing disc cells could be fixed, labelled for a mitotic marker and sorted by FACS, then analysed biochemically to determine the levels, subcellular fraction and phosphorylation parameters of Armadillo in mitotic versus interphase cells. Such protein analyses might also be performed on cells that were enriched for either mitotic or interphase cells by comparing heat shock-String and string<sup>9A</sup> (a temperature sensitive allele; Neufeld <i>et al.</i>, 1998) wing discs after appropriate temperature manipulations. More generally, the same kind of analyses could be applied to synchronized cell cycles in order to assess changes in
Armadillo levels and subcellular location through the entire cell cycle. This might be done in several ways, including using a simple heat shock approach (this work; Maldonado-Codina et al., 1993) or by more conventional means in cultured Drosophila cells.

Finally, on a cautionary note, it is possible that mitotic degradation of Armadillo is a fortuitous and unregulated event with no functional significance whatsoever, or that it facilitates successful mitosis but is not absolutely necessary for its execution. For example, perhaps the Shaggy kinase is transiently active in a separate mitotic role or maybe promiscuous proteolysis occurs. Arguing that mitotic destruction of Armadillo is not essential, C96-GAL4-mediated expression of non-degradable Armadillo did not obviously prevent cell divisions (Figures 5.8 and 5.9) while clones of cells that over-express Armadillo^^{3u-5} have been shown to divide and proliferate normally (Zecca et al., 1996). Moreover, it is well-documented that mutations in β-catenin or APC that abrogate β-catenin degradation and increase its cytoplasmic levels are frequently associated with human cancers (Polakis, 2000), clearly indicating that degradation of β-catenin (at least) is not absolutely necessary for cells to divide.

**Wider implications**

Is degradation of Armadillo during mitosis a general biological phenomenon? First, does it occur in other Drosophila tissues? This was not tested, but could be easily determined. However, a modifier screen that used an embryonic armadillo cuticle phenotype did not uncover string mutations as suppressors (Cox et al., 2000), and direct testing of this potential interaction also proved negative (not shown), indicating that String may not function during embryonic cell cycles to alter Armadillo levels. Nonetheless, this should be tested directly and in situ using the GFP-Armadillo protein, ideally using time-lapse technology in living embryos. Second, do Armadillo homologues behave similarly during mitoses in other organisms? This is not known at the present time, although one study in
cultured mammalian cells reported a decrease in junctional β-catenin but an increase in its cytosolic fraction during mitosis (Bauer et al., 1998b).

Might there be developmental situations where the effects described in this Chapter are important? Cells arrest in G2 during the initial eversion of the wing in the *Drosophila* prepupa (Schubiger and Palka, 1987) and during mesoderm invagination in the embryo (Seher and Leptin, 2000). One of the reasons for this may be to preserve strong cell-cell adhesion through these physically demanding processes, as mitosis would degrade junctional Armadillo and transiently reduce cell-cell adhesion (this study) which would be incompatible with these morphogenetic effects. Wnt/Wingless-mediated inhibition of mitosis may also be important in situations where cells are specified or differentiate in the G2 phase of the cell cycle (e.g. Usui and Kimura, 1992). In these cases, uninhibited passage though mitosis would reset β-catenin/Armadillo signalling to basal levels, or would prevent differentiation in G2.

Finally, it is worth considering whether other proteins are degraded during mitosis. Maybe several cellular junction components or signalling molecules are mitotically destroyed in order to regulate adhesion or signalling at this stage of the cell cycle? From this standpoint, it might have been wise to have analysed the mitotic stability of a second control molecule in the experiments described in this Chapter.
CHAPTER 6: DISCUSSION

"One knows so little. When one knows more, it is too late."

Agatha Christie, Three Act Tragedy (1934)

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Chapter 6: Discussion

Unresolved discrepancies, links to previously published work, and informative additional experiments were each addressed as they arose in the main text and in the individual Chapter discussions. Here, I confine my musings to two general topics. First, I review the lessons that I have learnt regarding the successful performance of dominant modifier screens and the analysis of the candidate interacting genes so identified. Second, I attempt to place cell cycle-intrinsic regulation of Armadillo in the context of the myriad of its other known regulatory mechanisms.

