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Review

Tumour suppressors—a fly's perspective

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Abstract

For a century, the little fruitfly *Drosophila melanogaster* has taught generations of geneticists about how genes control the development of a multicellular organism. More recently, *Drosophila* has begun to contribute more directly towards our understanding of human disease [Bernards A, Hariharan IK. Of flies and men—studying human disease in Drosophila. *Curr Opin Genet Dev* 2001, **11**, 274–278]. It is capable of doing this because it shares many disease-related genes with us. For example, the *Drosophila* genome sequencing project has revealed that two thirds of the genes implicated in human cancers have a counterpart in the fly genome [Adams MD, Celniker SE, Holt RA, *et al*. The genome sequence of Drosophila melanogaster. *Science* 2000, **287**, 2185–2195, Fortini ME, Skupski MP, Boguski MS, Hariharan IK. A survey of human disease gene counterparts in the Drosophila genome. *J Cell Biol* 2000, **150**, F23–30]. In particular, the fly has homologues of the Retinoblastoma protein (pRb) and of p53, two prototypical tumour suppressors which are inactivated in the majority of human tumours. Here, we will compare the fly's tumour suppressors with their human counterparts and we will review recent advances in our understanding of how these factors function in the fly.

Keywords: Tumour suppressor; pRb, p53; Cell cycle; *Drosophila melanogaster*

1. The pRb network in human cells

Entry and progression through the DNA synthesis phase (S-phase) of the eukaryotic cell cycle requires the orderly activation of an extensive set of genes (Fig. 1). Many of these are regulated by the E2F family of transcription factors [4,5]. Regulation of E2F activity is a major focus of cell cycle control and deregulated E2F activity is the hallmark of many cancer cells.

The network regulating E2F activity in human cells is highly complex and involves many players (Table 1): Six E2F proteins (E2F1-6) exist which heterodimerise with one of three DP proteins (DP1-3) [6–8]. Whereas five E2Fs (E2F1-5) can transactivate reporter genes with E2F binding sites in transient transfection assays, only E2F1, E2F2 and E2F3 are bound to their target genes *in vivo* when these are expressed during late G1 and S-phase [9,10]. E2F4 and E2F5, on the other hand, bind preferentially to their target genes in resting cells (G0) and during early G1 when transcription is turned off. Accordingly, E2F1-3 have been classified as 'activator E2Fs' which activate genes at the G1/S boundary. E2F4 and 5 have been termed 'repressor E2Fs' because they repress untimely gene transcription.

E2F factors associate with members of the pocket protein family which includes the prototypical Retinoblastoma tumour suppressor protein (pRb) and two related polypeptides, p107 and p130. Distinct E2F/pocket protein complexes are formed *in vivo*: pRb binds E2F1-4, p107 only associates with E2F4 and p130 complexes with E2F4 and 5 [8]. The pocket proteins repress E2F bound genes by several mechanisms (see below). To allow synchronised expression of E2F target genes at the G1/S boundary, cyclin/cyclin-dependent kinase (CDK) complexes initiate the disruption of pocket protein/E2F repressor complexes by hyperphosphorylation. Several distinct cyclin/CDK complexes are activated at this time: complexes containing CDK4 or CDK 6 and one of three cyclin D proteins (cyclin D1-3) as well as a cyclin E/CDK2 complex. These cyclin/CDK complexes act on pRb in a sequential fashion

