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Discovery of the First Endogenous Gain of Function Mutation in Drosophila RAS1 as a Dominant Suppressor of Apoptosis

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DISCOVERY OF THE FIRST ENDOGENOUS GAIN OF FUNCTION MUTATION IN DROSOPHILA *RAS1* AS A DOMINANT SUPPRESSOR OF APOPTOSIS

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Discovery of the First Endogenous Gain of Function Mutation in Drosophila *ras1* as a Dominant Suppressor of Apoptosis

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The development of multicellular organisms requires a tight coordination of cell proliferation, cell differentiation and cell death in order to correctly specify cell fate and number. According to the trophic theory of survival, this is achieved in part by a competition between cells in a tissue for a limited number of extracellular survival factors. Cells that do not receive sufficient quantities of these survival cues engage a default cell death program and are thereby eliminated. This 'social control' of cell survival ensures the integrity of tissues by matching the correct number of different cell types to each other. Apoptosis is one morphologically distinct, genetically programmed form of cell death by which cells in an organism are efficiently and rapidly removed. The proper execution of apoptosis is therefore critical to normal development and homeostasis in metazoans and defects in the regulation of apoptosis is known to contribute to the etiology of several major diseases. Initial insights into the complex molecular networks that regulate apoptosis derived largely from elegant genetic

analyses of invertebrate model organisms. These early studies identified several genes critical for the execution of the apoptosis and established an evolutionarily conserved core cell death pathway. To further elucidate the molecular mechanisms underlying the control of apoptosis, we conducted several mutagenesis screens in *Drosophila melanogaster* to identify genes that can modulate cell death phenotypes. One particularly interesting mutant isolated in these screens was recovered as a strong, specific and dominant suppressor of cell death induced by the RHG protein *hid*. We demonstrate that this mutant is a gain-of-function allele of *ras85D* (*ras1)*, the *Drosophila* homolog of mammalian oncogenes *H-ras*, *K-ras* and *N-ras*. We further establish that this viable allele, ras^{R68Q}, contains a mutation in the Switch II region of Ras and that it produces a GTPase protein with diminished enzymatic activity. Ras^{R68Q} is the first endogenous gain-of-function *ras1* allele to be identified in *Drosophila* and represents one of very few hypermorphic Ras mutations compatible with organismal viability to be isolated.

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CHAPTER 1.

Introduction

Programmed Cell Death

Animal development encompasses not only cell proliferation, but also highly regulated cell death. Biologists have recognized the occurrence of cell death in multicellular organisms as a normal physiological event for more than 150 years (Glucksmann, 1951). The term programmed cell death (PCD) was first introduced in 1964 following the characterization of intersegmental muscle degeneration during pupation in the silkmoth. Noting the "carefully timed" and ecdysone dependent nature of cellular dissolution in this organism, the authors posited the concept that cell death during development is not of an accidental or random nature, but rather follows a sequence of precisely controlled steps that ultimately lead to spatially and temporally defined cell deaths (Lockshin and Williams, 1964).

The manner in which a cell dies can vary tremendously from paradigm to paradigm, rendering it difficult to study the general phenomena of cell death in a systematic manner. The framework for scientific inquiry into the mechanisms regulating PCD was significantly advanced in 1972 when the Scottish pathologists Andrew H. Wyllie, John F. Kerr and A.R. Currie coined the term "apoptosis" to describe a common and stereotypic subset of cell deaths (Kerr et al., 1972). Apoptosis describes a morphologically distinct form of cell death that is accompanied by rounding-

up of the cell, retraction of pseudopodia, reduction of cellular volume (pyknosis), condensation of the chromatin, fragmentation of the nucleus (karyorhexis), little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing, and maintenance of an intact plasma membrane until late stages of the process (Bellairs, 1961; Kroemer et al., 2005). Unlike necrosis, which typically occurs as a result of toxic cellular insults, apoptosis was observed to occur in a regulated manner as a normal part of animal development. Kerr et al further noted that, in contrast to necrosis, cells eliminated by apoptosis were removed rapidly and efficiently without eliciting an inflammatory response. In addition to the above morphological criteria, apoptosis has subsequently become associated with a number of biochemical changes including loss of membrane phospholipid asymmetry, DNA fragmentation, activation of caspases and activation of nucleases (Hengartner, 2000).

It is now appreciated that PCD is an active, gene-directed process essential for the proper growth, morphogenesis and homeostasis of metazoans. Apoptosis is used extensively in animal development for the removal of unnecessary cells and structures, the sculpting of tissues, the adjustment of cell numbers and as a defensive strategy to remove infected, mutated, or damaged cells (Jacobson et al., 1997; Vaux and Korsmeyer,

1999). Classic examples of the use of apoptosis in developmental biology include; deletion of the tadpole tail during metamorphosis into an adult frog (Tata, 1994), formation of free and independent digits by removal of interdigital mesenchymal cells during limb pattern formation (Mori et al., 1995), culling of greater than 50% of neurons by apoptosis during maturation of the vertebrate brain (Yuan and Yankner, 2000), and formation of vertebrate reproductive organs by deletion of the Wolffian duct in females or of the Müllerian duct in males (Meier et al., 2000a).

Given the integral role of apoptosis in animal development and homeostasis, it is perhaps not surprising that defects in the regulation of apoptosis can contribute to the etiology of several major diseases. Excess apoptosis is a characteristic of many neurological diseases which exhibit the gradual loss of specific sets of neurons, resulting in disorders of movement and CNS function (Sastry and Rao, 2000). Diseases for which excess apoptosis is believed to play a causal role include Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa, several forms of cerebellar degeneration, spinal muscular atrophy and Alzheimer's disease (Thompson, 1995). In contrast, a reduction in apoptosis is associated with cancer and autoimmunity. In cancer for example, inappropriate activation of the negative regulator of apoptosis, Bcl-2, is associated with non-Hodgkin's

lymphomas (Tsujimoto et al., 1985). Similarly, inactivation of Bax, a positive regulator of cell death, is associated with colon, gastrointestinal and hematological malignancies (Meijerink et al., 1995; Rampino et al., 1997; Yamamoto et al., 1997).

Extensive research efforts conducted into the subject of programmed cell death over the last two decades have yielded a detailed understanding of many of the mechanisms and pathways involved in this vital biological phenomenon. It is now recognized for example, that programmed cell death can occur through several diverse mechanisms that lead to a variety of distinct cell death morphologies (Kroemer et al., 2005). In response to this greatly improved understanding, a multitude of novel terms have arisen to describe these varied types of cell death including, apoptosis, necrosis, autophagy (Levine and Klionsky, 2004), mitotic catastrophe (Castedo et al., 2004), anoikis (Frisch and Screaton, 2001), excitotoxicity (Orrenius et al., 2003) and cornification (Candi et al., 2005). Recently, there has been a surge of interest in type II or autophagic cell death, which is distinguished from apoptosis by a stereotypical degradation of the Golgi apparatus, polyribosomes, and endoplasmic reticulum prior to nuclear destruction (Bursch et al., 2000; Martin and Baehrecke, 2004). Apoptosis however, is by far the most studied and best understood form of programmed cell death,

with publications on the subject surpassing 70000 to date (Yuan and Horvitz, 2004).

The 'modern era' of apoptosis research and an exponential leap of interest in the field was heralded by the identification of several biochemical and genetic processes that govern it. That programmed cell death is genetically controlled was appreciated by the late 1960's pursuant to the demonstration by several labs that the inhibition of protein synthesis could prevent cell death (Lockshin, 1969; Makman et al., 1971; Tata, 1966). It was not until 1988 however that the first molecular component of apoptosis, Bcl-2, was identified as the product of a gene found to be activated by the t(14;18) chromosomal translocation in follicular lymphoma (Adams and Cory, 1998; Vaux et al., 1988). Unlike previously described oncogenes such as c-myc or abl which were known to be promoters of cell proliferation, bcl-2 did not stimulate cell division, but rather prevented cells from dying when growth factor was removed. Hence, in addition to identifying a molecular component of the apoptotic mechanism, this discovery established that inhibition of cell death could contribute to the development of cancer in humans.

The first direct evidence that a genetic program exists purely for physiological cell death came from groundbreaking screens in the model

genetic organism *Caenorhabditis elegans* (*C. elegans*), a nematode with an invariant, lineage-restricted development that renders this organism ideal for the genetic study of programmed cell death. During ontogeny of the adult hermaphrodite worm, precisely 131 of the 1,090 somatic cells predictably die by apoptosis, leaving an adult comprised of exactly 959 cells of known origin (Sulston and Horvitz, 1977; Sulston et al., 1983). Genetic screens in *C. elegans* subsequently identified three genes, *egl-1*, *ced-4* and *ced-3* that are essential for the execution of cell death in this organism. Loss-of-function mutations in any one of these genes disables the apoptotic program and leads to the survival of all somatic cells that normally die by apoptosis during wild-type *C. elegans* development (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986). Conversely, a fourth gene, *ced-9*, was discovered to be absolutely required for protection against unscheduled cell deaths in *C. elegans* since null mutations in *ced-9* result in extensive ectopic apoptosis during development (Hengartner et al., 1992). The remarkable power of such genetic analyses in *C. elegans* has led to the identification of more than 20 genes that function in programmed cell death and has permitted these genes to be ordered into a coherent genetic pathway. For their pioneering contributions to developmental genetics and programmed cell death, Sydney Brenner, Robert Horvitz and John Sulston received the Nobel Prize in

Physiology or Medicine in 2002 (Brenner, 2003; Horvitz, 2003; Sulston, 2003).

The independent discovery that human Bcl-2 could prevent programmed cell death in *C. elegans* indicated that apoptosis in mammalian cells and programmed cell death in the nematode were highly related processes. This insight rapidly progressed to the wider realization that apoptosis is in fact an ancient, evolutionarily conserved phenomenon that operates in virtually all multicellular organisms and thereby validated the use of genetic models to better understand the apoptotic process in human development and disease (Vaux et al., 1992). These genetic and other studies ultimately established that many components of the core apoptotic pathway originally described in *C. elegans* by Horvitz *et al.* are highly conserved amongst animals as diverse as the fly, the mouse and humans (Danial and Korsmeyer, 2004).

Discovery of Caspases

A critical advancement in our understanding of the biochemical mechanisms regulating apoptosis came in 1993 with the cloning of *ced-3* and the discovery that it encodes a protein similar to the mammalian cysteine protease, interleukin-1B-converting enzyme (ICE) (Yuan et al.,

1993). This finding firmly established a role for the cysteine aspartate-specific proteases (caspases) as cell death effectors and proclaimed the discovery of a molecular mechanism for apoptosis in *C. elegans*. The discovery that *ced-3* encodes a cysteine protease was completely unexpected and indicated a mechanism of action that had not been anticipated. Overexpression studies with the newly cloned protein determined that Ced-3 could induce the death of mammalian cells in a cell-autonomous fashion and suggested that both Ced-3 and mammalian caspases cause cells to die by a mechanism more direct than that of a hormone or a transcription factor (Miura et al., 1993).

Since the discovery of their role in apoptosis, expansive efforts have focused on the identification of caspases and on the analysis of their regulation and biological functions. Certain caspases, such as ICE (now known as caspase-1) had already been long examined for their non-apoptotic functions, such as in the regulation of inflammation (Cerretti et al., 1992). The regulatory functions of caspases in apoptosis and in a number of other biological processes have now also been studied in detail. Caspases constitute a family of cysteine aspartyl specific proteases that are highly conserved amongst metazoans and in addition to functioning as central regulators of apoptosis, caspases participate in the regulation and execution

of a number of critical cellular processes such as the cell cycle, DNA replication, DNA repair, inflammation and differentiation (Fischer et al., 2003; Kuranaga and Miura, 2007; Thornberry and Lazebnik, 1998).

There has been a clear evolutionary tendency to increase the number of caspases over phylogenetic time, from four in *C. elegans* to seven in *Drosophila*, ten in mice and eleven to twelve distinct caspases in humans (caspase 12 is a pseudogene in whites and is functional in a subset of individuals of African descent) (Lamkanfi et al., 2002; Shaham, 1998; Xue et al., 2006). Caspases that participate in apoptosis can be broadly classified into the initiator caspase group and the effector caspase group based on domain architecture and physiologic function (Fig 1.1). Initiator caspases typically occur in the cytosol as inactive monomers, contain long N-terminal prodomains that encode related homotypic oligomerization motifs such as the caspase recruitment domain (CARD) or the death effector domain (DED) and provide a link between cell signaling and apoptotic execution. In contrast, effector caspases often exist as dimers in their inactive form, contain a short prodomain that lacks death domains and are thought to act downstream of initiator caspases as the ultimate executors of cell death (Degterev et al., 2003; Turk and Stoka, 2007).

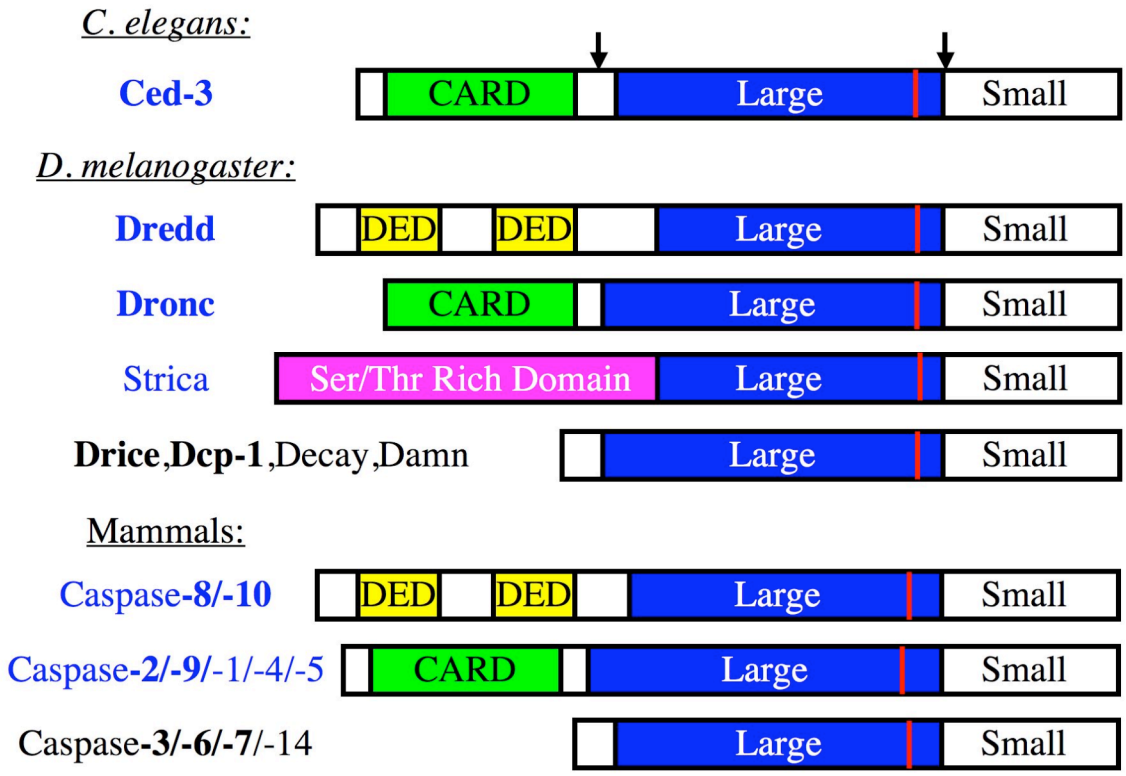


Fig 1.1. Domain architecture of caspases in flies, mammals and worm. Initiator caspases contain prodomains such the CARD or DED and are labeled in blue, whereas executioner caspases lack prodomains and are labeled in black. Those caspases for which a clearly defined role in cell death has been demonstrated are labeled in bold. Drosophila contains 7 caspases, of which 4 are known to be involved in cell death, Dredd, Dronc, Drice and Dcp-1. In mammals, 7 of the 11 identified caspases participate in apoptosis, including the initiator Caspases-2,-8,-9 and -10 and the executioner Caspases-3,-6 and -9. A possible role in cell death for other caspases however can not yet be excluded. Ced-3 behaves as both an initiator and executioner caspase. Approximate sites for proteolytic processing of zymogens are indicated by black arrows above Ced-3. CARD, Caspase Recruitment Domain; DED, Death Effector Domain.

Like most proteases, caspases are generally synthesized as weakly active proenzymes or zymogens consisting of an inhibitory N-terminal prodomain, followed by a large and then a small protease subunit. Overwhelming structural and biochemical evidence predicts that active caspases are obligate heterotetramers composed of two identical catalytic units, with each catalytic unit containing one active site. Currently, all three-dimensional structures of caspases in their active form reveal that each catalytic unit is composed of one large and one small subunit derived from the same precursor molecule (Fuentes-Prior and Salvesen, 2004). Subunits are generated by the sequential cleavage of precursors at specific aspartate residues delineated by a four-amino acid recognition motif within zones of the precursor protein termed the 'linker regions'.

These structural observations led to the assumption that caspases are activated and therefore regulated by proteolytic cleavage (Shi, 2002). Though this has been shown to hold true for the effector class of caspases, recent studies have revealed that cleavage is neither required nor sufficient for activation of the initiator caspases (Boatright et al., 2003). Instead, activation of initiator caspases is effected by an oligomerization process that brings multiple procaspase molecules into close proximity via formation of large multiprotein complexes. Evidence for this 'induced-proximity' model

of caspase activation comes from well-studied caspase complexes such as the apoptosome, the death inducing signaling complex (DISC), the PIDDosome and the caspase-1-containing inflammasome (Festjens et al., 2006). Activator proteins drive multimerization of initiator caspases via homotypic interactions between the death domains found in the long prodomains of initiator caspases and those found in the activators. In *C. elegans*, the CARD containing caspase Ced-3 is activated by its recruitment into a complex containing the activator Ced-4, while in Drosophila, the CARD containing initiator caspase Dronc is activated via recruitment to an oligomeric complex containing the activator Ark/Hac-1, which is homologous to Ced-4. Activation of mammalian DED containing caspase-8 and CARD containing caspase-9 is similarly mediated by recruitment into large multimeric complexes, namely, the DISC and the apoptosome, respectively. The 'induced-proximity' model of caspase activation is discussed in greater detail in the next section.

Once activated, caspases target and cleave various proteins in order to execute their apoptotic or nonapoptotic functions. In addition to autocleaving within activation complexes, a major target of initiator caspases are the effector (downstream) caspases, which in turn mediate the cleavage of a large number of cellular substrates. The execution phase of

apoptosis is thought to result from the limited caspase-dependent cleavage of hundreds of cellular proteins the sum of which results in the morphology characteristic of this form of programmed cell death. Interestingly, the death signals generated by the caspase proteolysis of target proteins are propagated in both an upstream and downstream fashion. For example, downstream effector caspases cleave initiator caspases and other upstream signaling molecules and in this manner generate positive feedback loops in the caspase signaling cascade. This combination of positive feedback with the irreversible nature of caspase cleavage results in a highly efficient molecular mechanism for executing cell death that is both rapid and inexorable once initiated (Turk, 2006). The efficacy of the apoptotic program can be visualized by time-lapse videomicroscopy, which has demonstrated that apoptotic cell death, from initiation to phagocytic removal of cell corpses, can be extremely rapid, often being completed in 20 min or less. This rapidity can regularly lead to a substantial underestimation of apoptotic rates in many experimental paradigms (Evan et al., 1992).

Understanding the many discrete and interacting signaling pathways mediated by caspases will require identification of the natural substrate repertoire for each caspase. Although more than 280 caspase substrates have thus far been identified in humans, it appears that many more apoptotic

caspase targets have yet to been revealed, a task complicated by the overlapping substrate specificities of multiple caspase family members (Fischer et al., 2003). Recent advances in technology, such as mRNA display, have allowed for enhanced identification of natural caspase substrates with improved delineation of caspase substrate repertoires and should improve our understanding of the molecular pathways underpinning programmed cell death (Ju et al., 2007).

Intrinsic Activation of Caspases

Although there are four caspase-like proteins in *C. elegans*, Ced-3 is the only one that has been shown to be required for apoptosis and in this regard, Ced-3 uniquely behaves as both an initiator and executor caspase (Ellis and Horvitz, 1986; Shaham, 1998). Genetic screens and epistasis experiments in *C. elegans* have established that the central and most downstream step in the execution of cell death is the activation of Ced-3 and that Egl-1, Ced-9 and Ced-4 act as upstream regulators for essentially all developmental cell deaths (Shaham and Horvitz, 1996). In addition to these genetic studies, most of the protein complexes that are postulated to be involved in the activation of Ced-3 have been crystallized, allowing for a detailed mechanistic analysis of apoptosis activation in *C. elegans*. These

structural and biochemical data demonstrate that initiation of the apoptotic cascade is achieved through a series of direct protein-protein interactions. According to the 'induced proximity' model of caspase activation, active Ced-3 is generated by recruitment of several proCed-3 molecules into a tetrameric Ced-4 complex (Yan et al., 2005; Yang et al., 1998). In the absence of apoptotic stimuli, Ced-4 is sequestered as an inactive dimer on the outer surface of mitochondria by a direct interaction with mitochondria-bound Ced-9 (Spector et al., 1997; Wu et al., 1997). In cells destined to die, developmental or external cues lead to Egl-1 expression, which binds to and induces a conformational change in Ced-9, thereby disrupting the Ced-4-Ced-9 interaction and discharging Ced-4 from the mitochondrial surface into the cytosol (Conradt and Horvitz, 1998). Once liberated, Ced-4 dimers oligomerize into the tetrameric complex to which proCed-3 molecules are recruited, cleaved and activated (del Peso et al., 1998).

Despite a disparity in many of the details, several components and functional aspects of this core *C. elegans* pathway are evolutionarily conserved in flies and mammals as part of the intrinsic or mitochondrial pathway of caspase activation. In mammals, a member of the initiator caspase family, caspase-9, is a critical mediator of the intrinsic apoptotic pathway (Hakem et al., 1998; Kuida et al., 1998). Likewise in *Drosophila*,

the initiator caspase Dronc is required for virtually all programmed cell death during embryogenesis (Chew et al., 2004; Daish et al., 2004). Ced-4, an adaptor protein of the P-loop ATPase family, is homologous to mammalian apoptotic protease-activating factor-1 (Apaf-1) and *Drosophila* Ark/Hac-1. These adaptor proteins contain a CARD followed by a nucleotide-binding/oligomerization domain and directly bind the CARD of initiator caspases to mediate apoptosome formation and caspase activation. Ced-9 is an anti-apoptotic member of the Bcl-2 family of proteins, containing four Bcl-2 homology (BH) domains and sharing homology with several mammalian Bcl-2 family proteins that regulate apoptosis at the level of the mitochondria (Danial and Korsmeyer, 2004; Hengartner and Horvitz, 1994). Finally, Egl-1 is a pro-apoptotic BH3-only protein that again shares significant homology with several mammalian apoptotic regulators (Cory et al., 2003). In *Drosophila*, the multidomain Bcl-2 family members Debcl and Buffy have been shown to localize to mitochondrial and ER membranes respectively and to have pro- and anti-apoptotic effects in certain contexts, however evidence for their role in the regulation of apoptosis remains limited (Doumanis et al., 2007; Igaki et al., 2000; Igaki and Miura, 2004; Quinn et al., 2003). In any event, the study of apoptosis in *C. elegans* has clearly been instrumental in the identification of several key components of

an apoptotic cascade that is now known to be highly conserved throughout evolution.

It is equally evident however, that distinct mechanisms and modalities of control over caspase activity have evolved amongst various organisms, typically with an increasingly complex network of regulators being utilized over phylogenetic time. The *C. elegans* genome, for example, encodes only three Bcl-2 family members (*ced-9*, *ced-13* and *egl-1*) whereas mammals possess a panoply of more than 20 (Cory et al., 2003). One apparent difference between species in the regulation of caspase dependent cell death concerns the role of mitochondria and their release of apoptogenic intermembrane space proteins. In mammals, mitochondria have been well substantiated as a critical control point for apoptosis induction, regulating death signals via a mitochondrial outer membrane permeabilization event that discharges into the cytosol several putative pro-apoptotic factors including cytochrome c, SMAC/Diablo, Omi/HTRA2, endonuclease G, apoptosis inducing factor (AIF) and ARTS (Green and Kroemer, 2004; Larisch et al., 2000; Wang, 2001). The various pro- and anti-apoptotic Bcl-2 family members in turn regulate this critical permeabilization event through mechanisms that remain controversial.

Perhaps the best studied role for mitochondria in the regulation of mammalian apoptosis concerns the release of cytochrome c. Upon its discharge from mitochondria into the cytoplasm during a permeabilization event, cytochrome c binds to the WD40 repeats of Apaf-1, inducing a conformational change in Apaf-1 that permits its oligomerization with and subsequent activation of Caspase-9 (Li et al., 1997). This is in marked contrast to Ced-4 in *C. elegans*, which lacks a WD40 domain and does not require cytochrome c for its activity in vitro (Yan et al., 2005). Likewise in *Drosophila*, biochemical and structural evidence to support a role for either mitochondria or cytochrome c in Ark/Hac-1 dependent cell death has not been forthcoming despite the fact that Ark/Hac-1 does contain C-terminal WD40 repeats and is able to bind cytochrome c (Yu et al., 2006). However, recent genetic data from both *C. elegans* and *Drosophila* does support at least some role for mitochondria in caspase dependent cell death in these organisms. Two mitochondrial proteins, CPS-6 and WAH-1, which are the *C. elegans* homologues of mammalian endonuclease G and AIF respectively, are released from mitochondria during apoptotic stimuli and synergize to promote DNA degradation (Parrish et al., 2001; Wang et al., 2002). In *Drosophila*, a homolog of the mitochondrial serine protease Omi/HTRA2 was recently cloned and shown to efficiently promote cell

death in a manner analogous to its mammalian counterpart (Igaki et al., 2007). Additional *in vivo* evidence from *Drosophila* indicates that a particular form of cytochrome c, cyt-c-d, is required for caspase activation during spermatid differentiation and for the proper regulation of developmental apoptosis in the pupal eye (Arama et al., 2006; Mendes et al., 2006). Mitochondrial disruption, which is a conserved aspect of apoptosis involving the mitochondrial fission mediator Drp1, has been observed in both *C. elegans* and *Drosophila* and has been found to affect programmed cell death (Goyal et al., 2007; Jagasia et al., 2005). Finally, there is evidence that the *Drosophila* cell death inducers *rpr*, *hid* and *grim* (the so called RHG proteins - see below) require mitochondrial localization via a mitochondrial targeting sequence referred to as the GH3 domain for full apoptotic activity (Freel et al., 2008).