6.1 Precautions to take when performing dominant modifier screens

Throughout this study, many lessons were learnt regarding the use of GAL4-UAS-based dominant modifier screens and the subsequent analysis of interacting genes. Most importantly, the significance of candidate interactions must be judged in a fair and unbiased manner. Thus suitable controls must to be performed in parallel to each set of interaction tests. Likewise, valid comparison of the extent of phenotypic modification between different interactors can only be made when all tests are performed simultaneously under the same conditions. In each case, an appropriate quantitative analysis facilitates unbiased judgement of the presence and extent of interaction. Of course, bona fide interactions should also be shown to be reproducible.

Steps should be taken to address the specificity of interaction between the identified mutation and the over-expressed factor. This can be done by testing multiple alleles of differing ‘strengths’ from different genetic backgrounds, and by testing interactions in a second sensitized phenotype that is similar to the first, but is generated by using a different GAL4 driver.
It can be useful to use different UAS insertions that express a given transgene at different levels to generate both ‘strong’ and ‘weak’ sensitized phenotypes. In this way, one should be able to detect all possible interactions, whether robust or subtle, or causing enhancement or suppression.

The use of two complementary sensitized phenotypes can be a useful way to address the functional relevance of genetic interactions. That is, biologically interesting interactors are predicted to suppress the one and enhance the other. However, this may not be a valid argument when overtly ‘opposite’ phenotypes actually stem from very different cellular effects or biogenetic histories. On this theme, one should not be tempted to over-interpret genetic interactions identified in dominant modifier screens: functional relationships and the wild type roles can only be determined after further genetic, molecular, cellular, and biochemical analyses.

Finally, it is important to remember that dominant modifier screens will only ever detect factors that are dosage-sensitive or limiting for the phenotype or pathway under investigation. Thus the absence of a dominant interaction does not mean that a particular gene is irrelevant to the pathway of interest.

6.2 Multifactorial regulation of β-catenin/Armadillo activity

β-catenin/Armadillo is now recognized as a dual-functional protein that participates both in the formation of adherens junctions and in Wnt/Wingless signalling (Miller and Moon, 1996; Chapter 1). In the former case, β-catenin/Armadillo is required for strong intercellular adhesion, while in the latter it can be said to function in the specification of cell fates and in directing cell proliferation. It is for these reasons that β-catenin/Armadillo has often been speculated to act as a cellular integrator, co-ordinating the three most
important forces that direct metazoan development (see Chapter 1.1). Thus it is not surprising that many diverse mechanisms have been shown to impinge on the function of this protein, including regulated flux between junctions, cytoplasm and nucleus, regulated proteolysis, and as yet uncharacterized effects that are independent on protein stability (Miller and Moon, 1996; Chapter 1). Proteins implicated in these steps encompass the serine/threonine kinase Shaggy and other members of the Wingless signalling cassette, components of adherens junctions, Src family tyrosine kinases, the integrin-linked kinase, insulin-like growth factor 1, and a miscellany of other factors (Chapter 1).

The results described in this thesis serve to add a new string to the bow of Armadillo regulation during Drosophila development. Many cell cycle regulators were shown to interact genetically with an Armadillo loss of function phenotype, a finding that implies a functional connection between the cell cycle/Armadillo activity and in vivo when taken at face value. However, several apparent inconsistencies and caveats serve to cast doubt on their direct biological significance (Chapter 4). String, on the other hand, interacted reproducibly and robustly with an Armadillo gain of function phenotype. Further investigations confirmed String as a novel inhibitor of Armadillo function and established its role as a cell cycle-intrinsic promoter of Armadillo destruction through the induction of mitosis (Chapter 5). Therefore Wingless signalling is transiently down-regulated during mitosis, a finding that may have important implications for the developmental effects of the Wingless signal in proliferating tissues. The cell-biological reason for this phenomenon is not understood, but one possibility is that Wingless/Armadillo inhibits the G2-M transition. In this view, String and Armadillo have antagonistic roles in the induction of mitosis and activation of String drives mitotic entry by stimulating both Cdk1 kinase activity and Armadillo degradation (Figure 5.23).

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"It could be said of me that in this book I have only made up a bunch of other men’s flowers, providing of my own only the string that ties them together”

Montaigne (1533-92)


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