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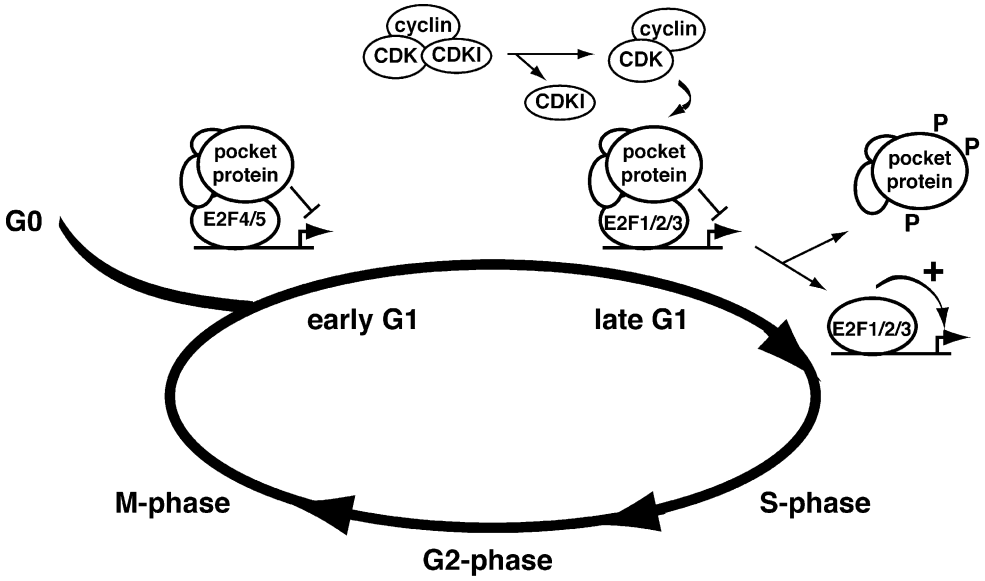

Fig. 1. Transcriptional control by E2F during the cell cycle. G0, G1, G2: Gap phases; S: DNA synthesis; M: Mitosis; CDK: cyclin-dependent kinase; CDKI: cyclin-dependent kinase inhibitor; P: phosphate group. See text for details.

leading to progressive disassembly of the repressor complex [11]. Prior to their activation, cyclin/CDK complexes are inactived by CDK inhibitors, such as p16 (INK4a), p15 (INK4b), p21 (Cip1), p27 (Kip1), and p57 (Kip2) [12]. In total, this regulatory network involves more than twenty key players.

Considering the importance of this network for cell proliferation, surprisingly few components are associated with tumours. For example, whereas pRb is inactivated in a variety of cancers, its relatives p107 and p130 are not. Moreover, none of the six E2Fs has been found mutated in tumours to date. These observations argue that the pRb network contains a high degree of redundancy and that in many cases loss of one regulator can be compensated for by another. Indeed, all three activating E2F proteins need to be ablated simultaneously to prevent mouse embryonic fibroblasts from entering S-phase [13].

2. The RBF network in the fly

The complexity and redundancy of the pRb network poses problems for scientists attempting to unravel the

Table 1
Comparison of pRb network components between humans and flies

	Human	Fly
G1 Cyclins	Cyclin D1, cyclin D2, cyclin D3 Cyclin E1, cyclin E2	Cyclin D Cyclin E
CDKs	CDK5, CDK6 CDK2	CDK4 CDK2
CDK inhibitors	p15, p16 (INK) p21, p27, p57 (CIP/KIP)	dacapo (CIP/KIP)
Pocket proteins	pRb, p107, p130	RBF1, RBF2
E2Fs	E2F1, E2F2, E2F3a, E2F3b E2F4, E2F5, E2F6	dE2F1 dE2F2
DPs	DP1, DP2, DP3	dDP
E2F/pocket protein complexes	E2F1/pRb, E2F2/pRb, E2F3a/pRb, E2F3b/pRb, E2F4/pRb, E2F4/p107, E2F4/p130, E2F5/p130	dE2F1/RBF1, dE2F2/RBF1 dE2F2/RBF2

CDK, cyclin-dependent kinase. Note that the number of human E2F/pocket protein complexes is further increased by the ability of E2F proteins to heterodimerize with different DP proteins.