Extrinsic Activation of Caspases

A major point of divergence between *C. elegans* and higher organisms in the regulation of caspases relates to the evolution in the latter of a second, alternative pathway for caspase activation. This pathway, referred to as the extrinsic cell death pathway, is mediated by transmembrane death receptors of the tumor necrosis factor receptor (TNFR) superfamily. In mammals this

includes Fas/CD95/Apo1, TNF-R1, TNF-R2, DR3/WSL-1/TRAMP, DR4/TRAIL-R1, DR5/TRAIL-R2, and DR6, all of which are characterized by the presence of a cytoplasmic death domain (DD). Activation of this pathway is initiated by ligand-induced receptor trimerization when members of the TNF superfamily of ligands, either soluble or membrane bound, bind their cognate death receptors. The recruitment and activation of initiator caspases is subsequently achieved by adapter molecules that bridge activated death receptors to initiator procaspases via homophilic death domain and death effector domain (DED) contacts (Ho and Hawkins, 2005; Park et al., 2007). Fas, upon ligand stimulation by FasL, recruits the adapter protein FADD to its cytoplasmic tail via a homotypic DD:DD interface. FADD in turn recruits caspase-8 or caspase-10, via homotypic interactions with the tandem DED motifs found within the prodomain of these caspases, to generate the ternary death-inducing signaling complex (DISC). In the case of TNFR1 activation by TNF-α, the multifunctional adapter protein TRADD is first recruited to the activated receptor again via a DD interaction. TRADD in turn engages receptor-interacting protein (RIP) and the TNF receptor-associated factors (TRAFs) to form the membrane-bound 'complex I', essential for IKK and NF-κB activation. Subsequently, TRADD dissociates from TNFR1 and associates with FADD and caspase-8 to

generate the cytoplasmic 'complex II', a platform for caspase activation. The regulated assembly of these two different TRADD complexes may underlie the ability of TNF to induce either cell death or cell survival under different cellular contexts (Micheau and Tschopp, 2003b). Ultimately, recruitment of procaspases into these high molecular mass complexes induces the proteolytic autoprocessing of zymogens via an induced-proximity mechanism similar to that described for the apoptosome. This liberates active caspase-8 or caspase-10 into the cytoplasm to cleave and activate downstream effector caspases such as caspase-3 and caspase-7, generating a caspase signaling cascade. Induction of apoptosis via the extrinsic pathway is used extensively in cells of the immune system to eliminate immune effector cells that have fulfilled their function and defects in components of this pathway manifest as an autoimmune lymphoproliferative syndrome (ALPS) (Rieux-Laucat et al., 2003). Additionally, mutations in caspase-8 have been linked to a variety of human cancers, suggesting that caspases-8 can act as a tumor suppressor.

The fly genome clearly encodes an ortholog of FADD, designated dFADD, that like its mammalian counterpart, binds to and activates an apical caspase, Dredd (Hu and Yang, 2000). Also encoded by the *Drosophila* genome are two TRAF homologs, dTRAF1 and dTRAF2, a

single TNF ligand, Eiger and a single putative TNFR homolog, Wengen, which contains an extracellular TNFR homology domain but lacks the intracellular DD motif characteristic of the death receptor family (Igaki et al., 2002; Kanda et al., 2002; Liu et al., 1999). Despite the expression of this TNF-like axis in *Drosophila* however, current evidence argues that it probably does not engage the dFADD/Dredd module to launch an apoptotic caspase cascade. Instead, the dFADD/Dredd module predominantly regulates innate immune responses triggered by Gram negative bacteria (Tanji and Ip, 2005). Nevertheless, Eiger is a potent inducer of apoptosis and represents the first TNF cytokine superfamily ligand to be isolated in an invertebrate. Originally identified in a gain-of-function screen for inducers of apoptosis, Eiger is a type II transmembrane protein with a C-terminal TNF homology domain, is predominantly expressed in the nervous system, and can be cleaved and released from the cell surface as a soluble factor. Genetic analysis of Eiger mutants revealed that the apoptotic effect of Eiger does not require the activity of dFADD/Dredd, but instead is completely dependent upon on its ability to activate the Jun-N-terminal kinase (JNK) pathway. Precisely how Eiger achieves activation of JNK and the role of Wengen in this pathway remains controversial. Recent evidence suggests that dTRAF2, the *Drosophila* homolog of the human tumor suppressor and

deubiquitinating enzyme CYLD, dCYLD, and the ubiquitination activity of Diap1, all play a critical role in transduction and modulation of the Eiger signal (Xue et al., 2007). It seems clear from an phylogenetic perspective that the extrinsic pathway of apoptosis induction represents a relatively recent evolutionary event, largely confined to mammals, whereas the intrinsic pathway is the more ancient, evolutionarily conserved mechanism of caspase activation.

Discovery of the RHG Proteins

Another pointed example of how evolution has led to the emphasis of discrete regulatory points of control over the execution of programmed cell death was imparted by the discovery in *Drosophila* of a novel class of apoptotic regulatory proteins referred to as the RHG proteins. *D. melanogaster* has proven an excellent model in which to examine programmed cell death, with many advantages, including a well documented developmental plasticity, a rapid life cycle, sophisticated genetic tools, well-developed misexpression systems, a largely complete and annotated genome sequence and a wealth of current and historical research literature. Furthermore, multiple tissues undergo programmed cell death in a globally patterned yet dynamically stochastic manner throughout *Drosophila*

development, affording the opportunity to analyze the complex regulatory decisions that control these cell deaths. Cell death has been characterized in the *Drosophila* embryonic central nervous system, embryonic head region, embryonic epidermis, larval salivary glands, larval midgut, larval wing and eye imaginal discs, pupal retina, adult nervous system and adult female germ line (Gorski and Marra, 2002). *Drosophila* was propelled to the forefront of apoptosis research in 1994 when a deficiency screen conducted using the vital dye Acridine Orange, a marker of dying cells, identified a chromosomal region essential for virtually all embryonic cell deaths, as well as ectopic deaths induced by irradiation and developmental defects (White et al., 1994). Subsequent analysis of this region, spanning 75C1-2 and deleted in the H99 deficiency, led to the identification of three genes that function in the activation of cell death: *reaper* (*rpr*), *head involution defective* (*hid*) and *grim* (Chen et al., 1996; Grether et al., 1995; White et al., 1994). The proteins encoded by these genes vary considerably in size and share little homology amongst each other or with any other known proteins. They do however contain a common 14 amino acid motif at the N-terminus, termed the RHG motif or the IAP-binding motif (IBM) which has been demonstrated to be critical for their pro-apoptotic function (Wing et al., 2001) (Fig 1.2). On the basis of this motif, three other pro-apoptotic

"reaper-like" genes have been characterized in *Drosophila*. *Sickle* (*skl*) lies immediately adjacent to the H99 locus and like *rpr*, is acutely upregulated in response to certain apoptotic stimuli such as ionizing radiation (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002). The thioredoxin peroxidase Jafrac2 is an IBM containing protein that normally resides in the endoplasmic reticulum of healthy cells, but is rapidly released into the cytosol following apoptotic stimuli (Tenev et al., 2002). Finally, as previously mentioned, a *Drosophila* homolog of the mammalian mitochondrial serine protease HtrA2/Omi was recently cloned and characterized (Igaki et al., 2007). DmHtrA2 is a developmentally regulated mitochondrial intermembrane space (IMS) protein that undergoes processive cleavage, in situ, to generate two distinct IBM motifs. In response to apoptotic stimuli DmHtrA2 is translocated to the extramitochondrial compartment in a manner reminiscent of its mammalian homolog and other pro-apoptotic intermembrane space proteins (Challa et al., 2007). Despite the virtual lack of homology between RHG proteins outside of the IBM motif, an abundance of data indicates that, at least in part, they function mechanistically in a similar fashion by disrupting the function of the same key regulatory molecule, Diap1. This inhibition of Diap1 function in turn allows for activation of caspases and engagement of the cell death program.

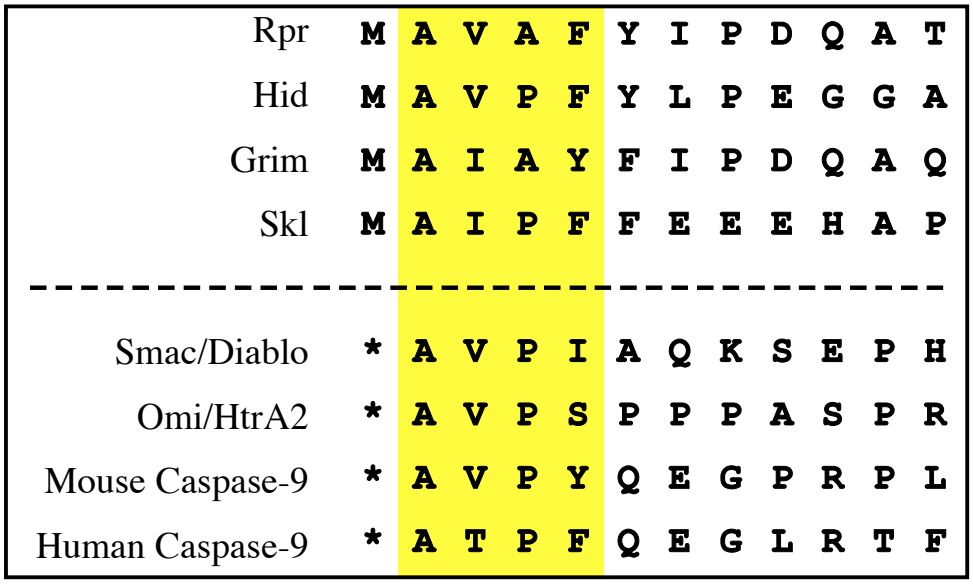


Fig 1.2. The RHG motif is a N-terminal region conserved among the H99 genes (above the dashed line) and to a lesser extent in several mammalian proteins. The tetrapeptides highlighted in yellow (IBM) are sufficient for binding to Xiap. The Drosophila RHG domains are located immediately carboxy-terminal to the initiation methionine, which is presumably removed by methionine amino peptidase activity *in vivo*. All mammalian IBM-containing proteins that have been discovered so far undergo proteolytic processing to expose the IBM.

Interestingly, heterologous expression of RHG proteins in mammalian cells was found to efficiently induce apoptosis in these cells, pointing yet again to a mechanistic conservation between fly and mammalian cell death pathways and suggesting the existence of mammalian RHG protein homologues (Claveria et al., 1998; Haining et al., 1999; McCarthy and Dixit, 1998). Though no mammalian counterparts with extensive sequence similarity to any of the Drosophila RHG proteins have been found, three mammalian proteins have been identified that do contain an N-terminal RHG tetrapeptide motif and appear to function in part via a molecular mechanism similar to that of the RHG proteins. Two of these proteins, Smac/Diablo and HtrA2/Omi, are among the mitochondrial IMS proteins released into the cytosol in response to apoptotic stimuli whereas the third, GSPT1/eRF3, is a proteolytically processed isoform of an endoplasmic reticulum-associated protein whose normal role is to act during translation as a polypeptide chain release factor (Du et al., 2000; Hegde et al., 2003; Suzuki et al., 2001a; Verhagen et al., 2000). In addition, recent screens have identified several other putative molecules that interact with IAPs via N-terminal IAP binding motifs (Verhagen et al., 2007). The validity of these mammalian IBM containing proteins as bona-fide regulators of apoptosis,

however, remains controversial and the search for legitimate RHG homologs is ongoing.

Inhibitor of Apoptosis Proteins (IAPs)

Diap1 belongs to the highly conserved class of cell death suppressors known as the Inhibitor of Apoptosis Proteins (IAPs). Discovery of this protein family came from virologists originally studying a mutant form of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) that resulted in premature cell death during infection of *Spodoptera frugiperda* (SF-21) insect cells. Characterization of this spontaneous viral mutant determined that the baculoviral protein p35 was responsible for blocking the apoptotic response in the infected host cell (Clem et al., 1991; Friesen and Miller, 1987). With no homology to proteins outside of Baculoviridae, p35 was subsequently shown to be a broad caspase inhibitor in several species and was quickly adopted as an invaluable tool for apoptosis research (Bump et al., 1995; Xue and Robert Horvitz, 1995). The only other caspase inhibitor known at the time, cytokine response modifier A (CrmA), was also discovered by virologists, before caspases were termed 'caspases', and before caspases were known to be the key executioners of the cell death program (Pickup et al., 1986). Originally identified on the basis

of its ability to produce hemorrhage in developing chick embryos, CrmA was determined to efficiently block inflammatory responses by specifically inhibiting Interleukin-lβ Converting Enzyme (ICE), now known as Caspase-1 (Palumbo et al., 1989; Ray et al., 1992). Unlike p35, CrmA contained extensive homology to other proteins that immediately placed it into the large and ancient serpin family of serine protease inhibitors. Although they have no structural similarity, CrmA and p35 both inactivate their cognate proteases in a mechanism-based manner by behaving as 'suicide substrates'. The reactive-site loop of the inhibitor binds to the active site of the caspase and is cleaved, inducing a conformational change that irreversibly locks the protease in an inactive conformation (Simonovic et al., 2000; Xu et al., 2001). It is believed that baculoviruses express these inhibitors to suppress and escape an apoptotic host response that would otherwise limit viral replication (Clem and Miller, 1994).

To identify additional genes involved in the inhibition of virally induced apoptosis, Miller *et al*. conducted a screen for genes that could functionally complement for loss of p35. This approach successfully identified such a gene from another baculovirus, *Cydia pomonella* granulosis virus (CpGV) that could also block actinomycin D induced apoptosis in SF-21 cells. Named inhibitor of apoptosis or Cp-iap, this gene remarkably

turned out to encode a protein with zinc finger motifs (the BIR domains) homologous to those found in several human proto-oncogenes and insect embryonic development genes (Crook et al., 1993). Orthologous proteins that also function as cell death inhibitors have subsequently been identified in a wide variety of organisms including insects, mammals and plants making Cp-iap the founding member of an evolutionarily conserved IAP family of apoptosis inhibitors (Salvesen and Duckett, 2002) (Fig 1.3).

All IAPs are characterized by the presence of between one and three tandem BIR domains, each approximately 70 amino acids in length and comprising a zinc-binding fold (Deveraux and Reed, 1999). The BIR domains of IAPs are critical for their anti-apoptotic properties and in flies and mammals, endogenous IAPs can inhibit active caspases by direct binding of their BIR domains to caspase catalytic sites, by promoting the degradation of active caspases or by sequestering caspases away from target substrates (Hinds et al., 1999; Riedl et al., 2001; Tenev et al., 2005). IAPs exhibit specificity towards a subset of caspases. Mammalian Xiap, Ciap1 and Ciap2, for example, can bind and through diverse mechanisms inhibit Caspase-3, -7 and -9 but do not interact with Caspase-1, -6, -8 or -10 (Deveraux et al., 1997; Roy et al., 1997). Moreover, the individual BIR domains of those IAPS with multiple BIR domains, fold into functionally

independent structures that target and inhibit distinct caspases. Xiap, which contains three BIR domains, requires the BIR2 domain and a small N-terminal extension of BIR2 for the inhibition of Caspase-3 and -7, whereas the BIR3 domain of Xiap is essential for the inactivation of Caspase-9 (Chai et al., 2001; Riedl et al., 2001; Shiozaki et al., 2003). Similarly in *Drosophila*, the BIR1 domain of Diap1 was found to specifically bind the effector caspases Dcp-1 and Drice, while BIR2 was found to be essential for binding the initiator caspase Dronc (Meier et al., 2000b; Zachariou et al., 2003).

Not all BIR containing proteins are IAPs however, as some of these proteins appear not to function as bona fide inhibitors of apoptosis, but rather seem to have roles in other vital cellular processes. *C. elegans* encodes two proteins, CeBir1 and CeBir2 that possess BIR domains, but neither are considered veritable IAPs because they have not been found to play a role in regulating apoptosis. Instead, CeBir1, it's yeast homolog Bir1p and its mammalian homolog Survivin, are primarily involved in cytokinesis as members of the chromosomal passenger complex (Fraser et al., 1999; Lens et al., 2006; Li et al., 1998; Rajagopalan and Balasubramanian, 2002; Speliotes et al., 2000).

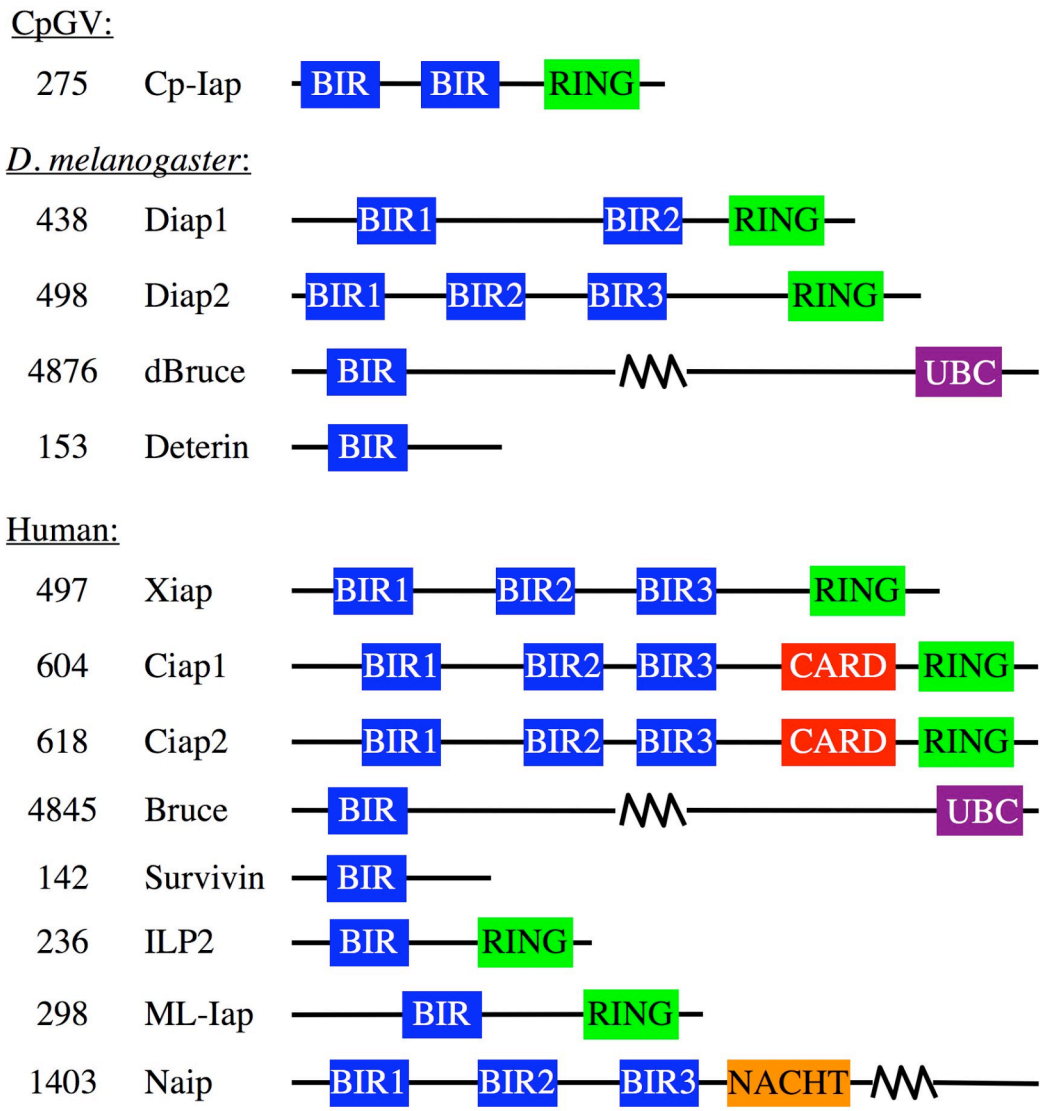


Fig 1.3. Domain architecture of *Drosophila* and human IAPs. The drosophila genome encodes four IAP genes; the human genome eight. Cp-Iap, the first IAP discovered, is also shown. Current evidence indicates that only Diap1 and Xiap1 are direct inhibitors of caspase activity. In addition to at least one baculoviral IAP repeat (BIR) domain, most IAPs have other distinct functional domains. RING, Really Interesting New Gene; UBC, Ubiquitin-conjugation; NACHT, nucleotide-oligomerization domain. Numbers to the left indicate the length in amino acid residues.

Ring Domains and Ubiquitination

In addition to BIR domains, IAPs with clearly defined roles in apoptosis also contain a second highly conserved zinc-binding motif at their carboxyl terminus called the RING domain (for Really Interesting New Gene.) The RING domain can behave as an E3 ubiquitin ligase and functions to recruit E2 ubiquitin-conjugating enzymes to target proteins, which are subsequently ubiquitylated by the transfer of a 76-amino-acid ubiquitin peptide (Joazeiro and Weissman, 2000). In contrast to the multisubunit RING E3 ligases, IAP E3 ligases combine a substrate-binding domain (the BIRs) and a RING domain within the same protein. Since ubiquitylation of proteins has emerged as a fundamental regulatory mechanism in eukaryotic cells, it follows that ubiquitin-mediated protein regulation is involved in IAP function.

The importance of the RING domain for the regulation of caspases and apoptosis *in vivo* was revealed by *Drosophila* screens that identified disruptive point mutations in the *diap1* RING that are embryonic lethal (Lisi et al., 2000; Moore et al., 1998). Contrary to much early *in vitro* and overexpression data, *in vivo,* Diap1 requires not only its BIR2 domain to bind the initiator caspase Dronc, but also a structurally intact RING domain to neutralize it (Chai et al., 2003; Wilson et al., 2002). The complexity of

Diap1 mediated apoptosis control and the critical role of the RING in this task are further underscored by evidence demonstrating that Diap1 promotes the ubiquitylation of a number of proteins including Rpr, Hid, Grim, dTRAF1 and of itself by way of an autoubiquitination reaction (Kuranaga et al., 2002; Olson et al., 2003; Ryoo et al., 2002). Similarly, Xiap has been demonstrated to ubiquitinate several proteins in a RING dependent manner including Smac/Diablo, Caspase-3, Caspase-9, MURR1 (a factor recently implicated in copper homeostasis), AIF and itself via autoubiquitination (MacFarlane et al., 2002; Morizane et al., 2005; Suzuki et al., 2001b; Wilkinson et al., 2007; Yang et al., 2000). Genetic evidence implicating the Xiap RING domain in apoptosis regulation has recently been furnished by Schile et al., who generated a knock-in mouse expressing a RING-deleted Xiap. Mouse embryonic fibroblasts (MEFs) derived from this mouse are strongly sensitized to TNF-α induced apoptosis. Furthermore, deletion of the RING in Xiap lessens the incidence of leukemia and prolongs the survival of mice on a Eu-myc lymphoma background. The authors demonstrated using irradiated MEFs that deletion of the Xiap RING domain results in reduced caspase ubiquitination and concluded that the ubiquitinating activity of Xiap is important for its role as a negative regulator of apoptosis (Schile et al., 2008).

The functional significance of IAP RING-mediated ubiquitylation of target proteins is often unclear however. In general, a target protein can be subjected to several possible ubiquitylation outcomes such as monoubiquitylation, multi-monoubiquitylation, polyubiquitylation through a K48 linkage or polyubiquitylation through a non-K48 linkage. Only substrates that are polyubiquitylated by a K48-linked chain of four or more ubiquitins are rapidly recruited to the 26S proteasome and degraded (Vaux and Silke, 2005). Therefore, even though an important role of the ubiquitin system is to regulate the half-life of proteins by targeting them for degradation by the 26S proteasome, there are many ubiquitin modifications that do not result in protein degradation but instead alter the activity of the modified protein. A prime example is provided by the regulation of Dronc by Diap1 in *Drosophila*. Despite a requirement of the Diap1 RING finger for Dronc ubiquitylation and the suppression of apoptosis, Diap1 does not target Dronc for degradation, indicating an inhibitory, but non-degradative, polyubiquitylation event (Wilson et al., 2002). Similar observations in a mammalian system suggest a regulatory mechanism involving IAP-mediated non-degradative mono-ubiquitylation of Caspases-3 and -7 (Huang et al., 2000). The ubiquitylation of caspases in this manner could, for example,

suppress them by blocking their recruitment into apoptosome complexes or modifying their subcellular localization.