molecular details underlying cell cycle regulation. Often, results obtained depend to a considerable extent on the cell line used arguing that certain members of the pRb network are more important in some cell types than in others, a notion that is supported by the analysis of various knock-out mice (for a detailed discussion see [8]). The identification in the mid-1990s of cyclins, CDKs, CDK inhibitors, E2F, DP and pRb-related proteins in *Drosophila* came therefore as a welcome surprise for it suggested that a pRb network is also operating in a much simpler organism [14–20]. An abundance of genetic experiments support this hypothesis: Inactivation of *Drosophila* E2F1 (dE2F1) prevents DNA synthesis during later stages of embryogenesis [21]. Conversely, overexpression of dE2F1 and dDP can drive cells into S-phase and apoptosis [22]. If this overexpression is directed to the composite eye, it can result in a "rough eye" phenotype that is suppressed by concomitant overexpression of the pRb related factor 1 (RBF1) [15]. These early genetic experiments have shown that dE2F1 is important for cell cycle progression and that the fly's pRb homologue RBF1 does repress dE2F activity. This notion was confirmed by transient transfection experiments in cell lines demonstrating that RBF1 specifically represses dE2F1/dDP dependent reporter genes [15]. Moreover, RBF1 is under the negative control of cyclin/CDK complexes: Cyclin D/CDK4, the *Drosophila* counterpart of the major pRb kinase in human cells, and RBF1 display antagonistic activities in genetic experiments [23–25]. In addition, cyclin E, one of several genes upregulated by dE2F1, forms a complex with the *Drosophila* CDK2 kinase which can phosphorylate RBF1 *in vitro* [15,26]. Accordingly, ectopic expression of cyclin E triggers S-phase onset and a loss-of-function cyclin E mutation enhances an RBF1 overexpression phenotype [15,27]. RBF1, dE2F1 and cyclin E constitute a negative feedback loop similar to the one operating in human cells (Fig. 2). There is also evidence that CDK inhibitors control the RBF network: Expression of Dacapo, a *Drosophila* CIP/KIP-type CDK inhibitor homologue, arrests the cell cycle in G1 and dacapo and RBF1 overexpression synergise in affecting cell cycle arrest [14,18]. Most strikingly, the phenotype of RBF1-deficient flies is remarkably similar to the phenotype of $pRb^{-/-}$ knock-out mice: Both mutants display ectopic S-phases, upregulation of E2F target genes and increased apoptosis [28]. Collectively, these and other results show that the fly's pRb, E2F, cyclin, CDK and CDK inhibitor homologues function to control entry into S-phase and that they display hierarchical relationships that are similar to their human counterparts.

But if the *Drosophila* pRb network is so similar to the human one, is it really simpler? The completion of the *Drosophila* and human genome sequencing projects allows us to answer this question with some authority. *Drosophila* has genes coding for E2F and DP proteins, pocket proteins, cyclins, CDKs and CDK inhibitors indicating that the fly's pRb network consists of the same hierarchical levels. However, the number of proteins operating on each level is much smaller (Table 1). As we will see, this greatly simplifies the analysis of cell cycle regulation in flies.

3. Activating and repressing E2Fs

Human cells express a group of activating E2Fs (E2F1, E2F2 and E2F3) and a group of repressing E2Fs (E2F4, E2F5 and E2F6), with significant functional overlap within each group. *Drosophila* has only two E2F factors, one of the activating (dE2F1) and one of the repressing type (dE2F2) [29,30]. This lack of redundancy facilitates the generation of mutant animals lacking activating, repressing or even both E2F functions. Flies lacking the repressing dE2F2 activity suffer from decreased fertility and viability, but clearly, dE2F2 is not absolutely essential for development [30]. As expected,