Given that ubiquitylation might arguably demonstrate as diverse a regulatory repertoire as phosphorylation, the regulation of caspases and other proteins by RING containing IAPs could prove enormously complex. It has been hypothesized that RING-mediated autoubiquitination of IAPs leads to their degradation and that this process is a key regulatory event in the apoptotic program, perhaps illustrating a paradigm in which levels of IAPs are carefully governed (Ryoo et al., 2002; Yang et al., 2000). Some recent data suggests however that this may be an oversimplification. In some contexts, RING-mediated cross ubiquitination by paralogous IAPs may be required to elicit degradational targeting to the proteasome. Silke *et al*. have shown, for example, that mammalian Ciap1 binds directly to Xiap via a homotypic RING-RING interaction, leading to the ubiquitination and subsequent proteasomal degradation of Xiap (Silke et al., 2005). It has also been demonstrated that Ciap2 is a direct target of Ciap1-mediated RING-dependent ubiquitination and degradation (Conze et al., 2005). Similarly, Herman-Bachinsky *et al*. have recently provided evidence that the RING-finger-mediated autoubiquitinating activity of Diap1 does not involve formation of the Lys48-based polyubiquitin chains necessary for targeting to

the proteasome, but rather produces chains linked via Lys63 that serve only to attenuate the ligase activity of Diap1 towards its exogenous substrates. They argue therefore, that it is Diap2 that ubiquitinates Diap1 with the Lys48-based polyubiquitin tag required for proteasomal degradation (Herman-Bachinsky et al., 2007). Despite these interesting observations however, this scenario seems improbable given that Diap2 null mutant flies, recently generated by two independent groups, exhibit essentially no apoptotic phenotype (Huh et al., 2007; Leulier et al., 2006).

It is clear that the RING is an important regulatory domain for IAP stability but the precise mechanisms and role of this regulation remain elusive at present. Some authors have questioned the importance of IAP degradation for apoptosis regulation all together, pointing out that IAP-antagonist can inhibit IAPs and promote caspase activation irrespective of whether or not IAPs are degraded (Ditzel and Meier, 2002). These authors suggest that Diap1 degradation is not a decisive event in the initiation of apoptosis, but rather a method to destroy low levels of unscheduled IBM protein production in healthy non-apoptotic cells. Further study of IAPs and their interacting proteins is required to clarify the role of RING domains in apoptosis regulation.

Mechanisms of IAP Mediated Caspase Inhibition

Of the eight known human BIR containing proteins, current data indicates that Xiap may actually be the only bona fide caspase inhibitor. Xiap is a 497-amino acid polypeptide with a predicted mass of 57 kD that is ubiquitously expressed in human tissues. It has been the most intensely studied IAP and is also the most potent inhibitor of cell death *in vitro* (Duckett et al., 1996; Holcik et al., 2001; Listen et al., 1996). Given the high sequence and domain conservation amongst IAPs, it had been assumed that they all neutralize active caspases through the same BIR-dependent mechanism. Though there are indeed conserved aspects of this mechanism, recent biochemical and structural studies have precisely mapped the elements of Xiap required for caspase inhibition and surprisingly some of these elements are not conserved among IAPs. This has precluded a universal mechanism of inhibition by this protein family and it is now appreciated that IAPs can inhibit caspases and apoptosis through a variety of distinct mechanisms (Tenev et al., 2005).

Employing a unique strategy, which differs from that described previously for viral caspase inhibitors, both the BIR2 and BIR3 domains of Xiap use a two-site binding mechanism for potent inhibition of their respective caspases. One binding site is a conserved surface groove

characteristic of many BIR domains that has a preference for binding the extreme N terminus of short peptides of defined sequence. The peptide sequence preferred by BIR domains, AXPX where X represents a hydrophobic amino acid, is termed the IAP-binding motif (IBM) (Shi, 2002). Activation of Caspase-3, Caspase-7 and Caspase-9 involves proteolytic processing that generates such an IBM in the small-subunit amino terminus of the caspase and crystal structures have confirmed that Xiap BIR domains bind to this IBM via its conserved IBM-interacting surface groove. This conserved interaction surface of Xiap, referred to as an exosite-anchoring motif is not sufficient for potent caspase inhibition however. A second non-conserved interaction between Xiap and caspases is required. For inhibition of effector Caspases-3 and 7 by BIR2, residues directly preceding the BIR2 domain provide such an interaction. This peptide loop stretches across the catalytic-binding cleft of the caspase in a reverse orientation relative to that of a substrate protein thereby generating a steric blockade prohibitive of substrate binding (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001). This unusual reverse-binding mechanism had previously been described for members of the papain family of proteases which use their own amino terminal prosegment to inhibit their enzyme activity (Coulombe et al., 1996). In the case of Caspase-9, rather than

targeting the enzyme active site directly, the BIR3 domain of Xiap abolishes activity using a fundamentally different mechanism. Structural analysis of a BIR3/Caspase-9 complex has revealed that a helix found immediately after the BIR3 domain packs against the dimer interface of Caspase-9, sequestering Caspase-9 in a monomeric state and forcing the protease into a dormant conformation with a collapsed active site (Shiozaki et al., 2003). Xiap is the first example of a natural protease inhibitor that uses this kind of allosteric mechanism (Eckelman et al., 2006).

The closest paralogues of Xiap, Ciap1 and Ciap2, also contain three BIR domains and a RING domain and similar to Xiap, Ciap overexpression protects cells from apoptosis (Listen et al., 1996; Uren et al., 1996). Ciap1 and Ciap2 also contain a CARD domain and were originally identified through their ability to interact directly with the TNF receptor-associated factor TRAF2 (Rothe et al., 1995). The BIR domains of Ciaps contain IBM-interacting grooves that are highly conserved with those in Xiap and Ciaps can bind caspases *in vitro*. However critical residues surrounding the BIR domains as revealed by the crystal structures of Xiap are not conserved in Ciaps and as a consequence they are incapable of directly inhibiting the enzymatic activity of caspases (Eckelman and Salvesen, 2006). On the basis of these structural arguments, it is probable that the other human BIR-

containing proteins, ML-Iap, Ilp2, Naip, Survivin and Bruce are not direct caspase inhibitors either. In several instances this has been demonstrated. Vucic and colleagues showed that ML-Iap is not a tight inhibitor of Caspase-9, Shin and colleagues have shown that Ilp2 cannot inhibit Caspase 9 in a physiological way on its own and Survivin is now thought to primarily be a regulator of the mitotic spindle (Lens et al., 2006; Shin et al., 2005; Vucic et al., 2005). Earlier studies concluding that IAPs other than Xiap were direct caspase inhibitors have largely been revised in light of problematic experimental techniques, principally concerning the use of GST tags.

The mechanisms by which Ciaps and other mammalian IAPs attenuate apoptosis possibly include: binding to IAP antagonists to reduce the amount available to antagonize Xiap, influencing signaling by NF-κB and MAP kinases, or targeting caspases for ubiquitylation and proteasomal degradation (Tenev et al., 2005). For example, through TRAF2 interactions, Ciaps are recruited to TNFR1 and TNFR2 associated complexes where they regulate receptor-mediated apoptosis via modulation of NF-κB activity and suppression of Caspase-8 activation (Micheau and Tschopp, 2003a; Shu et al., 1996; Wang et al., 1998). This interaction is conferred by the first two alpha-helices in the BIR1 domain of Ciap1 and Ciap2 (Samuel et al., 2006). Engagement of TNFR2 by TNF-α results in TRAF2 ubiquitination in a

manner that depends on the RING domain of Ciap1 (Li et al., 2002). Ciap1 and Ciap2 also promote proteasomal degradation of NF-κB inducing kinase (NIK), a highly labile ser/thr kinase that is a critical regulator of the noncanonical NF-κB pathway (Varfolomeev et al., 2007). Similarly, Xiap has been demonstrated to induce NF-κB and MAP kinase activation in a BIR1 dependent manner during TGF-ß and BMP receptor signaling through engagement of the NF-κB regulator TAB1 (Lu et al., 2007). Xiap has also recently been shown to directly bind copper and to be involved in copper homeostasis (Mufti et al., 2006). It is clear from these and other studies that IAPs are complex molecules with the capability to impinge on cell survival at multiple points in various signaling pathways.

Since knockout studies of mammalian IAPs (Xiap,Ciap1 and Ciap2) have failed to reveal significant apoptosis phenotypes, possibly due to redundancy or compensatory mechanisms, the strongest *in vivo* evidence for a direct role of IAPs in the regulation of caspases and apoptosis comes from genetic studies in the fruit fly (Conte et al., 2006; Conze et al., 2005; Harlin et al., 2001). *Drosophila* contains four IAPs of which Diap1 appears to be the most critical. Genetic loss of *diap1* leads to uncontrolled caspase activation, resulting in premature and widespread unscheduled apoptosis which culminates in embryonic lethality (Goyal et al., 2000; Wang et al.,

1999). This suggests that *diap1* provides an essential requirement for the inhibition of apoptosis and is consistent with the fact that *diap1* has been shown to directly interact with at least three *Drosophila* caspases, the apical caspase Dronc and the effector caspases Drice and Dcp-1 (Meier et al., 2000b; Zachariou et al., 2003).

In contrast to Xiap, structural data for Diap1, which contains only two BIRs and a RING, is limited to complexes containing the BIR1 domain bound to a ten-residue peptide derived from the N terminus of Rpr or Grim, and the BIR2 domain alone or bound to a ten-residue peptide derived from the N-terminus of Hid or Grim. In addition, the BIR2 domain of Diap1 has been crystallized with residues 114-123 of the initiator caspase Dronc. These structures reveal that the Diap1 BIR motifs contain the same conserved IBM-interacting surface groove found in Xiap and that this pocket is the site for mutually exclusive contact between caspases and the RHG proteins. In a manner reminiscent of Xiap, Diap1 requires non-conserved residues adjacent to its BIR1 domain to efficiently bind caspases. Biochemical data indicate that the BIR1 domain and the adjacent carboxy-terminal linker region of Diap1 are necessary and sufficient for direct inhibition of the effector caspases Drice and Dcp-1 (Tenev et al., 2005; Yan et al., 2004). Due to a lack of structural information for Diap1 complexes,

the precise molecular mechanism of this inhibition remains unknown. It may be that Xiap and processed Diap1 use a similar two-site binding strategy for potent inhibition of effector caspases, both using a BIR domain as a platform to correctly deploy an adjacent inhibitory peptide.

The mechanism for inhibition of the initiator caspase Dronc by Diap1, however, is completely distinct from that of Xiap mediated caspase-9 inhibition. First, Diap1 binds Dronc not via an N-terminal IBM motif, but through a 12 amino acid fragment between the CARD and the protease domain of Dronc (Chai et al., 2003). Consequently, Diap1 is uniquely able to bind both active Dronc and the unprocessed Dronc zymogen. Secondly, Diap1 does not directly inhibit Dronc activity, but rather regulates the activity of Dronc through a mechanism that is dependent on its RING finger. Strong *in vivo* evidence has demonstrated that the E3 ubiquitin ligase activity of Diap1 is required for Dronc ubiquitination and is indispensable for Dronc inhibition and apoptosis regulation (Wilson et al., 2002).

The activity of Diap1 itself may be regulated in several ways not yet observed for mammalian IAPs. Diap1 contains an N-terminal fragment that some authors believe is autoinhibitory and must first be cleaved before Diap1 can interact with and inhibit effector caspases. According to this model, cleavage of the N-terminus not only renders Diap1 competent for

caspase binding, but it also converts Diap1 into a highly unstable, Asn-bearing N-degron of the N-end rule degradation pathway that is rapidly degraded (Ditzel et al., 2003).

Far less is known about the other *Drosophila* IAPs, Diap2, dBruce and Deterin and evidence implicating these genes in apoptosis is largely limited to overexpression studies (Hay et al., 1995; Jones et al., 2000; Vernooy et al., 2002). *Dbruce* is the *Drosophila* ortholog of the mouse *bruce* and human *apollon* genes and is predicted to encode an enormous protein of 4852 amino acids with an N-terminal BIR domain and a C-terminal ubiquitin conjugation (UBC) domain. Some *in vivo* data supporting a role for dBruce as an apoptotic regulator has come from genetic screens designed to identify components of the *rpr* and *hid* apoptotic pathways. These screens isolated 11 loss-of-function alleles of *dbruce* that enhance *rpr*- and *grim*- but not *hid*-induced cell death and one gain-of-function allele that suppresses *hid*- but not *rpr*- or *grim*-induced death (Agapite, 2002). This differential pattern of enhancement and suppression is highly reminiscent of that observed for Diap1 mutants, raising the intriguing possibility that perhaps Dbruce and Diap1 function together in an E2/E3 complex to ubiquitinate target proteins. Unlike Diap1 however, dBruce null mutants are male sterile but viable, indicating that dBbruce has a more

restricted role than Diap1 during development. Intriguingly, dBruce has recently been shown to bind Klh10, a component of the testis-specific Cullin-3-Roc1b-dependent ubiquitin ligase complex that is required for caspase activation in spermatids (Arama et al., 2007). This interaction led the authors to speculate that dBruce may be the IAP that spatially and temporally restricts caspase activation during sperm differentiation and that the Cullin-3 enzyme complex activates caspases by degrading dBruce in response to developmental cues.

The physiological function of Diap2 has been investigated recently by two groups that generated *diap2* null alleles (Huh et al., 2007; Leulier et al., 2006). *Diap2* mutant animals develop normally, are fully viable and show no defects in developmental or stress-induced apoptosis, suggesting that *diap2* is dispensable for cell survival. Instead, *diap2* was found to be essential for the innate immune response to Gram-negative bacterial infection. *Drosophila* melanogaster lacks an adaptive immune system and relies exclusively on innate immune reactions for its defense against microbial infection. The immune deficiency (Imd) signaling pathway is activated in response to gram-negative bacteria and triggers nuclear translocation of the NF-κB like transcription factor Relish, which in turn induces expression of antibacterial genes (Hoffmann, 2003). Loss of Diap2

results in a profound defect in Relish nuclear translocation and antimicrobial peptide (AMP) expression, rendering mutant flies acutely sensitive to infection by gram-negative bacteria. These results suggest that Diap2, like the Ciaps in mammals, may function as an E3 ubiquitin ligase in a receptor signaling cascade rather than as an inhibitor of caspase activation.

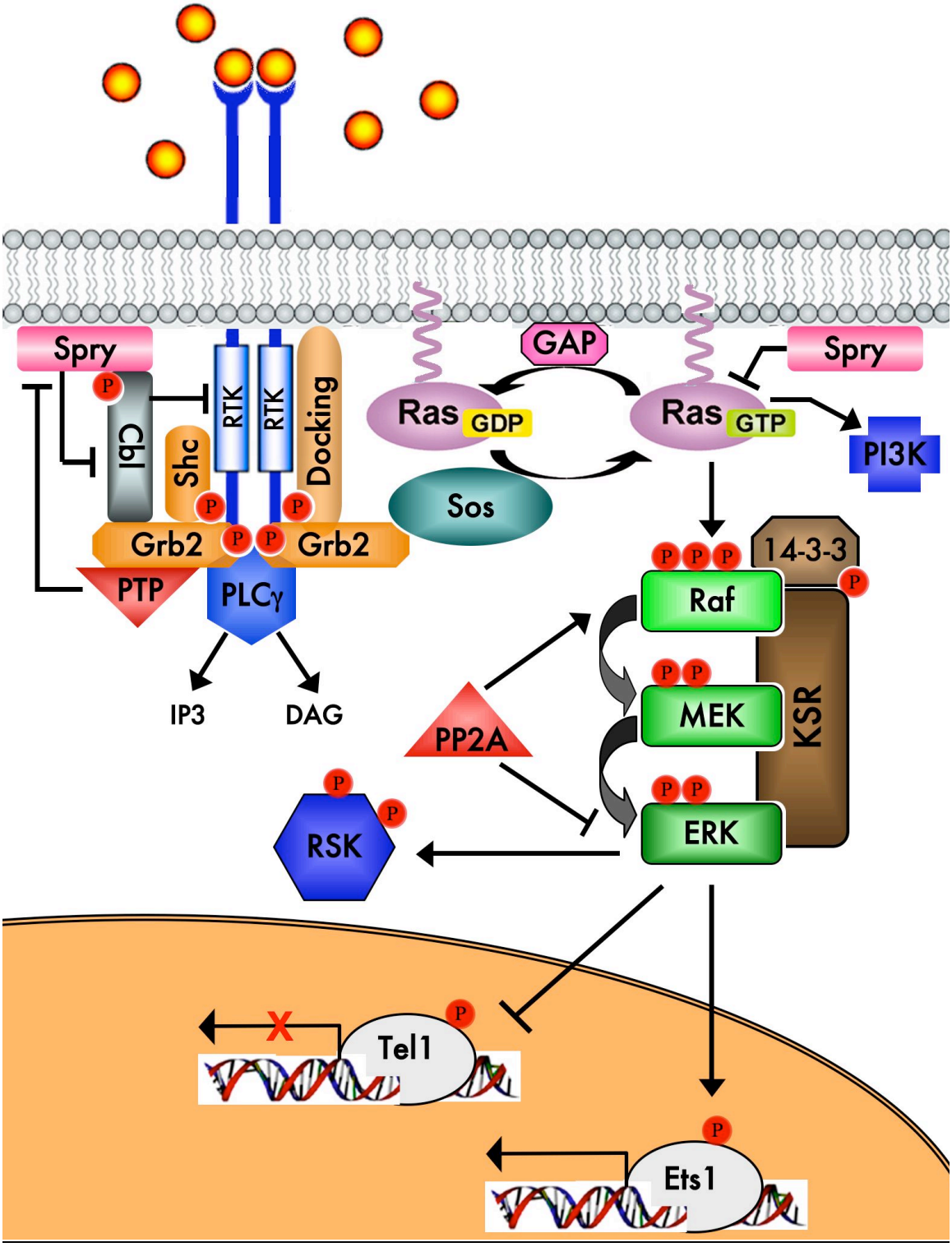
The MAPK Pathway and Apoptosis

Cell survival is regulated by a multitude of extracellular and intracellular signals and in most tissues, suppression of apoptosis is dependent upon a constant supply of exogenous survival signals. These signals are furnished by neighboring cells and the extracellular matrix as either immobilized or soluble peptide factors (Raff, 1992). Growth factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) are classic examples of survival factors that inactivate the intrinsic cell death program, thereby promoting cell survival. These growth factors bind to and activate receptor tyrosine kinases (RTKs), such as the EGF receptor (EGFR), at the cell surface and initiate a survival signal that is propagated throughout the cell via a number of effector pathways (Downward, 1998) (Fig 1.4). The mitogen-activated protein kinase (MAPK) cascade is one such effector pathway that responds to extracellular cues and transduces

signals from the cell surface to the nucleus via a protein phosphorelay system consisting of three sequentially activated kinases. This succession of kinases provides a signaling framework that is amenable to feedback regulation and signal amplification (Fig. 1.4).

MAPK signaling cascades are known to modulate a number of critical cellular activities including gene expression, mitosis, proliferation, motility, metabolism and programmed cell death (Johnson and Lapadat, 2002). Among the three subfamilies of MAPK modules that have been well characterized in multicellular organisms, it is the extracellular signal-regulated kinase (ERK) branch which has been implicated in promoting cell survival (Wada and Penninger, 2004). The canonical ERK-MAPK module consists of three successive serine/threonine kinases; Raf, MEK and ERK. Once activated, Raf initiates a phosphorylation cascade, whereby Raf phosphorylates and activates MEK, and MEK in turn phosphorylates and activates ERK. Activated ERKs then phosphorylate and regulate the activities more than 160 proteins, the majority of which are nuclear proteins, including several transcription factors such as c-Myc, Elk-1 and Ets-2 (Yoon and Seger, 2006). A central regulator of this signal transduction relay is the small GTPase Ras, which acts as a molecular switch in response to RTK activity to directly control the activity of Raf and therefore the MAPK

Fig 1.4. The RTK/Ras/MAPK signaling pathway. This illustration of the canonical MAPK signaling pathway highlights regulatory components that are conserved between humans and flies. Conserved RTK subfamilies that are known to employ canonical MAPK signaling include the Egfr, Fgfr, Pdgfr, Insr, Vegfr, Alk, Eph, Ret and Tie receptor subfamilies. Different receptors use various combinations of adaptor (Shc) and docking proteins such as Irs1 (Dme: Chico) to recruit Grb2 (Dme: Drk) and Sos to ligand activated receptor complexes. Sos catalyzes nucleotide exchange on Ras-GDP converting it to its active Ras-GTP form. Active Ras engages several downstream signaling molecules including the MAPKKK, Raf (Dme: Pole hole, Phl) and PI3-Kinase (Dme: PI3K92E/Dp110). Active Raf in turn phosphorylates and activates MEK (Dme: Dsor1), which phosphorylates and activates ERK (Dme: Rolled, rl). KSR and 14-3-3 (Dme: leonardo) are scaffolding proteins that facilitate Ras-dependent ERK cascade activation at the plasma membrane. Once active, ERK phosphorylates a large number of substrates including Ribosomal Protein S6 Kinase (RSK) in the cytosol and a number of transcription factors in the nucleus, including the activator Ets1 (Dme: Pointed, Pnt) and the repressor Tel1 (Dme: Yan). GTPase-activating proteins (GAPs) such as RasA3 (Dme: Gap1) and Nf1 terminate Ras signaling by accelerating the conversion of Ras-GTP to its inactive Ras-GDP form. Active Ras is also antagonized by Sprouty (Spry) proteins and active ERK is inactivated by the Ser/Thr phosphatase PP2A (Dme: Microtubule Star, Mts). PTP, protein tyrosine phosphatase. Dme above refers to the drosophila homolog if the component name is different than its mammalian counterpart.


Spry
Cbl
Shc
RTK
RTK
Docking
Grb2
Grb2
PTP
PLCγ
IP3
DAG
GAP
Ras GDP
Ras GTP
Sos
Spry
PI3K
14-3-3
Raf
MEK
ERK
KSR
PP2A
RSK
Tel1
Ets1

module. Recently, a number of ERK scaffolding proteins and signaling modulators have also been identified that play critical roles in determining the strength, duration and location of MAPK signaling (Fig 1.4). Together, these factors contribute to the diversity of biological responses generated by the RTK/MAPK signaling axis (McKay and Morrison, 2007).

Given the critical involvement of the ERK-MAPK module in transmitting cell proliferation and anti-apoptotic signals, the overwhelming frequency in which this pathway is aberrantly activated in human cancer is perhaps not too surprising. Studies using genetic or pharmacologic approaches have shown that the ERK-MAPK signaling cascade is required for the transforming activities of Ras, the most frequently mutated oncogene in human cancers, as well as for tumorigenesis associated with mutationally activated and/or overexpressed EGFR. Moreover, mutationally activated Raf has been identified in a considerable fraction and variety of human tumours (Davies et al., 2002). These observations suggest a critical role for MAPK activation in oncogenesis, making it an appealing pathway for drug development. This has stimulated intensive efforts by the research community and pharmaceutical industry to develop inhibitors of ERK-MAPK signaling for cancer treatment (McCubrey et al., 2007).

The MAPK signalling pathways are now understood in great detail at the molecular level as a result of two decades of intense study employing genetics, molecular and cellular biology, and encompassing organisms from yeast to man (Fig 1.4). Indeed, the *Drosophila* EGFR/MAPK pathway provides one of the best-characterised examples of a signaling cascade currently known. The high degree of homology between components of the *Drosophila* and mammalian MAPK signaling pathways has permitted many unique insights derived from examination of this pathway in *Drosophila* to be extrapolated to vertebrate systems. For example, the Sprouty (Spry) family of proteins is a highly conserved group of negative feedback loop modulators of MAPK activation that was originally discovered in *Drosophila*. Four mammalian orthologs of Spry have subesequently been identified (Hanafusa et al., 2002).

As in mammalian systems, the *Drosophila* ERK-MAPK module mediates a plethora of cellular functions during development, including proliferation, survival, cell fate choice and differentiation. Though activation of ERK-MAPK is has long been known to protect cells from apoptosis by suppressing the intrinsic cell death program, the molecular mechanisms by which this occurs remain poorly understood. New insights into understanding the regulation of apoptosis by survival signaling

pathways, however, has recently come from several genetic screens conducted in *Drosophila*, two of which are described in the next chapter. These studies revealed that activation of MAPK signaling inhibits the proapoptotic activity of the cell death inducer, *hid,* both by direct phosphorylation of the Hid protein and by downregulation of *hid* mRNA expression. This defines a novel mechanism by which MAPK signaling inactivates a critical component of the apoptotic machinery (Bergmann et al., 1998; Kurada and White, 1998).

It is now exceedingly clear that apoptosis and its regulation are highly relevant to many human diseases. Genetic lesions leading to diminished apoptosis play a general role in tumorigenesis and many cancer therapies result in elevated levels of cancer cell apoptosis (Ziegler and Kung, 2008). Over the course of the last two decades, many apoptosis effector mechanisms have been extensively characterized, allowing for the construction of elegant molecular models to explain the regulation of apoptosis (Fig 1.5). Nevertheless, our understanding of the pathways that signal and control developmental cell death is far from complete. Caspases have taken a leading role as key regulators of apoptosis, but caspase activation is not synonymous with cell death. A growing appreciation for the non-apoptotic roles of caspases in a number of critical cellular processes

such as differentiation, proliferation and cell migration is driving current efforts to understand how caspase activity is regulated and integrated to achieve these varied outcomes (Kuranaga and Miura, 2007). How, for example, is the extent of caspase activation within a cell temporally and spatially modulated to permit such specialized feats as dendritic pruning and spermatid individualization without eliciting self destruction (Arama et al., 2003; Kuo et al., 2006)? In general, we still do not understand very well how a particular cell chooses between life and death during development or disease, but it is clear that a multitude of distinct mechanisms are used to tightly regulate this decision. Examples for which some insight into the regulation of apoptosis has been garnered include; the transcriptional modulation of cell death proteins such as *egl-1*, activation of caspases by oligomeric complex formation, inhibition and degradation of caspases by IAPs and the ubiquitin–proteasome system, activation of caspases through inhibition of IAPs by RHG proteins and regulation of core cell death proteins by phosphorylation, microRNAs and modulation of subcellular localization (Domingos and Steller, 2007). Finally, recent evidence indicates that apoptotic cells themselves actively communicate with their cellular environment to stimulate cell proliferation and tissue regeneration in a process known as compensatory proliferation (Ryoo et al., 2004).

The aim of the research presented here was to use genetic and biochemical approaches in *Drosophila* to further elucidate the molecular mechanisms regulating the core, evolutionarily conserved caspase-dependent cell death pathway. Towards this end, we present here the partial characterization of a novel CARD containing serine/threonine kinase as putative regulator of apotosis and in addition we describe the identification and characterization of the first endogenous gain-of-function mutation in *Drosophila ras1*. We demonstrate biochemically that this mutant produces a Ras protein with deficient GTPase activity and therefore an enhanced signaling capacity. The phenotypic consequence of this in various developmental contexts is investigated.

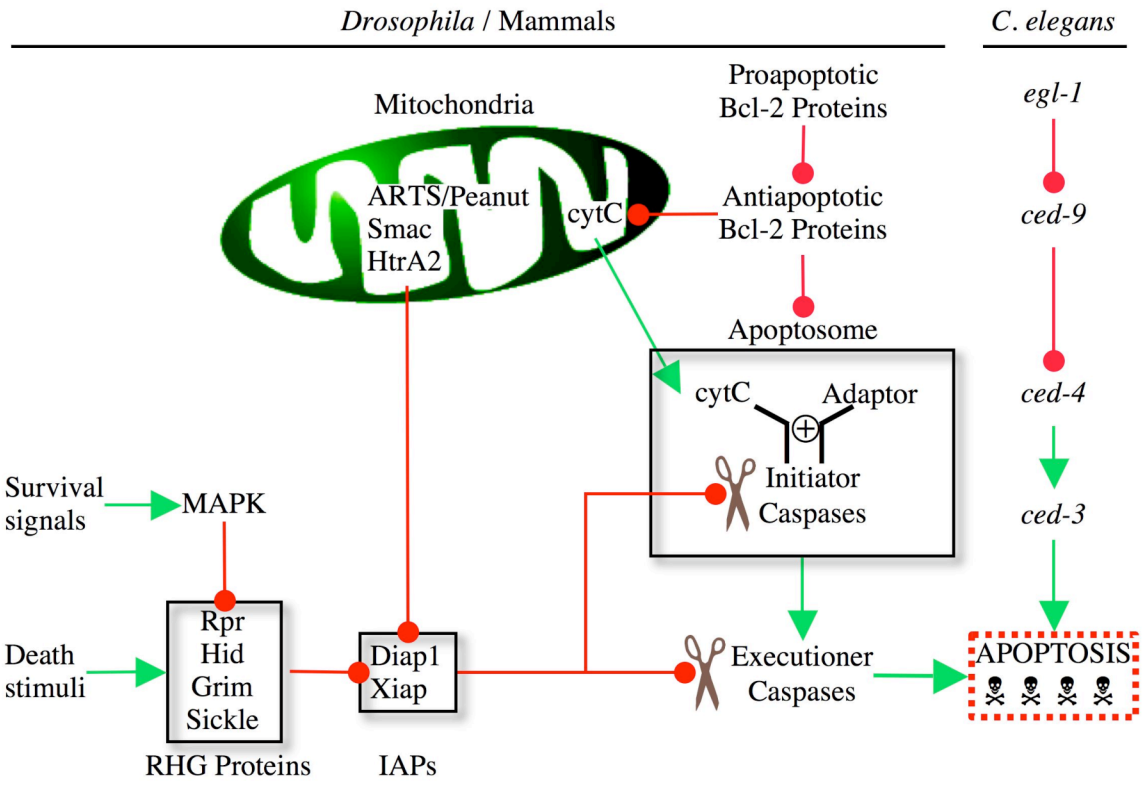


Fig 1.5. The "Gas and Break" model of intrinsic apoptosis control. The central components of the execution phase of apoptosis in worms, flies and mammals are members of the caspase protease family. In C. elegans the adaptor Ced-4 promotes activation of the caspase Ced-3. As in mammals, upstream decisions are integrated by pro- and anti-apoptotic Bcl-2 protein family members. In mammals and flies, two fundamental control points regulate caspase activation. The figure highlights the forward drive for zymogen activation by oligomerization of initiator caspases within the apoptosome (the Gas) and the inhibition of active caspases by IAPs (the Brakes). IAPs can be derepressed to release caspase activity by species specific IAP antagonists such as RHG proteins in flies or intermembrane space mitochondrial proteins discharged into the cytosol in mammals.

CHAPTER 2.

Preliminary Characterizations of the *GMR-hid* Suppressor *Su(21-3s)* and the Predicted Ser/Thr Kinase CG11870

The work presented in this chapter was built upon the efforts of Julie Agapite, Kim McCall, Chris Hynds and Andreas Bergmann who conducted the genetic screen from which the *Su(21-3s)* mutant is originally derived. All other data presented here represents original work.

Summary

In *Drosophila melanogaster*, the induction of apoptosis requires the activity of three closely related genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*. The proteins encoded by these genes induce apoptosis in part by inhibiting the anti-apoptotic activity of the caspase suppressor Diap1 and lead to activation of an evolutionarily conserved cell death pathway. Ectopic expression of *rpr*, *hid* or *grim* in the developing *Drosophila* eye elicits apoptosis and gives rise to a reduced eye phenotype. Genetic screens designed to isolate modifiers of this phenotype have been extremely successful at identifying genes that regulate apoptosis. In one such screen, a mutant, denoted *Su(21-3s)*, was recovered as a potent dominant suppressor of *hid* induced phenotypes. We sought to further characterize this mutant and to identify the affected gene. Preliminary data pointed to the unknown gene CG11870, predicted to encode a protein Ser/Thr kinase which, interestingly, is also reported to contain a putative caspase recruitment domain (CARD). Our analysis, reported here, reveals that the *Su(21-3s)* suppressor phenotype is most likely not due to a mutation in CG11870. Nevertheless, our partial characterization of this novel CARD containing kinase exposed a possible interaction with the *hid* cell death pathway.

Introduction

Programmed cell death is a fundamental aspect of metazoan development. Regulated cell death allows an organism to tightly control cell numbers and tissue size, and to protect itself from rogue cells that threaten homeostasis (Hengartner 2000). For example, apoptosis, a morphologically distinct and commonly observed form of programmed cell death, is used defensively by organisms to eliminate cells infected by viruses, cells undergoing unregulated proliferation and auto-reactive lymphocytes.

Many of the cellular changes associated with apoptosis are due to the actions of an evolutionarily conserved family of cysteinyl proteases termed caspases (Thornberry and Lazebnik 1998). The critical importance of caspases in the execution of apoptosis was initially revealed by the discovery that the *C. elegans* cell death gene *ced-3* encodes a protein similar to the mammalian caspase, Interleukin-1β-converting enzyme (ICE) (Yuan 1993.) This family of proteases has since grown to include 11 members in humans, 10 in mice, 7 in *Drosophila* and 4 in *C. elegans* (Aravind 2001, Shaham 1998.) Caspases are initially synthesized as inactive (or weakly active) zymogens, the activation of which is tightly regulated by both positive and negative inputs (Danial and Korsmeyer, 2004).

Caspases can broadly be separated into the initiator caspase group and the effector caspase group on the basis of domain architecture and physiologic function (Lincz 1998). Effector caspases contain a short prodomain, are activated by proteolytic cleavage and are thought to act downstream of initiator caspases to execute the cell death process by cleaving a large number of cellular proteins. In contrast, initiator caspases contain long prodomains that harbor regulatory motifs such as the caspase recruitment domain (CARD), are activated by an induced-proximity mechanism following recruitment into oligomeric complexes and are thought to act further upstream by cleaving a relatively limited number of substrates (Degterev et al., 2003).

The activity of caspases is negatively regulated by a second highly conserved class of proteins with members in all eukaryotic species, termed inhibitor of apoptosis proteins (IAPs) (Crook et al., 1993). Inhibition of caspases by IAPs is achieved through a number of diverse mechanisms including direct binding of IAPs to caspase catalytic sites and by targeting caspases for ubiquitinylation and proteasomal degradation (Tenev et al., 2005). Compelling *in vivo* evidence substantiating a role for IAPs in apoptosis regulation has come from genetic studies in *Drosophila* which

revealed that loss of *diap1* leads to uncontrolled caspase activation and widespread induction of apoptosis (Goyal et al., 2000; Wang et al., 1999).

Genetic analysis of programmed cell death in *Drosophila* also led to the discovery of three closely linked genes, *rpr, grim* and *hid*, whose gene products are required for the activation of developmental cell death that normally occurs during embryogenesis as well as the ectopic death induced by x-rays or developmental abnormalities (Chen et al., 1996; Grether et al., 1995; White et al., 1994). Although the proteins encoded by these genes do not show significant homology to each other or other known proteins, they do share a conserved 14 amino acid stretch at their N-termini (Chen 1996.) This conserved sequence, termed the RHG motif, has been shown in a number of paradigms to induce caspase dependent apoptosis in part by interacting with and inhibiting Diap1 (Vucic 1998, McCarthy and Dixit 1998, Vucic 1998).

To further define the mechanisms by which *rpr*, *hid* and *grim* activate caspases and induce cell death, Agapite et al. conducted a genetic screen in *Drosophila* to isolate dominant modifiers of *hid* and *rpr* induced eye phenotypes (Agapite, 2002). Approximately 500,000 flies were screened and 167 dominant modifiers recovered. Among these were components of the Ras/MAPK pathway and both gain and loss of function alleles of *diap1*

and *dbruce*. One mutant, designated *Su(21-3s)*, was recovered as a strong dominant suppressor of *hid* induced phenotypes. Contrary to what was initially reported in the screen, our analysis here found *Su(21-3s)* flies to be homozygous male and female sterile. Preliminary characterization of this mutant also identified a polymorphism in the previously uncharacterized gene CG11870, predicted to encode a putative CARD containing Ser/Thr kinase. Given the known role of CARD domains in caspase regulation, we sought to further characterize CG11870 with the aim of identifying a novel regulatory element in apoptosis. We have since determined that the suppressor phenotype of *Su(21-3s)* does not map to CG11870 but nevertheless present an initial characterization of this predicted kinase and present evidence that CG11870 may in some contexts negatively regulate *hid* induced apoptosis.

Results

Recovery of *Su(21-3s)* as a dominant suppressor of *GMR-hid* induced apoptosis

Eye-specific expression of *hid* or *rpr* under control of the GMR promoter induces apoptosis and results in a dosage sensitive eye ablation phenotype (Grether et al., 1995). Dominant modifier screens are designed to

detect pathway components for which small perturbations in gene dosage can alter such a sensitized phenotype. This strategy allows for the recovery of both loss-of-function and gain-of-function mutations and provides a facile method for screening a large number of genomes. This approach has been successful in defining a genetic pathway for R7 cell fate determination and identifying several core cell death genes (Dickson et al., 1996; Hay et al., 1995; Rebay et al., 2000; Simon et al., 1991).

The *Su(21-3s)* mutation was originally isolated in mutagenesis screens conducted by Agapite *et al*. as described in Fig. 1. The results are briefly summarized below for reference and presented in Tables 1 and 2 (Agapite, 2002).

Approximately 170,000 F1 progeny of ENU and EMS mutagenized *GMR-rpr* flies were screened for dominant modifiers of the rough eye phenotype leading to the recovery of 25 enhancers and 5 suppressors (Table 1). Similarly, about 300,000 F1 progeny of ENU, EMS and x-ray mutagenized flies were screened for suppression of the *GMR-hid* phenotype with the recovery of 128 dominant suppressors (Table 2). In sum total, 158 dominant modifiers were identified in these screens.

Modifiers were mapped by segregation, balanced and recessive phenotypes were assessed. Additionally, modifiers on the 3rd chromosome

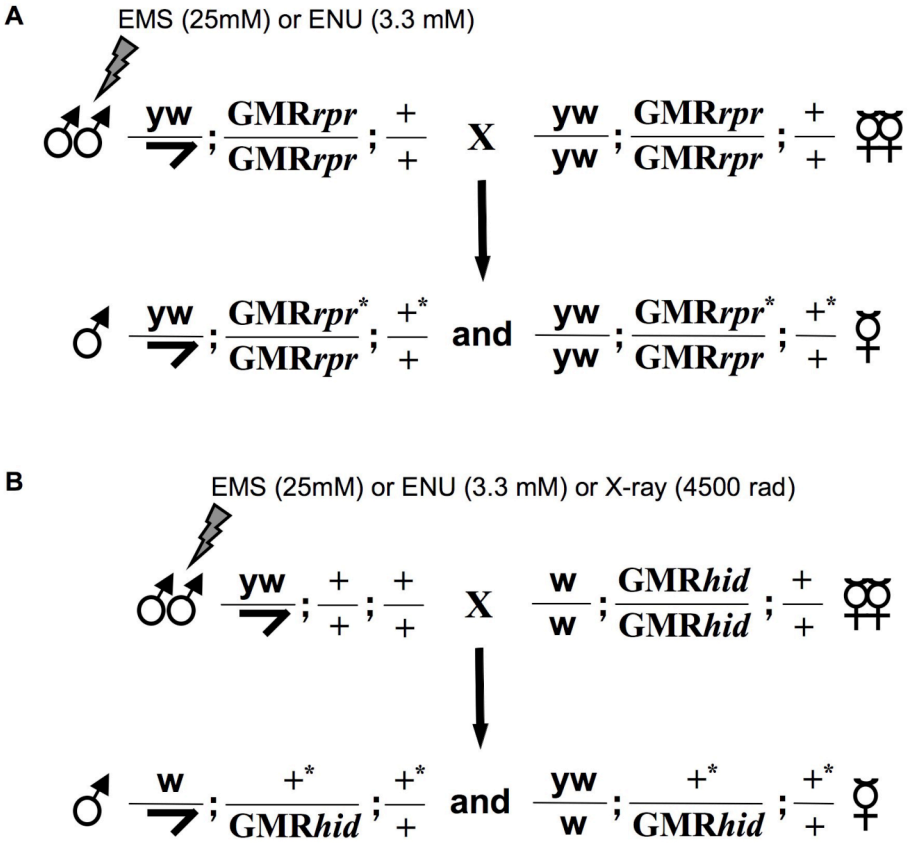


Fig. 2.1. Scheme for the dominant modifier screens conducted by Agapite *et al*. (A) *GMR-rpr* screen. *yw*; *GMR-rpr*^{81} homozygous males were fed either 0.25mg/ml ENU or 25 mM EMS. F1 progeny, were screened for suppression or enhancement of the parental rough eye phenotype. Of the 170,000 F1 progeny screened, ~95% derived from ENU treated males, (B) *GMR-hid* screen. *yw* males were treated as above or with 4500 rad x-rays and then crossed to *GMR-hid*^{10} homozygous females. F1 progeny were screened for suppression of the *GMR*-hid^{10} rough eye phenotype. Of the 300,000 F1 progeny screened, ~49% derived from EMS treated males, ~49% from x-ray treated males and 2% from ENU treated males.

Table 1. *GMRrpr* modifiers: Summary of genetic interactions

Groups	Map pos.	No. of alleles	Pheno-type	*GMR-rpr*	*GMR-hid*	*GMR-grim*	*GMR-rho1*	*GMR-phyl*
Star	21E4	13-2e 4	SemLth Lethal	Enh Enh	ND ND	ND ND	ND ND	ND ND
GMR-rpr81		2 11-1e 7-2s	Viable Rep Lethal	Sup Enh Sup	ND ND ND	ND ND ND	ND ND ND	ND ND ND
diap1	72D1	6-3s 11-3e	Viable Lethal	Sup Enh	Sup Enh	ND Enh	ND --	-- --
dBruce	86A7	10 2-3e	Mst Lethal	Enh Enh	-- --	Enh Enh	-- --	-- --
Delta	92A1	10-12e	Lethal	Enh	Enh	Enh	Enh	Enh
Other	*-th-st-* *sr-e* *sr-e*	5 5-2s 5-4e	Viable Viable Viable	Enh Sup Enh	-- Sup Enh	Enh ND Enh	-- ND Enh	-- Sup Lethal

Complementation groups are named for the known gene to which they correspond. The group named "other" consists of mutants that could not be placed into a complementation group. *-th-st-* indicates that the mutation was roughly mapped by meiotic recombination around the markers *th* and *st* and may be located on either side, whereas *sr-e* indicates that the mutation maps between *sr* and *e*. Alleles with the same map position and similar phenotypes are grouped together for simplicity. Single alleles are named. Sup, suppressor; Enh, enhancer; --, no effect; ND, not done; Mst, male sterile; SemLth, semi lethal.

Table 2. *GMR-hid* suppressors: Summary of genetic interactions								
Groups	**Map pos.**	**Alleles**	**Pheno-type**	**GMR-*rpr***	**GMR-*grim***	**GMR-*phyl***	**hs-*hid***	***vg-// hid***
sprouty	63D2	23-14s	Lethal	Sup	Sup	--	Sup	--
		25-1s	Lethal	Sup	ND	--	Sup	--
		27-1s	Lethal	Sup	Sup	W.su	Sup	ND
		28-4s	Lethal	Sup	Sup	W.su	Sup	--
		30-5s	Lethal	Sup	Sup	W.su	ND	--
Gap1	67C10	21-1s	Ro, wv	WS	Sup	--	Sup	--
		22-2s	Ro, wv	WS	Sup	--	Sup	--
		23-9s	Ro, wv	ND	ND	ND	Sup	--
		24-6s	Ro, wv	WS	Sup	W.su	Sup	--
		26-2s	Ro, wv	Sup	Sup	--	Sup	--
diap1	72D1	21-2s	SemLth	Sup	Sup	--	Sup	Sup
		21-4s	Lethal	Enh	ND	--	Sup	Sup
		22-8s	Lethal	Enh	Enh	ND	Sup	--
		23-4s	Lethal	Sup	Sup	W.su	Sup	Sup
		23-8s	Lethal	Sup	Sup	--	Sup	Sup
		33-1s	Lethal	Enh	Enh	W.en	Sup	Sup
		41-8s	Lethal	Enh	ND	--	Sup	ND
		45-2s	SemLth	Sup	ND	ND	ND	Sup
Su(GMR*hid*) 2A	2^{nd}	26-3s	Lethal	Sup	ND	ND	ND	ND
		32-1s	Lethal	Sup	ND	ND	ND	ND
		43-4s	Lethal	ND	ND	ND	ND	ND
Su(GMR)2A	2^{nd0}	22-1s	Lethal	Sup	ND	WS	ND	ND
		27-2s	Lethal	Sup	ND	WS	ND	ND
		29-4s	Lethal	Sup	ND	--	ND	ND
		30-2s	Lethal	Sup	ND	Sup	ND	ND
dBruce	86A7	23-6s	Lethal	Enh	Enh	W.en	Sup	Sup
glass	91A3	23-3s	Ro	Sup	Sup	Sup	--	--
Su(GMR*hid*) 3A	*sr-e* 3^{rd}	23-15s	Lethal	Sup	Sup	W.su	Sup	Sup
		29-2s	Lethal	Sup	ND	Sup	ND	--
Su(GMR*hid*) 3B	*sr-e* 3^{rd}	24-3s	Rep, ro	Sup	ND	WS	Sup	--
		38-5s	Rep, ro	Sup	ND	ND	ND	--
	-sr- *sr-e*	38-7s	Rep, ro	Sup	ND	Sup	ND	--
		38-8s	Rep, ro	ND	ND	Sup	ND	--
	-sr-e- *-sr-* *sr-e* 3^{rd}	38-11s	Rep, ro	Sup	ND	Sup	--	--
		38-13s	Rep, ro	Sup	ND	Sup	ND	--
		40-4s	Rep, ro	Sup	ND	ND	ND	--
		40-6s	Rep, ro	Sup	ND	Sup	ND	--
Su(GMR)3A	3^{rd}	24-9s	Lethal	Sup	ND	W.su	--	ND
		28-1s	Lethal	Sup	ND	W.su	--	--
		30-6s	Lethal	Sup	Sup	Lethal	ND	ND
		32-3s	Lethal	Sup	Sup	Sup	ND	ND

Table 2. *GMR-hid* suppressors: Summary of genetic interactions

		32-8s	Lethal	Sup	Sup	Sup	W.su	ND
Other	*-th-st-*	24-4s	Viable	ND	ND	ND	Sup	--
	th-st-	41-1s	Viable	ND	ND	ND	ND	--
	st-cu	27-17s	Lethal	ND	Sup	ND	W.su	ND
	-cu-	21-3s	Viable	Sup	Sup	--	Sup	Sup
	-cu-	39-1s	Ro	Sup	ND	--	Sup	--
	cu-sr	23-5s	Lethal	Sup	Sup	W.su	W.su	W.su
	-sr-	22-6s	Wv	Sup	ND	Sup	Sup	--
	-sr-	24-8s	Viable	Sup	ND	W.su	Sup	--
	-sr-	30-4s	Viable	ND	ND	ND	ND	--
	-sr-	41-4s	Ro	ND	ND	ND	ND	--
	sr-e	24-2s	Rep, ro	Sup	ND	Sup	W.su	--
	sr-e	28-7s	Rep, ro	Sup	ND	Sup	Sup	--
	sr-e	40-5s	Lethal	Sup	Sup	--	W.su	Sup
	sr-e	41-2s	Lethal	Sup	Sup	Sup	ND	Sup
	sr-e	41-6s	Ro	Sup	ND	Sup	ND	--
	sr-e	41-7s	Lethal	Sup	Sup	Sup	ND	Sup
	sr-e	43-1s	Wv	ND	ND	ND	ND	--
	sr-e	43-5s	Rep, ro	ND	ND	ND	ND	--

Legend is as for Table 1. *-th-st-*, *-cu-* and *-sr-* indicate that the mutation maps around the designated markers and may be located on either side. *st-cu*, *cu-sr* and *sr-e* indicate that the mutation maps between the designated markers. The mutation characterized in this study, *su(21-3s)*, is highlighted in yellow. Rep, reduced eye pigmentation; Ro, rough eye; Wv, extra wing veins; W.su, weak suppressor; W.en, weak enhancer; --, no effect; ND, not done; Sup, suppressor; Enh, enhancer; SemLth, semi lethal. *vg-//hid* refers to *vg-Gal4;UAS-hid*.

were roughly mapped by meiotic recombination. Complementation analysis using phenotype and map information placed 133 of the modifiers into 13 complementation groups. The remaining mutants represented single hits or had no recessive phenotype and could not be placed into a complementation group. The location and phenotype of some of these, however, suggest that they may be viable alleles of identified lethal complementation groups.

To enrich for mutants that specifically affect *rpr* and *hid* induced cell death, rather than expression from the GMR promoter or general eye development, modifiers were subjected to a panel of secondary screens (Tables 1 and 2). The effect of modifiers against *GMR-phyl* or *GMR-rho* induced eye phenotypes, which are unrelated to cell death, were assessed. It was surmised that death specific mutants would not affect these phenotypes whereas those affecting GMR promoter expression or eye development would (Chang et al., 1995; Hariharan et al., 1995). Conversely, mutants involving apoptosis genes were expected to modify cell death phenotypes in alternative contexts, while those affecting GMR promoter expression or eye development, whose effects should be eye specific, would not. To this end, dominant suppressors from the *GMR-hid* screen were tested for their ability to suppress the lethality induced by *hs-hid* or the ablated wing phenotype resulting from *vg-Gal4*, *UAS-hid* expression. Finally, to compare the

similarity of *rpr*, *hid* and *grim* induced cell death pathways, modifiers were tested against *GMR-rpr*, *GMR-hid* and *GMR-grim* phenotypes. These secondary screens allowed for the elimination of several complementation groups including *glass*, which encodes the transcription factor that drives GMR expression, *Su(GMR)2A* and *Su(GMR)3A*, which are known to indirectly and non-specifically affect GMR promoter expression, and *Su(GMR-hid)3A* and *Su(GMR-hid)3B*, which have not been assigned to previously characterized genes (Barrett et al., 1997; Moses and Rubin, 1991). Also eliminated were 4 alleles linked to the parental *GMR-rpr* transgene. The remaining mutants comprised a cell death enriched subset of modifiers consisting of 40 mutants that fall into 6 complementation groups, plus 18 single alleles.

Of the 6 complementation groups identified in these screens, 3 corresponded to genes that regulate EGF receptor (EGFR) signaling. Five loss-of-function (lof) alleles each of *gap1* and *sprouty*, both negative regulators of EGFR/MAPK signaling, were recovered as strong, *hid* specific suppressors. These mutants have been further characterized and were used to demonstrate that EGFR/MAPK signaling specifically inhibits the proapoptotic activity of Hid by direct phosphorylation and to provide a mechanistic link between survival signaling and the apoptotic machinery

(Bergmann et al., 1998). Five lof *Star* alleles were isolated as enhancers of *GMR-rpr*. *Star* is required for the correct processing of Spitz, a stimulatory ligand of EGFR (Shilo, 2005). Though EGFR/MAPK signaling does not directly impinge on Rpr activity, *star* lof alleles exhibit a dominant rough eye phenotype, perhaps in part due to a reduced suppression of endogenous Hid activity and consequently appear as enhancers of *GMR-rpr*.

Anticipated was the recovery of mutations in *diap1*, a known regulator of *hid* and *rpr* induced cell death and accordingly, 10 *diap1* alleles were isolated in these screens. Mutations in *diap1* included both loss-of-function (lof) alleles that enhance *rpr*, *hid* and *grim* induced death and two classes of gain-of-function (gof) alleles that either potently suppress death induced by all three RHG proteins or, alternatively, potently suppress *hid* induced death but enhance *rpr* and *grim* induced death. This latter class of gof mutants represents RING domain mutations in *diap1* and highlights a significant distinction between the *hid* pathway and the *rpr* and *grim* pathways. The *diap1* mutants isolated in these screens have been pivotal in several structure-function analyses employed to construct our current models of apoptosis and are described extensively elsewhere (Goyal et al., 2000; Ryoo et al., 2002; Wilson et al., 2002).