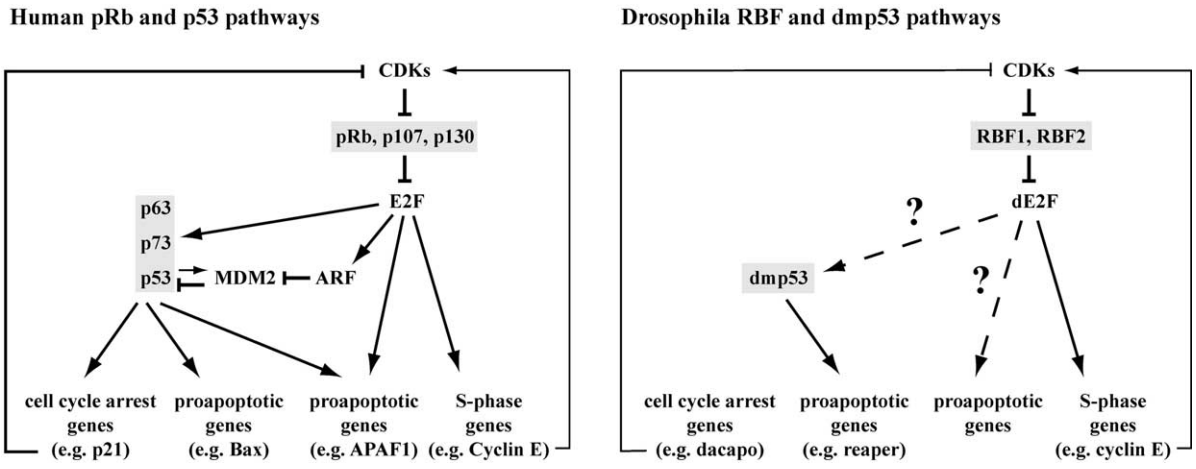

Fig. 2. Comparison of pRb and p53 pathways in humans and flies. See text for details.

several E2F target genes are upregulated in *de2f2*$^{-/-}$ flies. Embryos that do not express functional dE2F1 display much more severe defects, fail to develop into third instar larvae and never reach adulthood. E2F target genes normally upregulated at the G1/S boundary fail to be expressed, DNA synthesis is severely impaired during later stages of embryo development and the imaginal discs and the central nervous system (CNS) do not form properly [21,30,31]. Interestingly, these phenotypes are rescued when both dE2F1 and dE2F2 are knocked out simultaneously [30]: development proceeds through all larval stages and imaginal discs and the CNS develops normally suggesting that DNA synthesis is no longer perturbed. Double mutants die much later as mid- or late pupae.These genetic experiments suggest an antagonistic relationship between activating and repressing E2Fs. Indeed, dE2F2 overexpression reverses dE2F1-mediated transactivation of reporter genes in transient transfection assays. Chromatin immunoprecipitation experiments demonstrate that both factors are bound to the same E2F-responsive genes in asynchronously growing *Drosophila* cells. This led Dyson and colleagues to propose a model according to which dE2F2 represses genes throughout most of the cell cycle, but is replaced by dE2F1 near the G1/S boundary resulting in a burst of gene expression. This scenario is reminiscent of what has been found in the human system: As cells progress from G0 into G1 and then into S-phase, promoter bound E2F4 (a repressive E2F) is replaced by E2F1 and E2F3 (activating E2Fs) on several promoters [9,10]. It is conceivable that in both organisms cell cycle regulated genes are bound by repressing and activating E2F factors in a sequential manner as cells progress through the cycle. However, that this is the case in *Drosophila* remains to be firmly established.

4. Pocket proteins and repressor complexes

Most repressive E2Fs exert their effect on transcription by recruiting repressor complexes containing a pocket protein which masks the E2F transactivation domain and binds and inhibits both the RNA polymerase II (RpolII) transcription machinery and other transactivators bound in the vicinity. In addition, these complexes alter local chromatin structure by associated histone modifying (histone deacetylase and methylase), DNA methyltransferase and adenosine triphosphate (ATP)-dependent chromatin remodelling activities resulting in a repressed chromatin state [32–34]. Moreover, prior to the G1/S transition, the activating E2Fs are bound by a repressor complex containing pRb to prevent premature gene activation. Phosphorylation of pRb by CDKs at the G1/S boundary leads to dissociation of the repressor complex. This then allows the activating E2Fs to interact with the RpolII machinery and to bind histone acetylases which change chromatin structure back to a more active conformation [9,35]. The subunit composition of the various E2F/repressor complexes has so far not been unravelled. One problem is the sheer number of such complexes in human cells (Table 1). *Drosophila* has two pocket proteins, RBF1 and RBF2. Whereas RBF1 forms complexes with both dE2F1 and dE2F2, RBF2 interacts exclusively with dE2F2 thus limiting the number of dE2F/pocket protein complexes to three [36]. RBF1 is functionally analogous to pRb in that it complexes with both activating and repressing E2Fs. RBF2 exclusively interacts with the repressive E2F and in this respect resembles the behaviour of p107 and p130.