The two remaining complementation groups originally defined previously uncharacterized genes and *Su(GMR-hid)2A* remains as such. The other group, consisting of 12 alleles, was of particular interest because these mutants display a differential modulation of the *hid*, *rpr* and *grim* pathways in a manner reminiscent of *diap1* RING mutants, enhancing *GMR-rpr* and *GRM-grim* phenotypes but having no effect on or suppressing those of *GMR-hid*. These alleles were mapped using a combination of meiotic recombination, P-element induced male recombination and deficiency mapping to a 74 Kb interval on the right arm of the third chromosome. Two converging chromosome walks were then conducted to identify and clone *dbruce*. This enormous 4852 amino acid protein, containing an N-terminal BIR and C-terminal UBC domain, is the Drosophila ortholog of mouse Bruce and human Apollon (Hauser et al., 1998; Vernooy et al., 2002). The unique combination of a BIR domain and a UBC domain immediately suggested a model in which dBruce inhibits apoptosis by ubiquitinating, via its UBC domain, proapoptotic BIR binding factors such as caspases, Rpr or Grim. Evidence that dBruce is cytoprotective against caspases and partial characterization of some of the *dbruce* mutants isolated in these screens has been described (Arama et al., 2003). Molecular lesions in 9 of the *dbruce* alleles have been identified.

Of the remaining 18 modifiers that could not be placed into complementation groups, 1 was identified as an allele of *delta* and 5 others (9-4e, 9-5e, 14-1e, 14-2e and 16-3e) possibly represent weak hypomorphs of *diap1* as they have similar differential phenotypes and map close to the *diap1* locus.

The *Su(21-3s)* mutant was the only remaining uncharacterized allele found to potently and dominantly suppress all the *hid*-induced phenotypes tested (wing/eye/organismal lethality) without affecting *GMR-phyl* (Table 2). It was also reported in the screen to dominantly suppress *GMR-rpr* and *GMR-grim* induced eye phenotypes. The dominant suppressor phenotype associated with *Su(21-3s)* was roughly mapped by meiotic recombination to the right arm of the 3rd chromosome near the visible marker, *curled*. This is the same general area to which *dbruce* was roughly mapped. However, unlike *dbruce* homozygotes, which are male sterile, *Su(21-3s)* homozygotes were reported to be fertile with no obvious phenotype. In addition, all 12 *dbruce* alleles were found to enhance *GMR-rpr* and *GMR-grim* induced phenotypes and not to affect those of *GMR-hid* (excepting the lethal allele $dbruce^{23\text{-}6s}$). Since no cell death genes that could readily be mutated to explain the *Su(21-3s)* suppressor phenotypes were immediately obvious in

the area, the possibility that *Su(21-3s)* represented a mutation in a novel apoptotic regulatory element was considered.

Despite the differences in *Su(21-3s)* and *dbruce* phenotypes, it was also considered that *Su(21-3s)* might be an unusual allele of *dbruce* given their mutual proximity. Therefore, *Su(21-3s)* was included in the analysis that led to the cloning of *dbruce*. Southern blot analysis of the interval to which *dbruce* had been mapped unexpectedly revealed a polymorphism in *Su(21-3s)* relative to wildtype. Further localization of this polymorphism by PCR uncovered a 2Kb insertion within a presumptive intron of the unknown gene CG11870. This previously uncharacterized locus is predicted to encode a protein Ser/Thr kinase (Fig. 2). Interestingly, this kinase was also reported to contain a putative caspase recruitment domain (CARD), which is found in a number of known cell death regulators. Taken together, these data suggested that the phenotypes associated with *Su(21-3s)* might derive from a mutation in CG11870 and the possible presence of a CARD domain was sufficiently intriguing to warrant further study of this gene.

Recharacterization of *Su(21-3s)* phenotypes

Since a considerable amount of time had lapsed between the original characterization of mutants isolated in the genetic screens conducted by

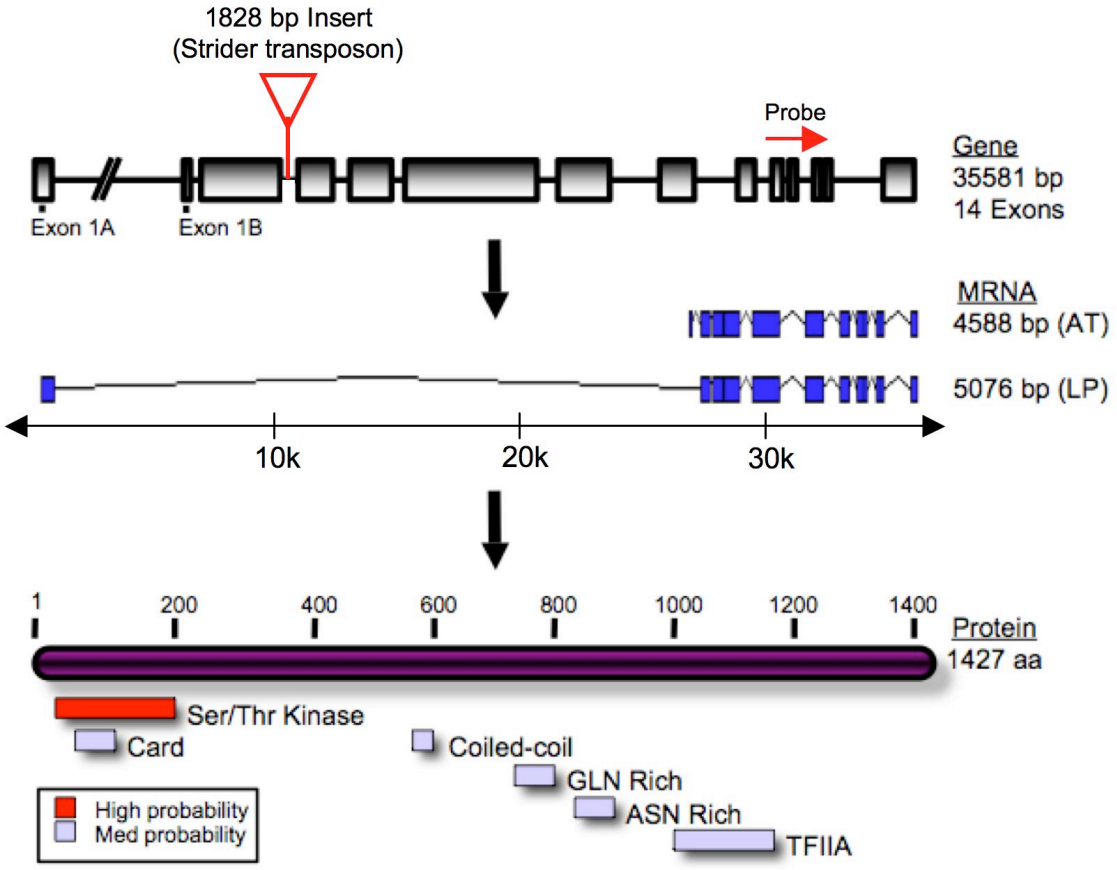


Fig. 2.2. Schematic diagram illustrating the predicted gene structure of CG11870. For simplicity, only two of four mRNAs are depicted in blue. Both are supported by EST data from adult testes (AT) and third instar larva (LP) and both are expected to encode a protein of 1427 amino acids. Along with a high probability Ser/Thr kinase domain, several other motifs are predicted with a lower probability, including a CARD domain within the kinase motif. The red triangle indicates the location of the strider transposon insertion in *Su(21-3s)* flies and the red arrow indicates the binding site of the DIG-labelled probe used for northern analysis.

Agapite *et al*. and initiation of the studies reported here, we first sought to recharacterize in greater detail the phenotypes associated with the *Su(21-3s)* mutant. This analysis led to a number of unanticipated findings that confounded somewhat are initial hypothesis concerning this mutant. As can be seen from the data in Table 2, *Su(21-3s)* had been classified as recessive viable with no obvious phenotypes. Specifically, *Su(21-3s)* was stated to be recessive fertile (Agapite, 2002). In our follow up examination, however, it was discovered that *Su(21-3s)* in fact harbored a mutation that rendered it recessive male and female sterile. Dissection of gonads from adult *Su(21-3s)* flies revealed severely atrophied testes and ovaries in heterozygotes and an even more marked degeneration of testes from male homozygotes. Ovaries in homozygous *Su(21-3s)* females could not be isolated, presumably because they were too deteriorated (Fig. 3). The reason for the discrepancy between our observations here and those first reported remains unclear. It is possible, given the nature of mutagenesis in general and the mosaicism associated with chemical mutagens in particular, that a second mutation in the background of *Su(21-3s)* flies initially went undetected and over time was fixed in the population (Rubin, 1990). Alternatively, a spontaneous mutation may have arisen at some point. Why in either case a recessive sterile mutation in a mixed population would persist is another matter for

speculation. Perhaps it conferred some sort of survival advantage in the context of other mutations located on the mutagenized chromosome. It also had to be considered that slight imprecisions can occur during the execution of such large scale screens involving a number of different individuals. In any event, no determination could immediately be made as to whether the sterility and suppressor phenotypes of *Su(21-3s)* were linked given that mutations with both features are known (Baum et al., 2007; Mendes et al., 2006). It was therefore resolved to map both phenotypes in order to clarify their relationship with each other and to the polymorphism identified in CG11870.

In addition to uncovering the sterility phenotype associated with *Su(21-3s)*, our reanalysis of its suppressor phenotype exposed another discrepancy with the data originally reported in the screens. Briefly, *Su(21-3s)* was originally reported to suppress *GMR-hid*, *GMR-rpr* and *GMR-grim* induced eye phenotypes. A careful reevaluation of these interactions however, clearly demonstrated that *Su(21-3s)* specifically suppresses only *hid* induced phenotypes and has no affect on *GMR-rpr* or *GMR-grim*. The details and consequences of this finding are presented in the next chapter and will not be discussed further here.

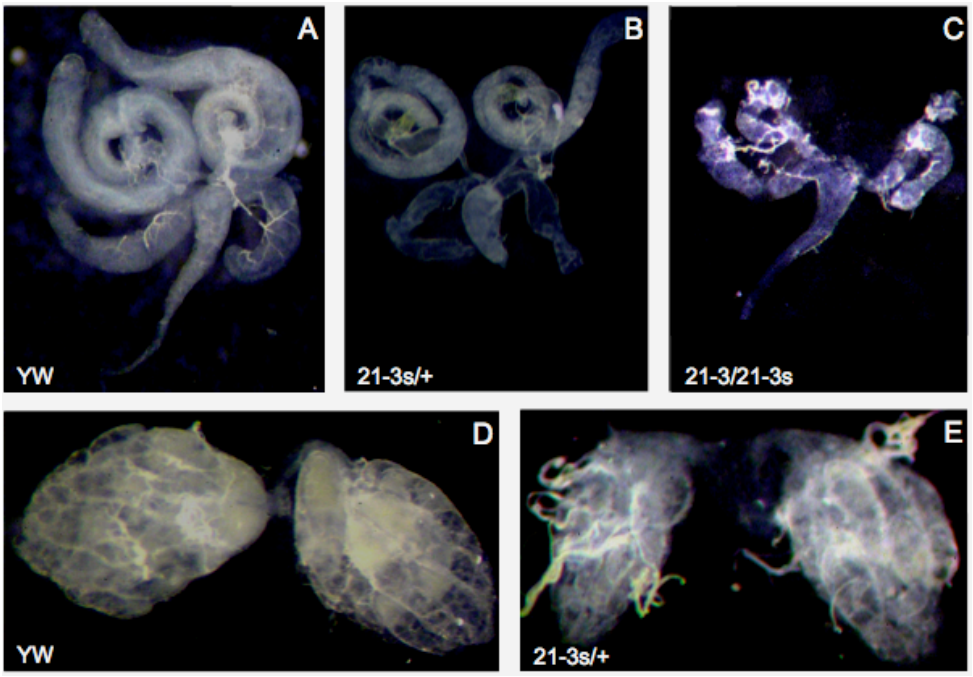


Fig. 2.3. The *Su(21-3s)* mutation results in atrophy of the testes in males (B and C) and the ovaries in females (E). Ovaries could not be found in *Su(21-3s)* homozygous females. *Yw* testes (A) and ovaries (D) are included for comparison. All images were taken at the same magnification.

Despite a partial reclassification of *Su(21-3s)* phenotypes, the evidence implicating CG11870 as a gene of interest remained unchanged. Accordingly, while the genomic mapping of *Su(21-3s)* phenotypes was underway, we preceded with a preliminary characterization of CG11870.

CG11870 is predicted to encode a protein Ser/Thr kinase

The Flybase annotation for CG11870 reports a gene length of 35581 bp with a cytological map location 86A3-86A6. Strong EST data derived from most tissues and stages indicate the production of four differentially spliced mRNAs ranging in size from 4357 bp to 5076 bp. The shortest mRNA consists of 12 exons, lacks coding exon 6 and is expected to encode a protein of 1180 aa. The remaining mRNAs all contain 13 exons including coding exon 6 and are predicted to encode a protein of 1427 aa. Both protein isoforms are electronically inferred to function as receptor signaling Ser/Thr protein kinases in cytoskeleton organization and biogenesis. At the time these studies were initiated, CG11870 was also predicted to comprise a putative CARD, albeit with low probability, as well as a number of other interesting features (Fig. 2). Interestingly, the short protein isoform of CG11870 alone includes a so called TREACLE or TCS fingerprint, implicated in nucleolar trafficking (Wise et al., 1997). The only proteins

with significant homology to CG11870 outside of the universally conserved kinase domain and the other recognized conserved motifs come from Aedes aegypti and Anopheles gambiae. Other than these electronically inferred insights, CG11870 remains largely uncharacterized.

Northern analysis of CG11870 mRNA

Su(21-3s) mutants were determined by PCR to contain an insert of approximately 2Kb within intron 3 of the CG11870 locus. Subsequent analysis by sequencing revealed this insertion to be a 1828bp degenerate fragment of the Strider or Juan non-LTR retrotransposon. To date, 9 of these elements have been detected in the Drosohophila genome, 6 of which are full length (Kaminker et al., 2002). Non-LTR retrotransposons are eukaryotic mobile genetic elements that transpose by reverse transcription of an RNA intermediate and can be mobilized during mutagenesis experiments. It was unclear whether the insertion identified in *Su(21-3s)* would disrupt proper splicing or expression of CG11870 and whether this was the cause of the dominant suppressor phenotypes observed in this mutant. We therefore performed a northern analysis of wildtype and *Su(21-3s)* mutant flies using a DIG-labeled probe directed against the last coding exon of CG11870. A faint but clearly visible band of about 5kb was detected in total RNA

prepared from adult wildtype and mutant flies and from wildtype embryos (Fig 4). Quantitation of the blot using *actin* mRNA as a loading control revealed no significant difference in expression level between wild type and mutant flies and no differences in transcript size were detected. This 2Kb insertion does not appear to affect CG11870 expression in the adult fly and argued against an involvement of CG11870 in *Su(21-3s)* phenotypes. However, the possibility remains that the insertion might have tissue or timing specific effects on expression or result in minor splicing defects not detectable by northern.

Overexpression of CG11870 in the fly eye suppresses *GMR-hid*

Though an attractive feature of dominant modifier screens is the ability to detect and therefore isolate weak hypermorphs, in general it is much easier to induce a loss-of-function mutation in a gene than a gain-of-function mutation, We presumed this to be the case for *Su(21-3s)* and assumed that, if a hypomorphic allele of CG11870 suppresses cell death, then a hypermorph may induce it. To test this, we overexpressed a cDNA encoding the long isoform of CG11870 in the fly eye using the GMR promoter. A total of 15 individual *GMR-CG11870* transgenic lines were generated and examined. No observable phenotypes were detected in any of

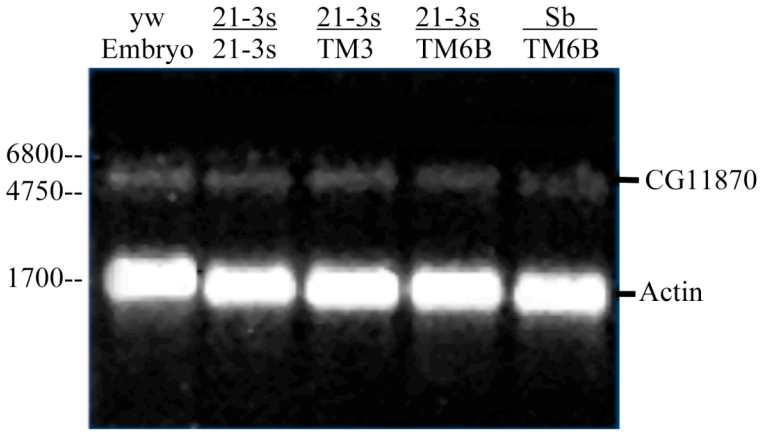


Fig. 2.4. Northern analysis of total RNA from adult flies with a Dig-labeled RNA probe made from the 3' exon of CG11870. The probe detects a faint band at the correct size of ~5 Kb confirming that CG11870 is an expressed gene. Quantitation of the blot using actin mRNA as a loading control revealed no significant differences in expression between wildtype controls (*yw* and Sb/TM6B) and *Su(21-3s)*. Molecular weight markers are indicated on the left.

the lines when either one or two copies of the transgene were present. This suggests that CG11870 is not a pro-apoptotic molecule. When these transgenic lines were placed in a *GMR-hid* background however, several lines over-expressing CG11870 were found to weakly suppress the *GMR-hid* induced rough eye phenotype (Fig. 5). A similar effect was observed when we used *GMR-Gal4* in conjunction with *UAS-CG11870* to drive expression in the eye. As with *GMR-CG11870*, *GMR-Gal4/UAS-CG11870* exhibits no observable phenotypes on its in own, but is able to suppress the rough eye phenotype induced with *GMR-hid* (Fig. 6). Taken together, these over-expression studies raise the intriguing possibility that CG11870 can inhibit *hid* induced cell death. In this case, the insertion present in *Su(21-3s)* flies would have to be a gain-of-function mutation. This seemed unlikely given the Northern results and for additional reasons discussed below. Instead, it seems we may have uncovered this feature of CG11870 strictly by serendipity.

The *Su(21-3s)* sterility phenotype maps to the zpg locus

Given that the sterility phenotype of *Su(21-3s)* was recessive, we assumed it to be loss of function and hence amenable to deficiency mapping.

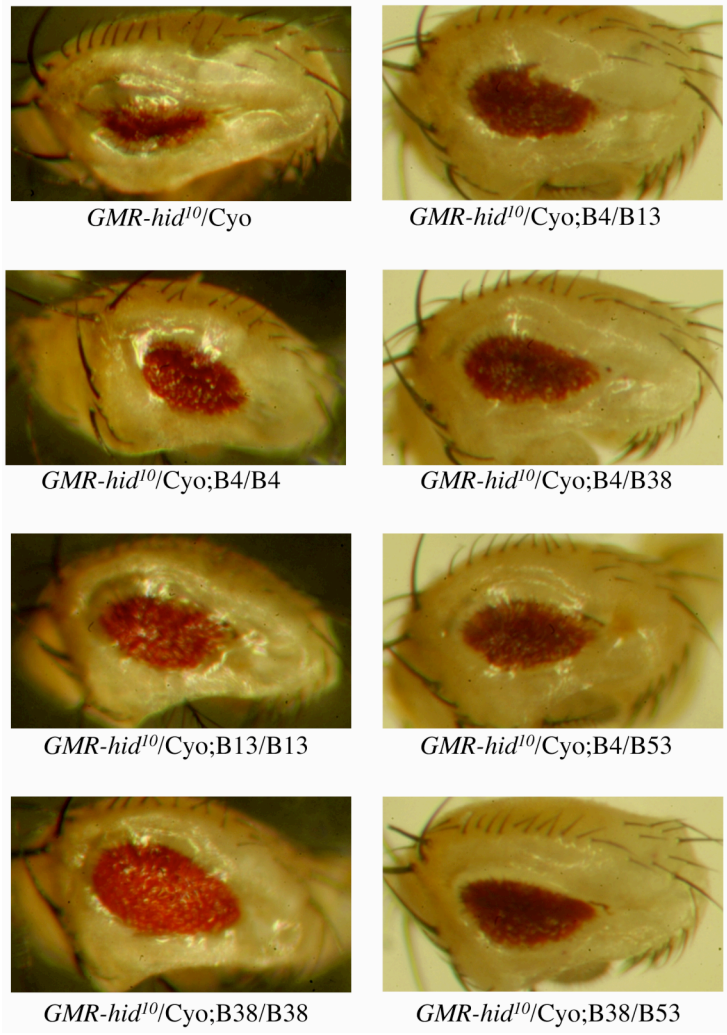
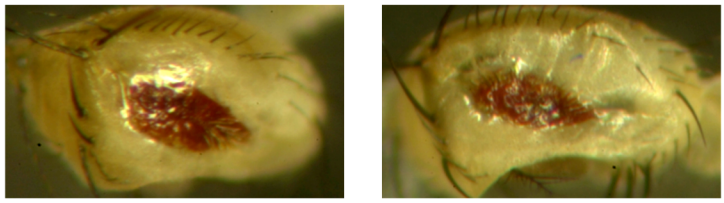


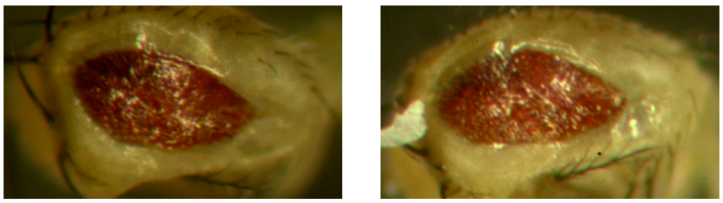
Fig. 2.5. Flies overexpressing the long isoform of CG11870 can suppress the *GMR-hid* induced rough eye phenotype. The genotype of each eye is indicated immediately underneath. B3, B4, B13, B38 and B53 represent independent *GMR-CG11870* transgenes. Transheterozygous allelic combinations were used to minimize transgene insertion affects.

To this end, we obtained 22 overlapping molecularly defined deletions from the Exelixis stock center covering the interval to which the *Su(21-3s)* suppressor phenotype had originally been roughly mapped in the modifier screens. This region, from 84B2 to 88C1 of the right arm of the third chromosome, also includes the CG11870 locus as well as *dbruce*. All 22 of these deletions complemented the sterility phenotype of *Su(21-3s)*. This strongly indicated that the sterility of *Su(21-3s)* flies was neither due to a mutation in *dbruce* nor related to the polymorphism identified in CG11870. Furthermore, these results demonstrated that the sterility phenotype of *Su(21-3s)* did not map to this region and therefore was not linked to the suppressor phenotype. This established that *Su(21-3s)* contained two separable mutations, one recessive that causes male and female sterility and the other a dominant suppressor of *hid* induced phenotypes.

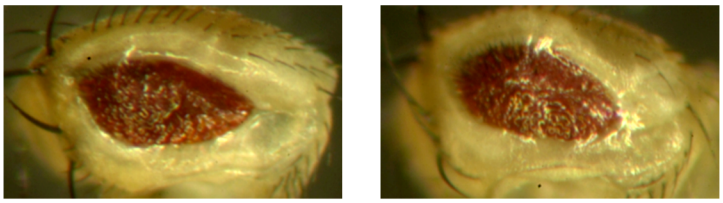
To further localize the gene responsible for the sterility phenotype, we first thought to continue with the deficiency mapping. However, while waiting for the arrival of a new batch of deletions, it was noticed that the atrophied gonads of *Su(21-3s)* resembled somewhat those of mutants under analysis in an unrelated study. This other investigation was focused on the



GMR-Hid^{10}*/GMR-Gal4*



GMR-Hid^{10}*/GMR-Gal4*;E69/E19



GMR-Hid^{10}*/GMR-Gal4*;E69 / E11

Fig. 2.6. Overexpression of CG11870 in the eye using the Gal4/UAS system also suppresses *GMR-hid* induced apoptosis. Two examples are given for each genotype, which is indicated immediately underneath each pair of eyes. E11, E19 and E69 represent independent *UAS-CG11870* transgene insertions.

involvement of caspases during Drosophila spermatogenesis and a collection of male sterile mutants with defects in spermatid individualization had been obtained from the Zuker stock of mutagenized flies. The third chromosome collection of this publicly available stock consists of 6,000 partially characterized lines derived from an EMS mutagenesis (Koundakjian et al., 2004). Remarkably, of these 6000 mutants, only 24 are reported to be both male and female sterile, suggesting that relatively few genes can be mutated to elicit the sterility of both sexes in Drosophila. When these 24 lines were crossed to *Su(21-3s)*, five failed to complement its sterility phenotype. A search of the literature fortuitously uncovered two of these Zuker mutants that had previously been identified as loss of function alleles of *zero population growth* (*zpg*) (Tazuke et al., 2002). The *zpg* locus in the *Su(21-3s)* mutant was subsequently sequenced and a mutation at position 662 of the cDNA resulting in an amino acid substitution (G221D) within a highly conserved region of the protein was identified. This confirmed that the sterility phenotype of *Su(21-3s)* was due to a loss of function mutation in *zpg* and that the two *Su(21-3s)* phenotypes were not linked. *Zpg* maps to 65B5 on the left arm of the third chromosome and the two *Su(21-3s)* phenotypes were easily separated by meiotic recombination. All subsequent analyses of the *Su(21-3s)* mutant, now recessive fertile as initially reported,

were conducted in this fresh genetic background and are discussed at length in the following chapter.

Discussion

In this chapter, we describe the initial isolation and characterization of the *Drosophila* mutant *Su(21-3s)*. We also present preliminary molecular data on the predicted protein Ser/Thr kinase CG11870 and provide evidence showing that a mutation in this gene is unlikely to give rise to the *Su(21-3s)* mutant phenotypes as originally hypothesized.

The dominant *hid* suppressor, *Su(21-3s)*, is the product of a genetic screen carried out to isolate genes that can modulate the eye ablation phenotypes elicited by expressing *hid* or *rpr* under the control of an eye-specific promoter. Roughly 500,000 flies were screened and 167 dominant modifiers recovered. Secondary screens allowed us to compile a cell death specific subset of 58 modifiers of which 40 could be placed into six complementation groups that define both known and unknown genes. This includes 3 genes, *Star*, *gap* and *sprouty* involved in EGFR/MAPK signaling, the known cell death regulator *diap1*, the enormous BIR and UBC containing protein *dbruce* and a gene, *Su(GMRhid)2A,* that remains to be identified. Analysis of these mutants have provided several valuable

insights concerning the regulation of apoptosis and generated a multitude of tools that have proven essential in a number of studies. For example, the large number of *diap1* mutants obtained in this study, both gain and loss of function, permitted structure function assessments of Diap1 that provided strong *in vivo* evidence for mechanistic models of IAP apoptosis inhibition (Goyal et al., 2000; Wang et al., 1999; Zachariou et al., 2003). Moreover, *diap1* RING mutants derived from this screen have been used to implicate the ubiquitin system in apoptosis regulation and to provide evidence that degradation of caspases as well as Diap1 itself are key regulatory events in cell survival and death (Ryoo et al., 2002; Wilson et al., 2002).

Su(21-3s), included in the cell death specific subset of modifiers described above, was isolated as a strong dominant suppressor of *GMR-hid*. This mutant was reported at the time the screen was originally conducted to be viable with no obvious recessive phenotypes, to dominantly suppress *hid* induced organismal lethality and wing phenotypes and to map near the marker *curled* on the right arm of the third chromosome. It was also reported to be a suppressor of *GMR-rpr* and *GMR-grim*. Finally, because the complementation group encompassing *dbruce* alleles mapped to a similar position on the third chromosome, *Su(21-3s)* was included in the molecular analysis that led to the identification and cloning of *dbruce*.

During the course of this analysis, a 2Kb insertion within the unknown gene CG11870, was identified in *Su(21-3s)* flies. This suggested the possibility that a mutation in CG11870 could be responsible for the phenotypes associated with *Su(21-3s)*. Our suspicions were bolstered by the fact that CG11870 was predicted to encode a protein Ser/Thr kinase with a putative CARD, a motif present in several known cell death regulators (Park et al., 2007).

Though the features of *Su(21-3s)* were considered interesting enough to warrant further study, it was a number of years until the investigation reported here was initiated. When the *Su(21-3s)* mutant was next examined, it was discovered to harbor a recessive sterility contrary to its initial characterization. We first showed using deletions that this sterility phenotype was not related to CG11870 or *dbruce* and that it was separable from the suppressor phenotypes associated with *Su(21-3s)*. Subsequent analysis revealed that the sterility phenotype was due to a mutation in *zpg*, a germline-specific gap junction required for the survival of early differentiating germ cells (Tazuke et al., 2002). Fearing this mutation might confound analysis of the suppressor phenotype, it was crossed out of the *Su(21-3s)* line by meiotic recombination. This yielded a *Su(21-3s)* line that is fully fertile. Though the detection and removal of this *zpg* mutation was

unexpected, it was not relevant to our supposition that the insertion in CG11870 might be responsible for suppressor phenotypes that remain associated with *Su(21-3s)*. We therefore preceded with a preliminary characterization of this predicted kinase.

Since the insertion found in CG11870 of *Su(21-3s)* falls within an intron, it could only be mutagenic by disrupting expression in some way. Northern analysis of CG11870 transcripts from adult flies detected a faint RNA band of the correct size (~5Kb), but failed to reveal any alterations in size or levels between wildtype and *Su(21-3s)*. This result argues that the insertion present in *Su(21-3s)* does not affect the transcription of CG11870 and that it can not account for the phenotypes observed in *Su(21-3s)* flies. In addition, the entire CG11870 ORF in wildtype and *Su(21-3s)* flies was sequenced but also revealed no differences, precluding the possibility that a mutation in the coding sequence of CG11870 was concomitantly induced by a transposition event.

When CG11870 was over-expressed in the fly eye, no cell death was observed. Instead, overexpression of CG11870 was able to moderately suppress the cell death induced by *GMR-hid*. Though interesting in its own right, this result in fact argues that CG11870 is not related to the suppressor phenotype of *Su(21-3s)*. For this to be the case, two improbable scenarios

would have to be invoked. First, the insertion in *Su(21-3s)* flies, which falls within an internal intron of CG11870, not upstream or within the first intron (Fig. 2), would have to be a strong gain-of-function mutation that significantly increases CG11870 expression to a level comparable to that achieved by GMR transgenes. Second, given the results of the northern analysis, this considerable increase in expression would have to be restricted to a pre-adult stage. Finally, *Su(21-3s)* is a potent endogenous suppressor of *GMR-hid,* whereas strong transgenic overexpression of CG11870 gives only a moderate suppression. Taken together, these data compellingly indicate that the insertion identified in *Su(21-3s)* flies does not give rise to the observed suppressor phenotypes and that another gene is responsible. As is described in the next chapter, this indeed turned out to be the case.

Experimental Procedures

Fly stocks and genetics

The following fly stocks were used for the dominant modifier screens: *GMR-rpr*^{81} (White et al., 1996), *GMR-rpr*^{34} Cyo/Sco (Bergmann et al., 1998), *GMR-hid*^{10} and *hs-hid*^{3} (Grether et al., 1995), *GMR-grim* (Chen et al., 1996), *GMR-phyl* (Chang et al., 1995), *GMR-rho*^{1} (Hariharan et al., 1995), *vg-Gal4* (F.M. Hoffmann, unpublished), *UAS-hid* (Zhou et al., 1997).

Stocks for meiotic recombination mapping (*ru*^{1} *h*^{1} *th*^{1} *st*^{1} *cu*^{1} *sr*^{1} *e*^{s} *ca*^{1} and *ru*^{1} *h*^{1} *th*^{1} *st*^{1} *cu*^{1} *sr*^{1} *e*^{s} *Pr*^{1} *ca*^{1}/TM6B, *Bri*^{1}, *Tb*^{1}) and *GMR-Gal4* were obtained from the Bloomington Stock Center (Bloomington, IN). Flies carrying *GMR-CG11870* and *UAS-CG11870* were generated by P element-mediated transformation according to standard protocols. All other lines were generated by meiotic recombination of the appropriate alleles.

Dominant modifier and reversion screens were performed as described in Fig. 1 (Agapite, 2002). Modifiers were mapped to a chromosome, balanced and their recessive phenotypes determined. Complementation analysis was performed on mutants of the same chromosome exhibiting similar recessive phenotypes. Dominant modifiers on the third chromosome were mapped by meiotic recombination using the *rucuca* mapping chromosome. Male sterility was assessed by mating twenty homozygous mutant males individually to Canton-S females. The mutants were considered to display some degree of sterility if fewer than 20% of the crosses gave rise to at least forty progeny.

All crosses and suppression experiments were carried out at 25°C except crosses with *vg-Gal4* and *UAS-hid*, which were performed at both 18°C and 25°C. Suppression experiments with hs*hid* were done by heat

shocking 1st instar larvae at 37°C for 15 minutes. Flies were raised on standard cornmeal-molasses medium at 25°C unless otherwise indicated.

For analysis of gonads, the testes and ovaries of 3-5 day old adult flies were dissected into PBS and immediately visualized. Fertility tests were performed by placing ten young adult males with five wild-type virgin females in a vial at 25°C, and vials were scored for offspring after ten days. Flies were raised on standard cornmeal-molasses medium.

Identification of a polymorphism in the *Su(21-3s)* mutant

The 2Kb polymorphism in *Su(21-3s)* flies was identified as previously described (Agapite, 2002). Briefly, the dominant suppressor phenotype associated with *Su(21-3s)* was roughly mapped by meiotic recombination to the right arm of the 3rd chromosome near the visible marker, *curled*. *Dbruce* mapped to a 74Kb interval in this general area and for this reason *Su(21-3s)* was included in the analysis that led to the cloning of *dbruce*. Genomic Southern was used to screen this interval using individual EcoR1 fragments as probes. One fragment revealed a polymorphism in *Su(21-3s)* relative to wild-type and was partially sequenced. PCR using primer pairs designed to give 1 Kb products that spanned this fragment was performed with both wildtype and *Su(21-3s)* genomic DNA and one primer pair yielded a 1Kb

product from wildtype and a 3Kb product from *Su(21-3s)* templates. These products were sequenced and revealed that *Su(21-3s)* contained an insertion in the third intron of the predicted gene CG11870.

Molecular Biology

A full length cDNA clone encoding the long isoform of CG11870 was obtained from the *Drosophila* Genomics Resource Center (clone ID: GM10858) and the entire ORF was subcloned into the pUAST (Brand and Perrimon, 1993), pGMR (Hay et al., 1994) and pSPT18 (Roche) vectors to generate *pUAST-CG11870, pGMR-CG11870* and *pSPT18-CG11870*, respectively. Plasmid DNA for each construct was isolated using the Qiagen Plasmid Maxi Prep kit (Qiagen).

For Northern analysis total RNA was extracted from 100 adult *yw* and *Su(21-3s)* flies using the TRIZOL reagent according to the manufacturer's instructions (Invitrogen). Northern analysis was performed by using 1ug of total RNA per sample and blotting with a DIG-labeled RNA probe complimentary to the last 300bp of CG11870 coding sequence according to the Dig-Northern Starter Kit (Roche). Probes were prepared with 1ug of linearized *pSPT18-CG11870* DNA and SP6 RNA polymerase using the DIG RNA Labeling Kit (Roche).

CHAPTER 3.

Molecular and Biochemical Analysis of *ras1*^{R68Q}, a Viable Gain of Function Mutation in the Switch II Region of Drosophila *ras1*.

Summary

Cells are continuously exposed to a multitude of environmental cues and are required to integrate the resulting signals into cell fate decisions, including whether to live or die. Among the many signaling pathways that control these fate decision, mitogen-activated protein kinase (MAPK) family members are crucial for the transduction of signals that mediate survival, proliferation and differentiation. This evolutionarily conserved pathway can respond to a number of extracellular inputs, such as growth factors, to promote cell survival by inhibiting the activation of apoptosis. A central regulator of these signal transduction processes is the small GTPase Ras, which is involved in virtually every aspect of cell biology. The critical nature of Ras in physiologic homeostasis is underscored by the fact ~20% of all human tumours contain an activating mutation in one the Ras genes. Much of our understanding of the role Ras proteins play in development has come from studies in genetic systems such as the developing *Drosophila* eye *C. elegans* vulva. Here we report the identification and characterization of a novel gain-of-function mutation in the switch II region of RAS85D (*ras1*), the Drosophila homologue of mammalian N-ras, K-ras and H-ras. Though several loss of function alleles have been described for *drosophila ras1*, this mutation represents the first endogenous *ras1* hypermorph to be isolated.

Introduction

The development of multicellular organisms requires the tight coordination of cell proliferation, cell differentiation and cell death in order to correctly specify cell fate and number. One model that describes how this can be achieved is the trophic theory of survival. Originally postulated to explain the massive neuronal cell loss during development of the vertebrate CNS, the trophic theory presumes that in the absence of extracellular survival factors, cells die by the engagement of a default cell death program. Cells compete for these trophic factors, secreted from neighboring cells in a limited amount, thereby ensuring that only an appropriate number survive (Raff, 1992). This 'social control' of cell survival ensures the integrity of tissues by matching the correct number of different cell types to each other.

The MAPK pathway is one of many conserved signaling modules that respond to extracellular cues and relay survival signals to the cell interior. The signaling relay consists of a transmembrane receptor that binds to extracellular factors, intracellular proteins that engage activated receptors and amplify the signal and effector molecules that transduce the signal to cytoplasmic and nuclear targets. Signals are propagated via a protein phosphorylation cascade by a series of protein kinases which act downstream of the small GTPase Ras (Seger and Krebs, 1995).

Ras proteins are guanine nucleotide binding proteins that act as molecular switches to integrate the signal transduction pathways involved in several aspects of normal cell growth and malignant transformation (Colicelli, 2004). The remarkable fact that ~20% of all human tumours have undergone an activating point mutation in one of the Ras genes emphasizes the necessity of understanding in detail the mechanistic workings of this signal transducer and the biological contexts in which it operates (Bos, 1989). Oncogenic mutations in Ras occur most frequently at codons 12,13 or 61 and result in an enzyme with a deficient GTPase activity that is refractory to stimulation by GTPase Activating Protein (GAP) (Scheffzek et al., 1997). Ras thus remains trapped in an active state because Ras is 'on' when bound to GTP and is switched 'off' by hydrolyzing bound GTP to GDP. Inhibition of Ras GTPase activity therefore stabilizes Ras in its active conformation, prolonging its recruitment and activation of downstream signaling components.

Much of our understanding of Ras-mediated signaling is derived from a combination of biochemical experiments conducted in mammalian tissue culture and screens conducted in the workhorses of developmental genetics, *Drosophila melanogaster* and *Caenorhabditis elegans* (McCormick, 1994). For example, the power of *Drosophila* genetics proved instrumental in

defining how Ras-mediated signaling regulates the specification and differentiation of R7 photoreceptors in the fly eye (Gaul et al., 1992; Rebay et al., 2000; Simon et al., 1991). Furthermore, findings from the genetic screens described in the previous chapter and from those conducted by others, have defined a mechanism by which Ras-mediated signaling can directly inactivate a critical component of the intrinsic cell death pathway (Bergmann et al., 1998; Downward, 1998; Kurada and White, 1998). Such successes have helped to garner credibility and visibility for *Drosophila* as a model organism in cancer research (Vidal and Cagan, 2006).

In *Drosophila*, *ras85D* (*ras1*) is the ortholog of mammalian *H-ras*, *K-ras* and *N-ras* (Neuman-Silberberg et al., 1984). As a complimentary approach to the use of genetic screens, Ras has also been extensively studied in Drosophila by targeted overexpression of activated Ras alleles in a variety of tissues and paradigms. For example, $ras1^{V12}$ has been expressed in imaginal discs and larval hemocytes to examine cell proliferation, differentiation and cell death (Asha et al., 2003; Fortini et al., 1992; Karim and Rubin, 1998). The use of such transgenic approaches in *Drosophila* has helped to elucidate the role of Ras in a variety of signaling pathways and developmental contexts.

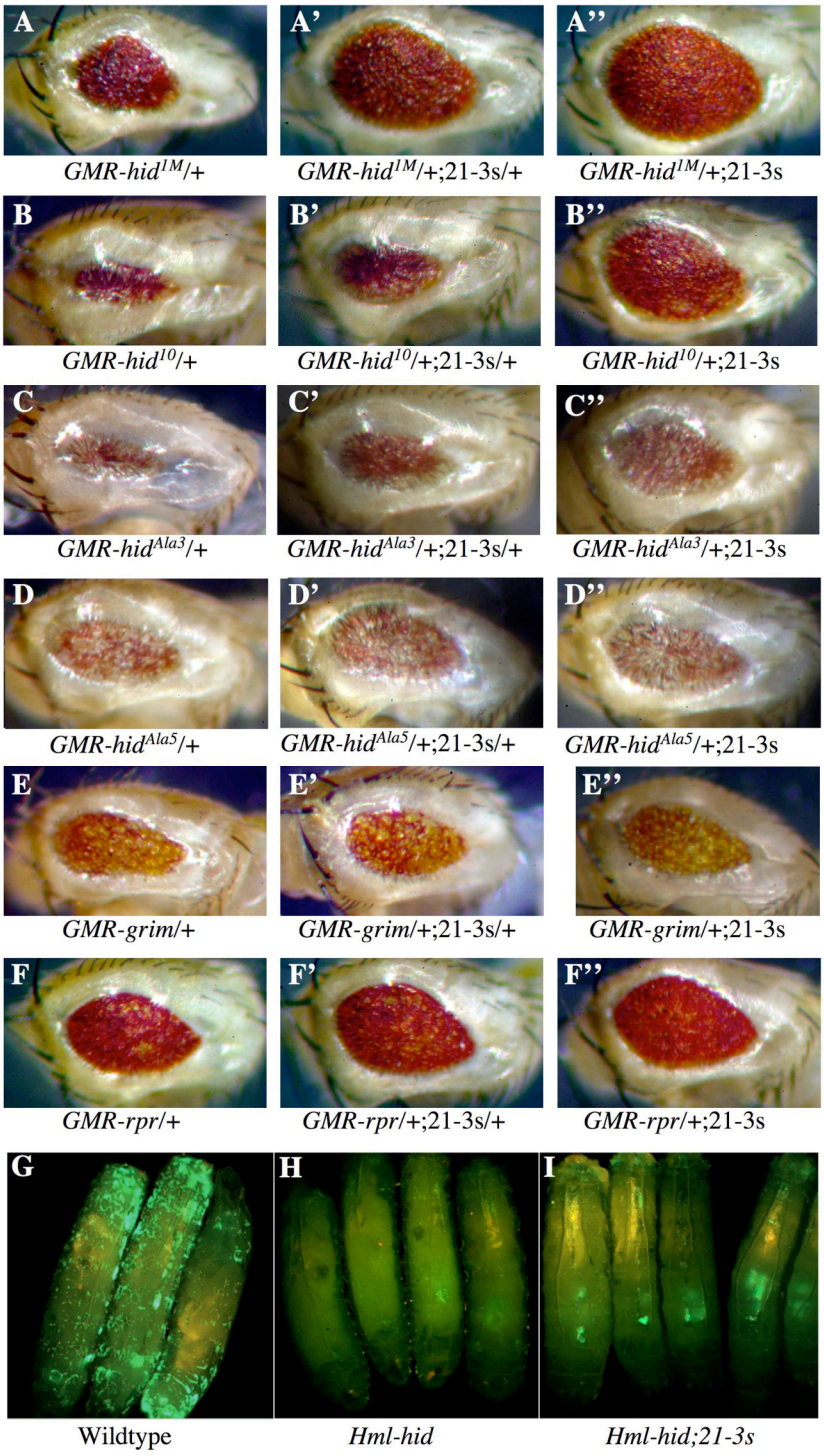
In the previous chapter, we described the isolation and preliminary phenotypic characterization of the *Drosophila* mutant *Su(21-3s)*. This mutant was identified as a dominant suppressor of *GMR-hid* induced cell death in a genetic modifier screen. Here we report on the further characterization of *Su(21-3s)* and show that its associated suppressor phenotypes derive from a hypermorphic mutation of *ras1*. Though numerous loss of function alleles have been described for *ras1*, the mutation described here represents the first endogenous gain of function allele to be identified. The phenotypic consequences of this hypermorph in various developmental contexts is investigated.

Results

Phenotypic characterization of *Su(21-3s)*

As stated in the previous chapter, we sought to re-examine more rigorously the suppression phenotypes of *Su(21-3s)* in the eye by testing the modifier effects of one or two copies of *Su(21-3s)* against various GMR expression constructs (Fig. 1). This analysis confirmed unequivocally that *Su(21-3s)* potently suppresses *GMR-hid* induced cell death in a dosage dependent manner (Fig. 1A,B). Unexpectedly, however, we found the suppression of *GMR-rpr* and *GMR-grim* phenotypes, though detectable as

Fig. 3.1. *GMR-hid* but not *GMR-grim* or *GMR-rpr* induced cell death is dominantly suppressed by *Su(21-3s)* in a manner that requires intact MAPK phosphorylation sites in *hid*. (A-F) *GMR* driven expression of IAP-antagonist induces cell death in the eye. Cell death is strongly suppressed by one (′) or two (″) copies of the *Su(21-3s)* chromosome when induced by either a weak allele, *GMR-hid*^{1M} (A) or strong allele, *GMR-hid*^{10} (B) of *hid*, but is very weakly suppressed by *Su(21-3s)* when induced *GMR-grim* (E) or *GMR-rpr* (F). In addition, *Su(21-3s)* suppresses cell death induced by a *hid* allele lacking 3 of 5 predicted MAPK phosphorylation sites, *GMR-hid*^{Ala3} (C) but not by *GMR-hid*^{Ala5} (D), a hid allele lacking all 5 MAPK consensus. sites (Bergmann et al., 1998). (G-H) Death of larval hemocytes induced by expression of *hid* under control of the hemocyte specific driver *Hml* is also partially suppressed by the *Su(21-3s)* mutation. (G) EGFP is used to visualize hemocytes in wildtype 3rd instar larva: *Hml-GAL4*, 2x*UAS-EGFP*. (H) Overexpression of Hid in hemocytes results in their complete ablation by the 1st instar larval stage: *Hml-Gal4*, 2x*UAS-EGFP*; *UAS-hid*. (I) *Su(21-3s)* is able to partially suppress hemocyte death induced by HID. Surviving hemocytes appear to be concentrated within the lymph glands: *Hml-Gal4*, 2x*UAS-EGFP*; *UAS-hid*, *Su(21-3s)*. Genotypes are as indicated except 21-3s refers to *Su(21-3s)*.


A
A'
A''
GMR-hid1M/+
GMR-hid1M/+;21-3s/+
GMR-hid1M/+;21-3s
B
B'
B''
GMR-hid10/+
GMR-hid10/+;21-3s/+
GMR-hid10/+;21-3s
C
C'
C''
GMR-hidAla3/+
GMR-hidAla3/+;21-3s/+
GMR-hidAla3/+;21-3s
D
D'
D''
GMR-hidAla5/+
GMR-hidAla5/+;21-3s/+
GMR-hidAla5/+;21-3s
E
E'
E''
GMR-grim/+
GMR-grim/+;21-3s/+
GMR-grim/+;21-3s
F
F'
F''
GMR-rpr/+
GMR-rpr/+;21-3s/+
GMR-rpr/+;21-3s
G
H
I
Wildtype
Hml-hid
Hml-hid;21-3s

initially reported in the screen, to be extremely weak, even in the presence of two copies of *Su(21-3s)* (Fig. 1E,F). Given that *hid* is highly expressed in the developing eye, we believe the small effect exerted by *Su(21-3s)* on *GMR-rpr* and *GMR-grim* is due to a suppression of endogenous Hid activity and not on Rpr or Grim (Grether et al., 1995). We therefore conclude that *Su(21-3s)* is a *hid* specific suppressor that again illustrates a distinction between the *hid*, *grim* and *rpr* pathways.

It has been demonstrated that Hid activity is regulated by the EGFR/MAPK pathway in a manner that depends on intact MAPK phosphorylation sites in Hid. Intriguingly, our analysis here reveals that *Su(21-3s)* readily suppresses *GMR-hid*^{Ala3}, a *hid* allele lacking 3 of 5 predicted MAPK phosphorylation sites, but fails to suppress *GMR-hid*^{Ala5}, which is missing all 5 MAPK sites (Fig. 1C,D) (Bergmann et al., 1998). This requirement for one or two of the predicted MAPK phosphorylation sites in Hid (Ser-121 and Thr-228) suggested that *Su(21-3s)* might be mediating its suppressive effects through the EGFR/MAPK pathway.

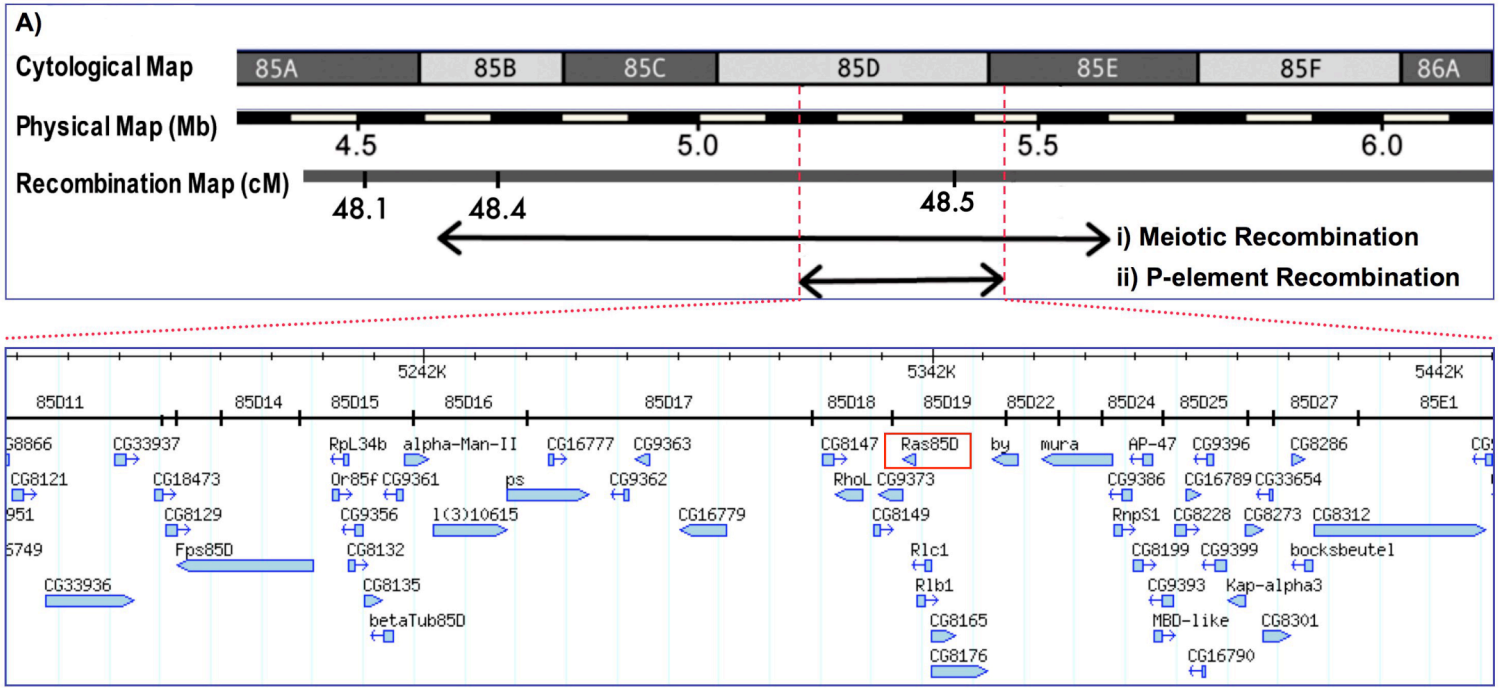
We further extended analysis of the *Su(21-3s)* suppression phenotype to the developmental context of larval hemocytes, an important model system for the study of vertebrate haematopoiesis (Jung et al., 2005; Wood and Jacinto, 2007). *Drosophila* hemocytes require trophic signaling from

multiple pathways for their survival and in its absence undergo caspase dependent cell death (Bruckner et al., 2004; Matova and Anderson, 2006). Using a hemocyte specific promoter to drive expression of EGFP, we visualized hemocytes in wandering 3rd instar larva (Fig. 1G) (Goto et al., 2003). Ectopically expressing Hid using the same driver results in complete ablation of hemocytes by the 1st instar larval stage (Fig. 1H). *Su(21-3s)* is able to partially suppress this cell death such that anterior hemocyte aggregates become readily visible, possibly due to "cannibalistic phagocytosis" by surviving hemocytes as described by Bruckner et al (Fig. 1I).