Biochemical purification and characterisation of multiprotein complexes from human cells involves the use of rapidly growing, transformed cell lines to allow the accumulation of sufficient starting material. However, most transformed cell lines either contain mutations in pRb (or other components of the pRb network) or express viral oncoproteins disrupting pRb complexes. *Drosophila* affords biochemists with the possibility of purifying pocket protein complexes from non-transformed cells. Embryos can be rapidly accumulated in large quantities and at little cost (a fly facility housing approximately 300 000 flies will routinely produce 100 g of embryos per day). Indeed, *Drosophila* embryo extracts contain several stable RBF complexes which are amenable to further purification and characterisation (M.Korenjak and A.Brehm, unpublished observation). dE2F/RBF repressor complexes appear to directly regulate DNA replication in certain contexts. dE2F1 and dE2F2 are critical for proper endoreplication of the chorion genes in follicle cells [37,38]. Furthermore, dE2F1 and RBF1 coimmunoprecipitate with subunits of the origin recognition complex (ORC) from ovary extracts [39]. It remains to be seen if dE2F/RBF plays a general role in regulating DNA replication or whether its role is limited to the special case of endoreplication.

The mechanism of RBF repressor complex action is not known. A genetic screen for modifiers of a dE2F1 mutation has identified subunits of the brahma chromatin remodelling complex [40]. Moreover, a weak physical interaction between RBF1 and the brahma chromatin remodelling complex has been reported recently [41]. These findings suggests that dE2F regulation in the fly also involves alteration of chromatin structure.

5. p53, dmp53 and their regulation

The regulation of p53 activity is highly complex and has been the subject of several recent reviews [42–45].

Here, we will concentrate on aspects that are relevant to the function of *Drosophila* p53. The human p53 tumour suppressor is the founding member of a family that comprises three proteins (p53, p63 and p73) [46]. Activation of p53 by DNA damage, hypoxia, nucleoside depletion or other conditions existing within tumour cells can have two consequences: Either further proliferation of the cell is arrested until the damage is repaired or the offending cell is eliminated by apoptosis. Activated p53 mediates its effects by both transcriptional and non-transcriptional mechanisms. Best understood is p53's role as a classical transcription factor which upregulates the expression of genes which serve to arrest the cell cycle (for example, the gene coding for the p21 CIP/KIP-type CDK inhibitor) or which elicit apoptosis (such as the *bax* gene). How p53 decides which type of genes (cell cycle arresting or apoptotic) to activate in a given situation is not well understood. Recently, it has been suggested that cofactors help p53 to decide. Cofactors such as JMY, ASPP1 and ASPP2 target p53 to apoptotic genes [47,48].

p53 activity is regulated on many levels including transient increases in protein stability, DNA binding activity and nuclear localisation. The MDM2 protein plays a prominent role in the regulation of p53 stability. MDM2 is a ubiquitin ligase that binds to the N-terminus of p53 and transfers ubiquitin moeties to several sites within the p53 C-terminus [43]. Ubiquitinated p53 is then rapidly exported from the nucleus and degraded by the proteasome. The gene coding for MDM2 is itself positively regulated by p53. This negative feedback loop ensures that p53 levels remain low in unstressed cells and that elevated p53 levels in stressed cells will go back to normal once the damage has been repaired. Many of the signals that lead to activation of p53 impinge on the p53/MDM2 interaction. For example, DNA damage induces phosphorylation of the MDM2 binding site on p53 by several kinases (42). These phosphorylations weaken the interaction between MDM2 and p53 resulting in p53 stabilisation. MDM2 itself is also subject to regulation both through phosphorylation and the binding of small regulatory proteins such as p14/p19 (ARF). ARF, expression of which is frequently silenced in human cancers, binds to MDM2 and inhibits its ubiquitin ligase function thereby increasing p53 stability. It should be noted, however, that MDM2-independent pathways of p53 stabilisation also exist.