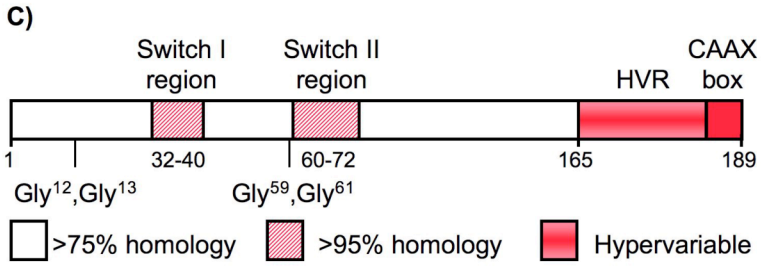
_Su(21-3s)_ is a gain-of-function allele of _ras85D_ (_ras1_)

In order to identify the gene responsible for the *Su(21-3s)* phenotype, we mapped it by a second, finer round of meiotic recombination to a 1 Mb interval between 85A and 85E, then further localized the mutation by P-element mediated male recombination to a 270 Kb interval between 85D11 and 85E1 (Fig. 2A). Given that *Su(21-3s)* differentially suppresses *hid*, but not *grim* or *rpr* in a manner reminiscent of EGFR/MAPK mutants, we suspected that *Su(21-3s)* might be a hypermorphic allele of *ras85D*(*ras1*), which is located within this interval. Therefore, we sequenced *ras1* in a

Fig. 3.2. *Su(21-3s)* is a gain of function allele of *ras85D*(*ras1*), the *Drosophila* ortholog of human N-*ras*, H-*ras* and K-*ras*. (**A**) The cell death suppression phenotype of *Su(21-3s)* mutants was mapped by meiotic recombination to the region of the 3rd chromosome indicated by the large horizontal arrow. This interval was further narrowed by P-element induced male recombination mapping to the region indicated by the short arrow. This shorter interval corresponds to 5.162-5.452 Mb on the physical map and an enlargement of this interval is shown below indicating the ORFs contained therein, including *ras85D* or *ras1*, outlined with a red box. The *ras85D* locus was subsequently sequenced in a candidate gene approach and a G to A transition in exon3 was identified. This transition results in an amino acid substitution at position 68 of the *ras1* protein ($Ras1^{R68Q}$.) (**B**) Amino acid alignment of the universally conserved Switch II region of Ras. The *Su(21-3s)* mutation is highlighted in yellow. (**C**) Schematic diagram of the Ras protein highlighting conserved functional regions as well as some well known naturally occurring activating point mutations that inhibit GTP hydrolysis and therefore lock the GTP-RAS complex in an active form. The Switch regions are known to undergo large conformational changes upon exchange of bound GDP for GTP (Souhami, 2002). Numbers indicate amino acid position. HVR, hypervariable region.


A)
Cytological Map
85A
85B
85C
85D
85E
85F
86A
Physical Map (Mb)
4.5
5.0
5.5
6.0
Recombination Map (cM)
48.1
48.4
48.5
i) Meiotic Recombination
ii) P-element Recombination
5242K
5342K
5442K
85D11
85D14
85D15
85D16
85D17
85D18
85D19
85D22
85D24
85D25
85D27
85E1
CG33937
RpL34b
alpha-Man-II
CG16777
CG9363
CG8147
Ras85D
by
mura
AP-47
CG9396
CG8286
CG8121
CG18473
Or85f
CG9361
ps
CG9362
RhoL
CG9373
CG9386
CG16789
CG33654
CG8129
CG9356
l(3)10615
CG16779
CG8149
RnpS1
CG8228
CG8273
CG8312
Fps85D
CG8132
Rlc1
CG8199
CG9399
bocksbeutel
CG33936
CG8135
Rlb1
CG9393
Kap-alpha3
betaTub85D
CG8165
MBD-like
CG8301
CG8176
CG16790


B)
R68Q
D. melanogaster LDILDTAGQEEYSAMRDQYMRTGEGFL
H. sapiens LDILDTAGQEEYSAMRDQYMRTGEGFL
M. musculus LDILDTAGQEEYSAMRDQYMRTGEGFL
R. norvegicus LDILDTAGQEEYSAMRDQYMRTGEGFL
F. rubripes LDILDTAGQEEYSAMRDQYMRTGEGFL
C. elegans LDILDTAGQEEYSAMRDQYMRTGEGFL
S. cerevisiae LDILDTAGQEEYSAMREQYMRTGEGFL
Switch II


C)
Switch I region
Switch II region
HVR
CAAX box
1
32-40
60-72
165
189
Gly12,Gly13
Gly59,Gly61
>75% homology
>95% homology
Hypervariable

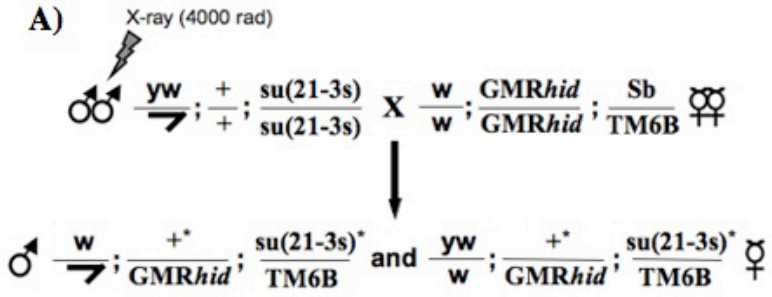
candidate gene approach and a G to A transition in exon3 was identified. This transition mutation results in an amino acid substitution at position 68 of the Ras1 protein, replacing a positively charged arginine within the universally conserved Switch II region of Ras1 with a neutral glutamine (Fig. 2B). The switch regions of Ras have been defined as regions that undergo a large conformational change upon transition from the GTP- to the GDP-bound state (Milburn et al., 1990). Detailed crystal structures have revealed that residues in the Switch II region of Ras contact and are stabilized by GAP, allowing them to participate in the catalysis of GTP (Scheffzek et al., 1997). Mutations in the Switch II may therefore interfere with Ras GTPase activity and possibly explains why several naturally occurring oncogenic Ras mutations occur in this area (Fig. 2C) (Brose et al., 2002; Lee et al., 2003). It therefore seemed feasible that the R68Q mutation identified in *Su(21-3s)* flies could similarly result in a Ras protein with enhanced signaling capacity.

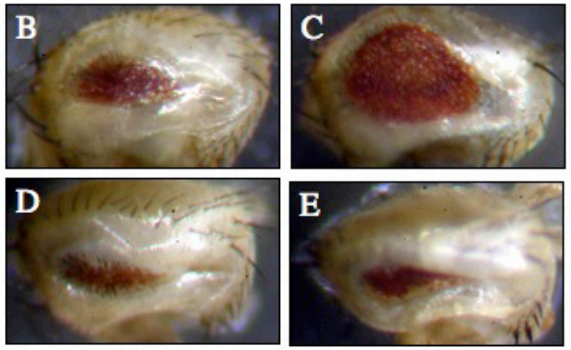
We reasoned that if the *Su(21-3s)* phenotype is due to a gain of function mutation in *ras1*, it should be revertible by introduction of a second, intragenic loss of function mutation. To test this, we conducted a reversion screen for loss of the *Su(21-3s)* suppression phenotype (Fig. 3A). From a total of 80,000 F1 progeny that were screened, 15 phenotypic

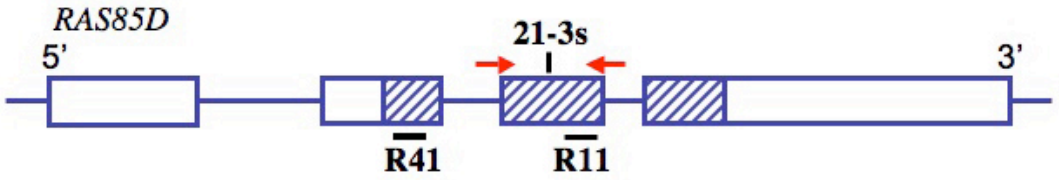
revertants were recovered. Based on lethality, 11 of these could be placed into one of 4 complementation groups. One of these groups, containing the revertants *Su(21-3s)*^{R11} and *Su(21-3s)*^{41}, failed to complement the lethal null *ras85D*^{e1B} allele and therefore corresponds to the *ras85D* (*ras1*) locus. Analysis of these two revertants revealed intragenic loss-of-function mutations in the *ras1* coding sequence (Fig. 3F). One revertant contains a 31bp deletion in *ras1* that results in a Ras1 protein truncated at amino acid 87. The second revertant contains an in frame 18bp deletion of *ras1* that eliminates amino acids 87-92 of the protein, which are known to be essential for Ras function (Willumsen et al., 1986). These revertants greatly resemble the null *ras85D*^{e1B} or *ras85D*^{e2F} alleles with regard to suppression of *GMR-hid* and lethality (Fig. 3B-E and data not shown) and provide genetic evidence for the hypothesis that *Su(21-3s)* is due to a revertible gain-of-function mutation in *ras1*.

Finally, as an allele of *ras1*, *Su(21-3s)* should interact genetically with other members of the MAPK signaling pathway in a predictable manner. We crossed *GMR-hid*^{10} flies in a *Su(21-3s)* background to mutants of MAPK signaling and observed the extent of cell death in the eye (Fig. 4). MAPK signaling mutants tested include *argos, ras1, rolled* and *EGFR*. In this analysis, the *Su(21-3s)* mutant behaves as expected for a gain-of-function

Fig. 3.3. Screen to revert the *Su(21-3s)* suppressor phenotype. A) Homozygous *Su(21-3s)* males were treated with 4000 rad x-rays and crossed to *GMR-hid*^{1M}; Sb/TM6B females. 80,000 F1 progeny were screened for loss of the *Su(21-3s)* suppression phenotype. A total of 15 phenotypic revertants were recovered, 11 of which could be placed into one of 4 complementation groups. One of these groups, containing revertants *Su(21-3s)*^{R11} and *Su(21-3s)*^{41}, corresponds to the *ras85D* (*ras1*) locus. (B-E) The suppression of the *GMR-hid*^{10} rough eye phenotype (B) by the *Su(21-3s)* mutation (C) is lost in revertants *Su(21-3s)*^{R11} (D) and *Su(21-3s)*^{R41}(E). Genotypes: (B) *GMR-hid*^{10}/+, (C) *GMR-hid*^{10}/+;*Su(21-3s)*/+ (D) *GMR-hid*^{10}/+;*Su(21-3s)*^{R11}/+ and (E) *GMR-hid*^{10}/+;*Su(21-3s)*^{R41}/+. F) A schematic of the ras85D locus with exons boxed and coding regions stippled, depicting the relative locations of the *Su(21-3s)* point mutation in exon 3 to the deletions identified in *Su(21-3s)*^{R11} and *Su(21-3s)*^{R41} (labeled R11 and R41 respectively). The red arrows correspond to PCR primers used in a diagnostic for the *Su(21-3s)* point mutation. As illustrated by the sequence alignments below, *Su(21-3s)*^{R41} contains a 31bp deletion compared to the wildtype *ras85D* locus, resulting in a frameshift at amino acid 6 and a truncation amino acid 87. The *Su(21-3s)*^{R11} mutant contains an 18bp in frame deletion that removes amino acids 87-92. Sequences are labeled on the left and numbers above correspond to basepair position in the *ras1* cDNA. Also shown is a PCR diagnostic confirming that *Su(21-3s)*^{R11} (R11) and *Su(21-3s)*^{41} (R41) retain the *Su(21-3s)* point mutation.





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                461                                                 512
ras85D          TCGTTGGAGCCGGAGGCGTGGGCAAGTCCGCGCTCACCATCCAGCTAATCCA
Su(21-3s)R41    TCGTTGGAGC----------------------------AGCTAATCCA

                697                                                 747
ras85D          GCGAAGTCCTTCGAGGATATCGGCACCTACCGTGAGCAGATCAAGCGCGTA
Su(21-3s)R11    GCGAAGTCCT----------------ACCGTGAGCAGATCAAGCGCGTA
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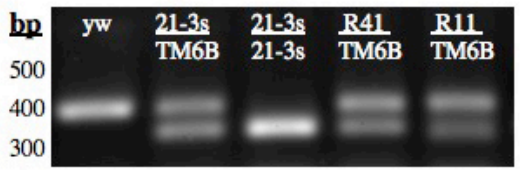


PCR diagnostic for the *Su(21-3s)* mutation

ras1 allele. For example, *Su(21-3s)* is not much affected by loss-of-function mutations in upstream components of MAPK signaling, such as *argos* or *EGFR* (Fig. 4B,C), but is strongly ameliorated by loss of downstream components, such as *rolled* (Fig. 4E). Additionally, when a dominant negative form of Ras1 (*sev-ras1^{N17}*) is expressed in the eye, the suppressive effects of *Su(21-3s)* are severely abrogated. Taken together, these data confirm that *Su(21-3s)* is an allele of *ras1* and we refer to *Su(21-3s)* from here on as *ras1^{R68Q}*.

Biochemical analysis of recombinant Ras1^{R68Q}

To test the hypothesis that exchanging a positively charged arginine with a neutral glutamine at position 68 of Ras1 results in a protein with a deficient GTPase activity, the intrinsic GTPase rates of wildtype and mutant Ras1 proteins were compared (Fig. 5). Full length wildtype Ras1 and mutant Ras1^{R68Q} proteins were bacterially expressed and purified as His-tagged fusion proteins, yielding large amounts of pure, catalytically active enzyme. Intrinsic GTPase activity was measured with a kinetic phosphate assay employing [γ-^{33}P]GTP as substrate. This sensitive assay revealed that Ras1^{R68Q} has an intrinsic GTPase activity that is approximately 1/3 that of wildtype Ras1, with enzymatic rates (k_{cat}) of 0.020 min^{-1} and 0.063 min^{-1},

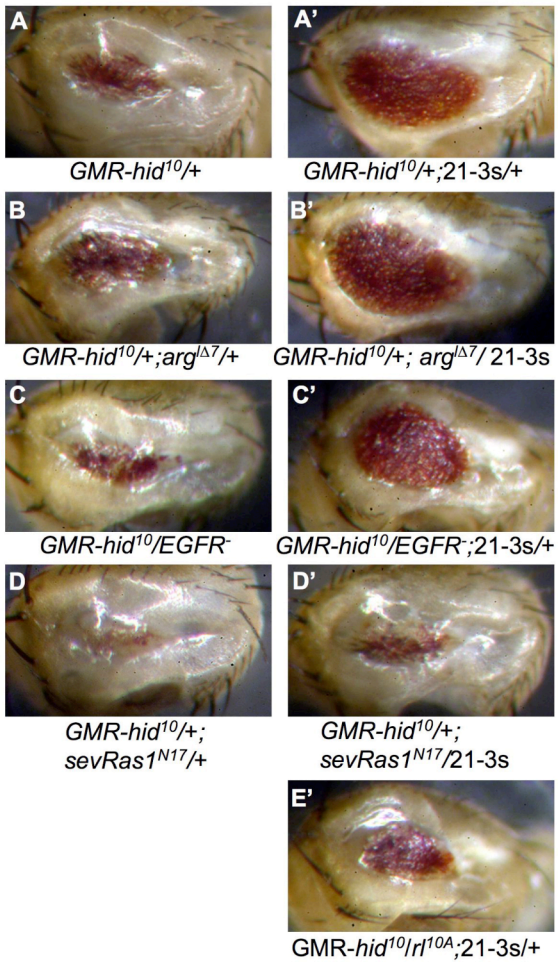
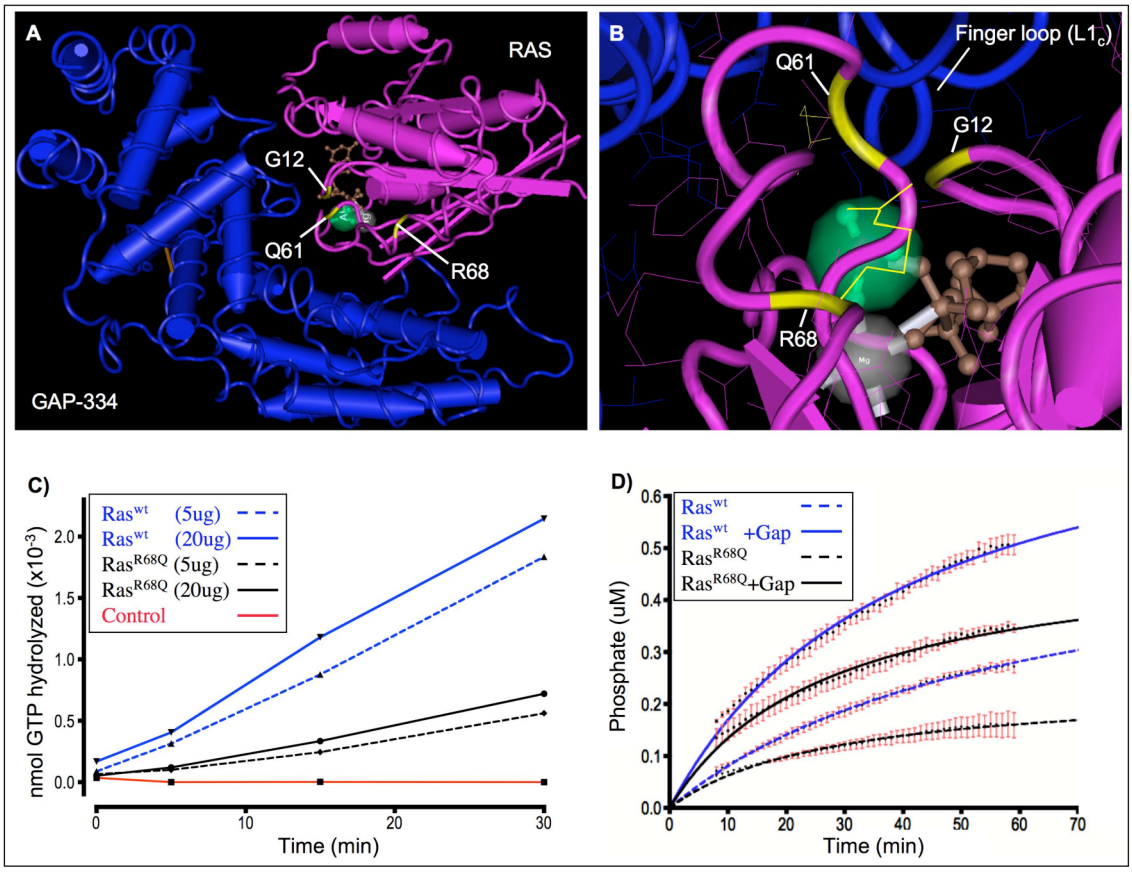


Fig. 3.4. The *Su(21-3s)/ras1*^{R68Q} mutant differentially interacts with components of the EGFR/MAPK pathway. Suppression of the *GMR-hid*^{10} induced eye ablation phenotype by *Su(21-3s)* (A vs A') is not much affected by loss of function mutations in upstream components of MAPK signaling such as *argos* (B vs B') or *egfr* (C vs C'), but is strongly ameliorated by loss of downstream components, such as *rolled* (A' vs E'). Additionally, when a dominant negative form of Ras1 (*sev-ras1*^{N17}) is expressed in the eye, the suppressive effects of *su*(21-3s) are completely abrogated (A' vs D'). Genotypes are as indicated except that 21-3s refers to *Su(21-3s)/ras1*^{R68Q}

Fig. 3.5. Structural and biochemical analysis of wildtype and mutant Ras1. (A-B) Three-dimensional crystal structure of human H-Ras (pink) bound to the GTPase-activating domain of human GTPase-activating protein $p120^{GAP}$ (GAP-334, blue) in the presence of aluminum fluoride (AlF_3, green.) The positions of oncogenic residues glycine-12 (G12) and glutamine-61 (Q61) as well as the mutant residue in *ras1*^{R68Q} flies, arginine-68 (R68), are shown in yellow. The Switch II region of Ras, of which Q61 and R68 are a part, is stabilized by GAP-334. (B) An enlargement of (A) showing the finger loop of GAP-334, which supplies an arginine side chain (arginine-789) into the active site of Ras to neutralize developing charges in the transition state (Scheffzek et al., 1997). R68, located proximally to the catalytic site of Ras, also extends a positively charged guanidinium group towards the active site. The images were constructed using the Entrez software Cn3D with mmdbId:51925 (Chen et al., 2003). Guanosine diphosphate (GDP,brown); Mg^{2+} (grey). (C) The intrinsic GTPase activities of affinity purified drosophila $Ras1^{wt}$ (blue) and $Ras1^{R68Q}$ (black) were determined using a kinetic phosphate assay employing [γ-^{33}P]GTP as a substrate. The conditions of the assay are such that the reaction proceeds with unimolecular kinetics and is insensitive to the amount of Ras protein employed (dashed vs. undashed lines). The mutant $Ras1^{R68Q}$ has an intrinsic GTPase activity that is approximately 1/3 that of wildtype Ras1 (k_{cat}= 0.020 min^{-1} and 0.063 min^{-1} respectively.) (D) Human GAP-285 protein was purified by affinity chromatography and its ability to stimulate wildtype and mutant Ras1 proteins was tested using a real-time fluorescent assay. $Ras1^{R68Q}$ is amenable to GAP stimulation, but to a lesser extent than is the wildtype Ras1 protein. Data is the average of three independent experiments. Error bars are in red. E) Typical intrinsic GTPase rates.



E)

Ras	Intrinsic GTPase Activity (min^{-1})
Wild type	0.028
G12V	0.002
G12D	0.001
G12R	0.0014
G13V	0.013
Q61H	0.0019
Q61L	0.0013
$dmRas^{wt}$	0.063
$dmRas^{R68Q}$	0.020

respectively (Fig. 5C). This supports the prediction that the gain of function nature of $Ras1^{R68Q}$ is due to a reduced GTPase activity. Additionally, since many activating Ras mutations result in an enzyme that is insensitive to GAPs, the ability of $Ras1^{R68Q}$ to be stimulated by GAP was also assessed. Recombinant human GAP-285 protein was purified by affinity chromatography and its ability to stimulate wildtype and mutant Ras1 proteins was tested using a sensitive real-time fluorescent assay. It was determined that $Ras1^{R68Q}$ remains amenable to GAP stimulation, although to a lesser extent than the wildtype Ras1 protein (Fig. 5D). Unlike the oncogenic, constitutively active mutant $Ras1^{V12}$, whose GTPase activity is completely refractory to stimulation by GAP, $Ras1^{R68Q}$ can be regulated by GAP and is able to cycle between on and off states (Trahey and McCormick, 1987). This biochemical data supports the hypothesis that $Ras1^{R68Q}$ has a reduced GTPase activity, remains in its active GTP-bound form for a prolonged period of time and therefore has an enhanced signaling capacity, but is still largely amenable to regulation, permitting nearly normal cellular function and organismal development.

Analysis of midline glia (MG) survival in *ras1*^{R68Q} embryos

Drosophila midline glia (MG) cell survival during embryonic development is exquisitely sensitive to MAPK activity levels (Bergmann et al., 2002; Stemerdink and Jacobs, 1997). During formation of the *Drosophila* central nervous system, there are initially about ten MG cells per segment at stage 13. Most of these undergo apoptosis in a *rpr*, *hid* and *grim* dependent manner such that by stage 17, only three MG per segment survive (Sonnenfeld and Jacobs, 1995; Zhou et al., 1997). We tested the effect of *ras1*^{R68Q} in this sensitive system. MG cells were visualized in wildtype and *ras1*^{R68Q} embryos using the MG-specific reporter fusion construct *pslit-lacZ* and *β-gal* immunohistochemistry and clearly marked MG cells were carefully counted. This analysis revealed an increase in the number of MG cells in *ras1*^{R68Q} embryos as compared to wildtype embryos (Fig. 6A,B). Stage 17 wildtype embryos contained an average of 2.8 MG cells per segment (n=448) whereas *ras1*^{R68Q} embryos contained an average of 3.3 MG cells per segment (n=420). This difference is statistically significant by an unpaired t-test ($p_{95} \leq 0.0001$) and is consistent with elevated MAPK survival signaling in *ras1*^{R68Q} flies (Fig. 6C).

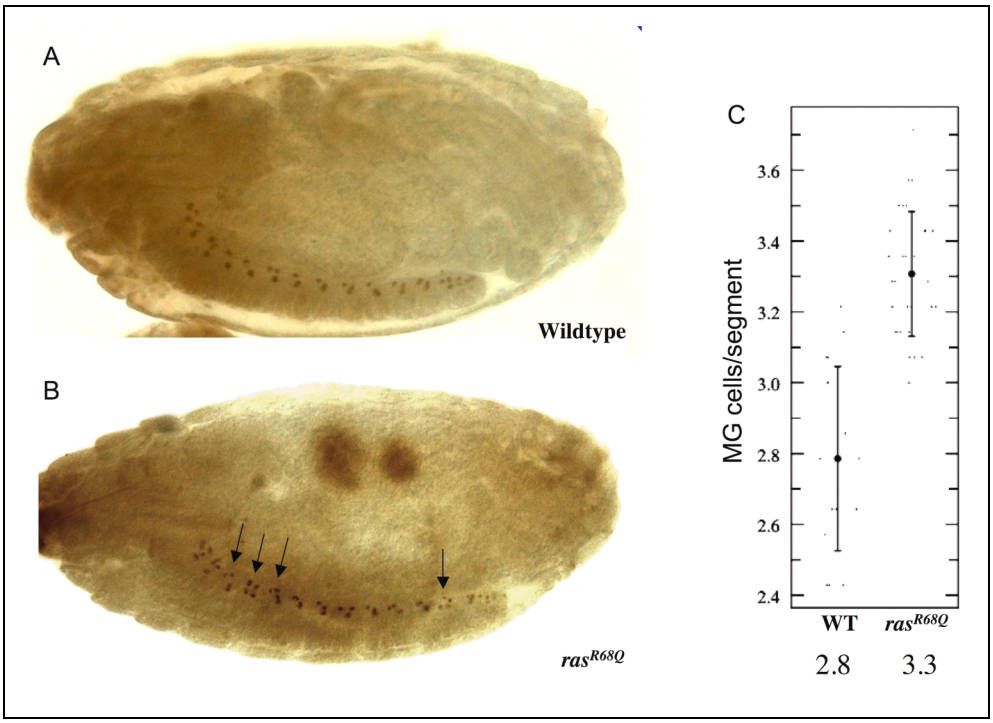


Fig. 3.6. *Ras1*^{R68Q} mutant embryos contain extra midline glial (MG) cells. MG were visualized in wildtype (A) and *ras1*^{R68Q} (B) stage 17 embryos using the MG-specific reporter construct P[*slit-1.0-lacZ*] and β-gal immunohistochemistry. During development, the majority of MG undergo apoptosis such that at this stage only about three MG per segment normally survive. This analysis reveals an increase in the number of surviving MG cells in *ras1*^{R68Q} embryos as compared to wildtype embryos. Arrows indicate segments that clearly contain more than three MG cells. (C) Wildtype embryos contained an average of 2.8 MG cells per segment (n=448) whereas *ras1*^{R68Q} embryos contained an average of 3.3 MG cells per segment (n=420). This difference is statistically significant by an unpaired t-test ($p_{95} \leq 0.0001$) and is consistent with enhanced MAPK signaling in *ras1*^{R68Q} mutants.

Assay for supernumery R7 cells in *ras1*^{R68Q} adult eyes

The adult Drosophila eye comprises about 800 ommatidia, each with a precise, reproducible structure consisting of eight photoreceptors and 12 accessory cells (Cagan and Ready, 1989; Morante et al., 2007; Tomlinson, 1988). Adoption of a neuronal cell fate by the precursor cell of R7 photoreceptors requires an inductive signal from the neighboring R8 cell and is dependent on EGFR/MAPK signaling (Gaul et al., 1992; Simon et al., 1991; Yang and Baker, 2001). In addition, cone cell precursors are capable of acquiring an R7 cell fate if MAPK signaling is ectopically activated in these cells, resulting in extra R7 cells that are easily visualized (Fortini et al., 1992). To determine if the *ras1*^{R68Q} mutation exerts effects in a paradigm other than apoptosis, such as cell fate determination, semi-thin plastic sections of adult eyes were prepared and analyzed for defects in ommatidia formation. This analysis revealed two clear defects in *ras1*^{R68Q} flies that are typical for mutations that enhance RAS/MAPK signaling during eye development. First, we detected ommatidia with supernumery R7 cells indicating that the *ras1*^{R68Q} mutation can provide a sufficiently strong inductive signal to drive cone cell precursors towards an R7 photoreceptor fate (Fig. 7B). Second, we observed ommatidia missing an occasional outer

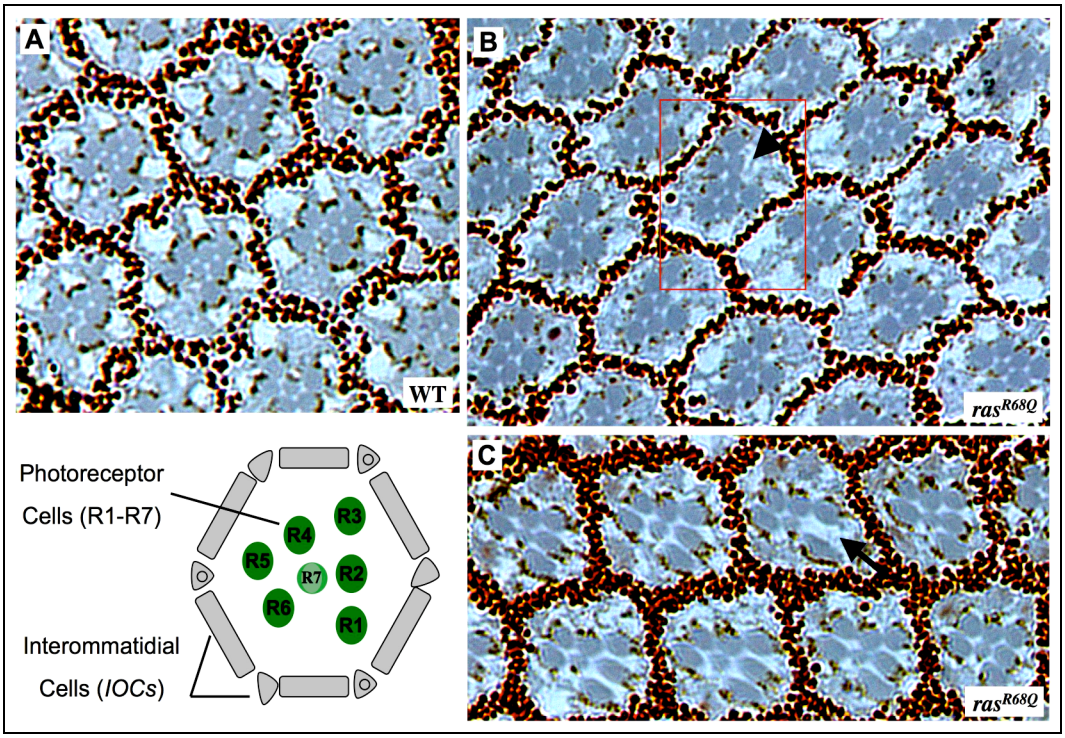


Fig. 3.7. Developmental analysis of the *ras1*^{R68Q} mutant adult eye phenotype. To determine if *ras1*^{R68Q} exhibits phenotypes in a paradigm other than apoptosis, semi-thin plastic sections of adult eyes were prepared and analyzed for defects in ommatidia formation. (A) Wildtype ommatidia contain one R7 cell and six outer photoreceptor cells. (B-C) *Ras1*^{R68Q} flies contain two types of differentiation defects typical of mutations that increase RAS/MAPK signaling during eye development, including supernumery R7 cells (arrowhead inside red outline, B) and missing outer photoreceptor cells (arrow, C). The developmental defects in retinal cell differentiation observed here supports our hypothesis that flies carry a gain of function *ras1* allele. The schematic illustrates the major cell types present in the ommatidia

photoreceptor cell, also reported to be a phenotypic consequence of elevated MAPK signaling (Fig. 7C) (Fortini et al., 1992).

The wings of *ras1^{R68Q}* flies contain ectopic vein material

In addition to defects in the eye and midline glial cells, *ras1^{R68Q}* flies also show abnormalities in adult wing tissues. Homozygous *ras1^{R68Q}* flies contain an additional longitudinal "veinlet" seen to branch off the posterior crossvein (Fig. 8). Additionly, an ectopic longitudinal vein appears directly beneath the posterior crossvein and an ectopic crossvein appears between the L4 and L5 wing veins near the hinge (Fig. 8B,F). These phenotypic defects are remarkably similar to those observed in the wings of *rl^{sem}* and *DER^{Ellipse}* flies which exhibit elevated levels of MAPK signaling in the wing as they are hypermorphic alleles (Brunner et al., 1994). When *UAS-ras1^{R68Q}* is overexpressed in the wing using *en-Gal4* an extensive amount of ectopic wing vein material develops and blisters also commonly appear (Fig. 8D). Overexpression of wildtype Ras1 in the same manner results in a significant but less severe phenotype (Fig. 8C). We also attempted to express the *ras1^{V12}* mutant in the wing using *en-Gal4* but found this induced lethality, a problematic feature of this ras1 allele.

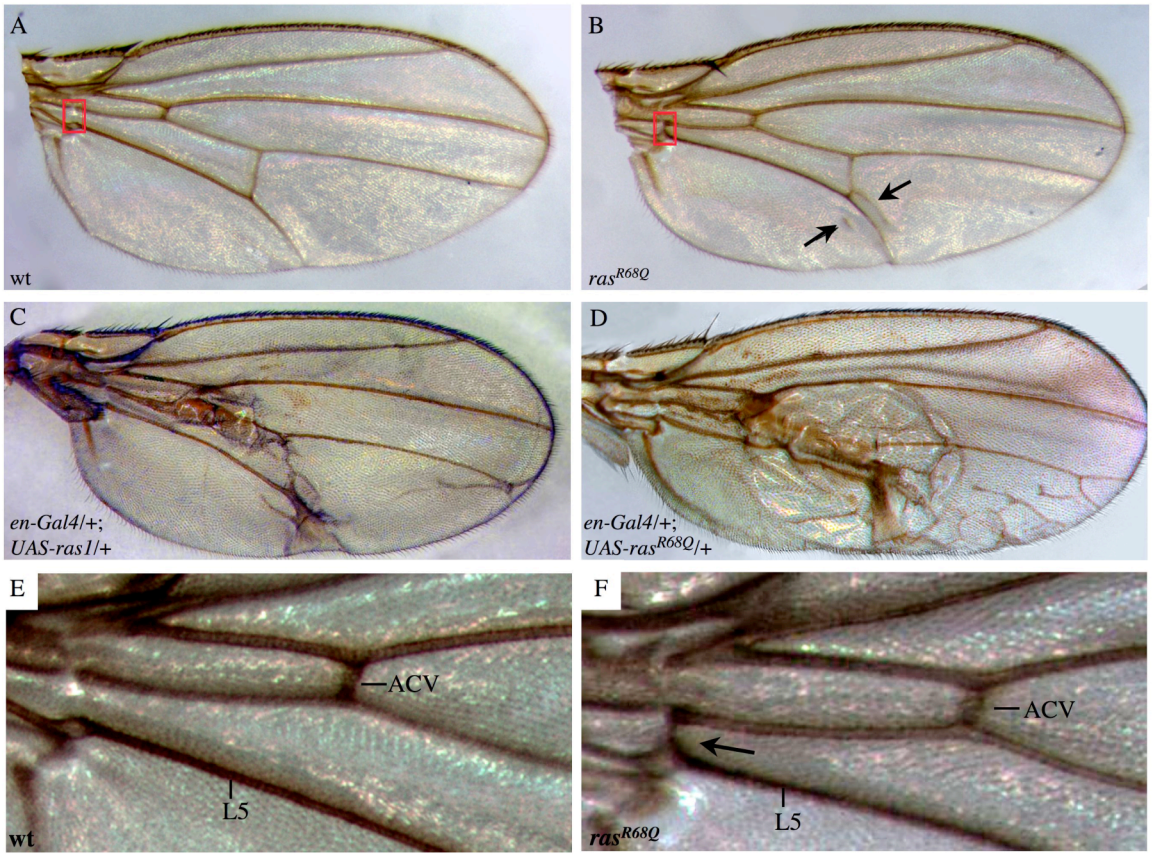


Fig. 3.8. Wing phenotypes associated with the *ras1*^{R68Q} allele. Flies bearing the *ras1*^{R68Q} allele develop ectopic wing material including extra longitudinal 'veinlets' near the posterior crossvein (arrows, B) and an extra crossvein near the wing hinge (red box, B and arrow, F). Overexpression of either wildtype *ras1* (C) or *ras1*^{R68Q} (D) using the *en-Gal4* driver results in the deposition of substantial amounts of ectopic vein material. (E,F) Magnification of (A) and (B) encompassing the area boxed in red. The arrow in (F) indicates an ectopic crossvein observed in *ras1*^{R68Q} flies. Genotypes are as indicated. ACV, anterior crossvein. L5, L5 wing vein. Anterior is up.

Overexpression in the eye of *ras1*^{R68Q}, but not wildtype *ras1*, induces severe overgrowth defects

To further establish that Ras1^{R68Q} is an activated version of the Ras1 protein and to observe the phenotypic consequence, we ectopically expressed *ras1*^{R68Q} in the developing *Drosophila* eye. Overexpression of wildtype Ras1 in various *Drosophila* tissues, even at the high levels obtained by transgene expression, often results in mild or no observable phenotypic effect (Fortini et al., 1992). For this reason, studies of elevated Ras1 signaling in *Drosophila* largely rely on a constitutively active, non-regulatable *ras1*^{V12} mutant allele. We similarly observed in eleven independent transgenic lines that wildtype *UAS-ras1* expression driven by *GMR-Gal4* was fully viable and had only minor effects on eye development (Fig. 9B). In sharp contrast, expression of *UAS-ras1*^{R68Q} in seven independent transgenic lines resulted in lethality for three and highly distorted eyes that appear to exhibit both overgrowth and cell death phenotypes for the remaining four (Fig. 9C-F). Similar results were obtained using *sev-Gal4* to drive ras expression (Fig. 9G,H). Interestingly, the anterior part of the eye appears to be much more sensitive to ras expression by the *sev-Gal4* driver since overgrowth is largely restricted to this region.

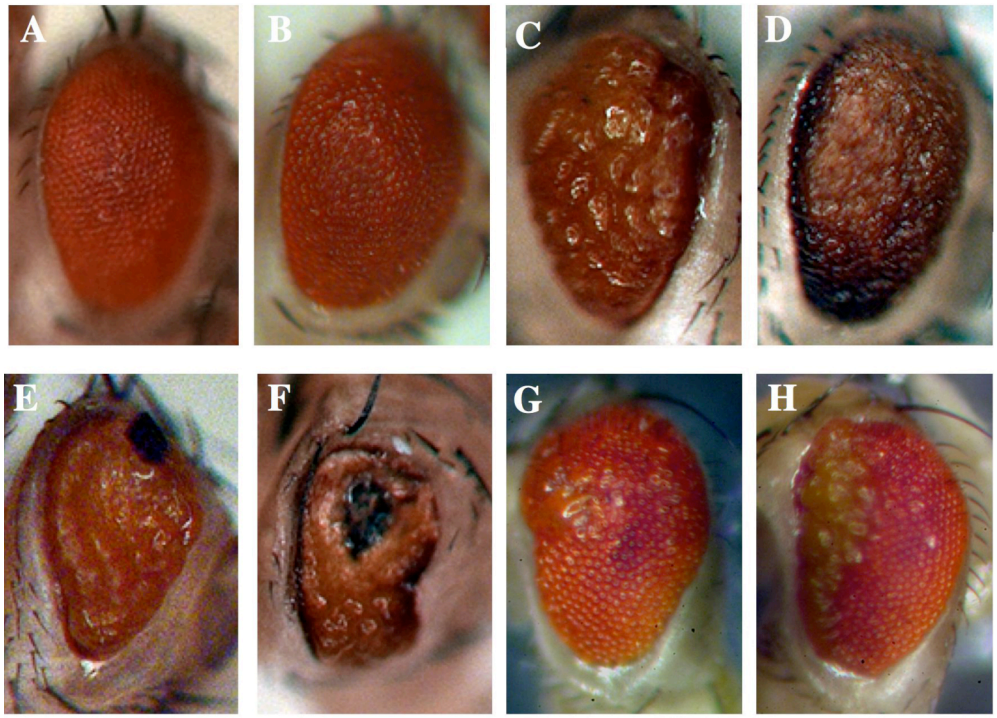


Fig. 3.9. Overexpression of ras in the eye induces developmental defects causing both overgrowth and cell death phenotypes. Flies overexpressing wildtype *ras1* (B,G) exhibit relatively minor disruptions in eye patterning and in the case of *sev-Gal4* driven expression, a small but significant amount of overgrowth occurs in the anterior part of the eye (G). In contrast, overexpression of $ras1^{R68Q}$ with *GMR-Gal4* (C-F) causes severe overgrowth and patterning disruptions. An example from each of four independent transgenic lines is shown to illustrate the range of phenotypes. Likewise, overexpression of $ras1^{R68Q}$ with *sev-Gal4* elicits a much more pronounced overgrowth phenotype in the anterior part of the eye (H) compared to that of wildtype *ras1* (G). Genotypes: (A) *GMR-Gal4*/+, (B) *GMR-Gal4*/+;UAS-*ras1*/+, (C-F) *GMR-Gal4*/+;UAS-$ras1^{R68Q}$/+, (G) *sev-Gal4*/+;UAS-*ras1*/+, (H) *sev-Gal4*/+;UAS-$ras1^{R68Q}$/+.

For purposes of comparison we attempted to express two different *UAS-ras1*^{V12} alleles in the eye with both these drivers, but unsurprisingly, again found this induced lethality (likely due to leaky expression and the fact that *ras1*^{V12} can elicit non-cell autonomous death when overexpressed) (Karim and Rubin, 1998). These overexpression experiments further support the notion that Ras1^{R68Q} is an activated Ras protein that, in contrast to the constitutively active Ras1^{V12} protein, remains amenable to negative regulation and therefore is less biologically potent than Ras1^{V12}. All else being equal, this will permit overexpression of the *ras1*^{R68Q} allele with a broader array of transgenic promoters in a wider range of physiologic contexts.

Discussion

The work presented here encompasses a genetic, molecular and biochemical characterization of the first endogenous gain-of-function *ras1* mutation to be identified in Drosophila. This hypermorphic Ras allele, Ras1^{R68Q}, ranks among one of a very few viable Ras hypermorphs to be identified in any multicellular organism. A curious fact considering the hundreds and perhaps thousands of genetic screens carried out over the last two decades or so with the express purpose of identifying components in the

Ras/MAPK signaling pathway. In fact, only two viable gain-of-function mutations in an essential Ras gene are well known, both of which are alleles of the *C. elegans* Ras homolog *let-60* (Sternberg and Han, 1998). One of these alleles, *let-60(G13E gf),* has been independently isolated more than 5 times and when taken into account with the countless loss-of-function and dominant negative Ras alleles that have been isolated across the animal kingdom, it is clear that the Ras locus has been well saturated in mutagenesis experiments. It appears that hypermorphs of Ras are very poorly tolerated in biological systems and likely reflects the critical and ubiquitous role played by Ras in biological systems.

Of course, many lethal gain-of-function Ras mutations have been identified, particularly in the context of human tumorigenesis. An activating mutation in one of the three human *ras* genes is found in ~20-30% of all tumours, and in up to 90% of some types of carcinomas (Bos, 1989). Invariably these oncogenic mutations occur at amino acid positions 12,13 or 61 and result in a very potent, constitutively active Ras protein. Normally, Ras proteins cycle between a GTP-bound state in which they able to productively engage downstream effectors and a GDP-bound state in which they are inactive. The interconversion between these two states is tightly regulated by two classes of enzymes: guanine nucleotide exchange factors

(GEFs) which catalyze the exchange of GDP for GTP thereby activating Ras, and GTPase activating enzymes (GAPs) which inactivate Ras by dramatically stimulating the rate of GTP hydrolysis by Ras. Like Ras, these regulatory proteins are highly conserved throughout many species (Colicelli, 2004).

In *Drosophila*, Ras1 has been implicated in a number of developmental processes, including the specification of ventral ectoderm fate in the embryo, imaginal disc cell growth, differentiation of wing vein and photoreceptor cells and regulation of embryonic midline glia survival by suppression of apoptosis (Bergmann et al., 2002; Diaz-Benjumea and Hafen, 1994; Miller and Cagan, 1998; O'Keefe et al., 2007; Yang and Baker, 2001). Genetic analysis in Drosophila has been pivotal in delineating the many functions of Ras during development and pathogenesis and many of the insights gleaned from these studies have proven applicable to other organisms, including humans.

We have described the isolation and characterization of $Ras1^{R68Q}$, a viable gain-of-function Ras allele that contains a mutation in the universally conserved Switch II region. Initially identified as a dominant suppressor of *hid* induced cell death in the eye, we extended the characterization of its suppression phenotype to larval hemocytes and in the setting of other MAPK

pathway mutants. Moreover, we describe the phenotypic consequences of this gain-of-function allele in several developmental contexts including its effect on midline glia survival in embryos, R7 photoreceptor differentiation in ommatidia and wing vein development. Finally, we showed biochemically that this Ras1 allele has a reduced intrinsic GTPase activity of about one third that of wildtype Ras1, but that it remains responsive to GAP stimulation. This latter fact is almost certainly why $Ras1^{R68Q}$ is viable.

Experimental Procedures

Fly stocks and genetics

The following fly stocks were used: *GMR-rpr*^{81} (White et al., 1996), *GMR-hid*^{1M}, *GMR-hid*^{Ala3} and *GMR-hid*^{Ala5} (Bergmann et al., 1998), *GMR-hid*^{10} (Grether et al., 1995), *GMR-grim* (Chen et al., 1996), *arg*$^{1\Delta7}$ (Freeman et al., 1992), EGFR^{-} = *flb*^{f2} (Nussleinvolhard et al., 1984), *rl*^{10a} (Peverali et al., 1996), *sev-Ras1*^{N17} (Karim et al., 1996), *en-Gal4*, *sev-Gal4* (Therrien et al., 1999), P[*slit-1.0-lacZ*] (Wharton and Crews, 1993), *Hml-Gal4, 2xUAS-EGFP* (J.A. Rodriguez, unpublished). Stocks for meiotic recombination mapping (*ru*^{1} *h*^{1} *th*^{1} *st*^{1} *cu*^{1} *sr*^{1} *e*^{s} *ca*^{1} and *ru*^{1} *h*^{1} *th*^{1} *st*^{1} *cu*^{1} *sr*^{1} *e*^{s} *Pr*^{1} *ca*^{1}/TM6B, *Bri*^{1}, *Tb*^{1}) and stocks for P-element induced male recombination mapping (*y*^{1} *w*; CyO, H{PDelta2-3}HoP2.1/Bc*^{1} *Egfr*^{E1} as a source of

transposase and all P-element insertion lines) were obtained from the Bloomington Stock Center (Bloomington, IN). Flies carrying *UAS-ras1* and *UAS-ras*^{R68Q} were generated by P element-mediated transformation according to standard protocols. All other lines were generated by meiotic recombination of the appropriate alleles.

Su(21-3s) was mapped by meiotic recombination using the *rucuca* mapping chromosome. Mapping of the *Su(21-3s)* mutant was further refined using P-element induced male recombination with the dominant markers *Ly* and *Pr* (Chen et al., 1998). Reversion screens were performed as described in Fig. 3. All crosses and suppression experiments were carried out at 25°C except overexpression studies with *en-Gal4*, *GMR-Gal4* and *sev-Gal4* used in conjunction with *UAS-ras1*^{R68Q} or *UAS-ras1*, which were performed at 18°C.

To visualize larval hemocytes, wandering 3rd instar larva expressing *UAS-EGFP* driven by *Hml-Gal4* were collected and immobilized on ice prior to imaging (Goto et al., 2003). MG cells in stage 17 embryos were visualized using P[*slit-1.0-lacZ*] and β-gal immunohistochemistry as previously described (Patel, 1994). The number of MG was averaged for segments T2 to A5. Tangential sections (1 μm) of adult eyes were prepared for analysis of ommatidia (Tomlinson and Ready, 1987).

Biochemistry

A cDNA clone encoding *Drosophila ras1* was obtained from the *Drosophila* Genomics Resource Center (clone ID: RE53955) and the entire *ras1* ORF was subcloned into pBluescript (Stratagene). Mutant ras^{R68Q} was generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). The Ras ORFs were then subcloned into pET-28a (Novagen) in frame for an N-terminal His tag for expression and into pUAST (Brand and Perrimon, 1993) for P element-mediated transformation. Catalytic human p120-Gap (GAP-285, amino acids 714-998, IMAGE Clone: 4829173**,** Open Biosystems) was subcloned into the pET41a vector (Novagen) to generate an N-terminal GST tag. Fusion proteins were expressed in BL21(DE3) *E.* coli (Invitrogen) and affinity purified on an AKTA Purifier (Pharmacia) using a HisTrap FF column (GE Healthcare) for Ras proteins and a GSTrap FF column (GE Healthcare) for GAP-285. Ras purification was performed according to the procedure described for human H-Ras (Boriack-Sjodin et al., 1998). GAP-285 was expressed by inducing cells for 16 hours at 30°C with 0.2 mM IPTG.

Intrinsic GTPase activities were measured using [γ-^{33}P]GTP (3000 Ci/mmol, NEN) and the EasyRad Phosphate Assay (Cytoskeleton) (Bollag and McCormick, 1995). GAP-stimulated GTPase activities were measured

with a real-time assay using the fluorescent substrate MDCC-PBP (Invitrogen) and 2 μM Ras protein, with or without, 0.02 μM GAP-285 (Shutes and Der, 2005).

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