In 2000, three groups reported the identification of *Drosophila* p53 (dmp53) [49–51]. Within the DNA binding domain, dmp53 and human p53 display significant homology (25% identity, 43% similarity). However, their N- and C-termini show little conservation. The *Drosophila* genome sequencing project has ascertained that there is only one p53-related protein in the fly [3]. Several point mutations in the DNA binding domain of human p53 have been identified in cancer patients and shown to result in a dominant-negative protein. Introduction of the corresponding mutations in dmp53 also creates a dominant-negative protein which can prevent wild-type dmp53 from binding to DNA. The three groups overexpressed wild-type and dominant-negative forms of dmp53 in flies to elucidate the function of dmp53 *in vivo*. Surprisingly, overexpression of wild-type p53 does not induce G1 arrest. Specifically, dmp53 fails to activate *dacapo*, the *Drosophila* homologue of the CIP/KIP-type CDK inhibitors. Moreover, expression of dominant-negative dmp53 does not interfere with cell cycle arrest induced by X-ray irradiation suggesting that this block is dmp53-independent. By contrast, overexpression of wild-type dmp53 does stimulate apoptosis. Furthermore, expression of dominant-negative dmp53 suppresses X-ray-induced apoptosis. Brodsky and colleagues have demonstrated that the proapoptotic gene *reaper* is regulated by a radiation inducible control element which binds dmp53 [49]. The model derived from these studies implies that irradiation results in activation of dmp53. Dmp53 then binds and activates proapoptotic genes such as *reaper*, but is not targeted to cell cycle arrest genes such as *dacapo*. The fact that dmp53 appears to be missing some of the activities attributed to human p53 affords scientists with an opportunity to study p53's apoptotic functions without interference from its cell cycle arrest functions.

Sequencing the *Drosophila* genome has uncovered a second major difference between dmp53 and p53: *Drosophila* lacks a homologue of the MDM2 protein, the master regulator of p53 stability [3]. ARF which negatively controls MDM2 activity is also missing [3]. Accordingly, the N-terminus of dmp53 lacks a MDM2 binding site. Nevertheless, ectopic expression of human MDM2 does lead to apoptosis, but it is not clear what the targets of MDM2 in this context are [52]. How then is dmp53 activated following cellular insults ? Although several antibodies have been raised against dmp53, none of them has been shown to recognise endogenous dmp53 protein (they do recognise overexpressed dmp53), likely indicating a low level of endogenous protein. These findings and the apoptotic effects of overexpressing dmp53 would be consistent with a (MDM2-independent) stabilisation of dmp53 following cellular stress, but this remains to be firmly established. It is also unclear how dmp53 levels would be kept low in the absence of a dmp53 ubiquitin ligase. It should be noted that proteasome-independent p53 degradation pathways do exist: Calpains, a family of ubiquitous cysteine proteases that is conserved between flies and man, degrade p53 and calpain inhibitors can induce apoptosis under certain circumstances [53–55]. In human cells, stabilisation of p53 in response to DNA damage is in part mediated by the kinases Chk1, Chk2, ATM and ATR [42]. These kinases phosphorylate residues within the MDM2 binding region and so weaken

the interaction between p53 and MDM2. As a result, p53 is no longer subjected to polyubiquitination and degradation leading to a transient increase in stability. Interestingly, overexpression of the *Drosophila* Chk2 kinase ascerbates the apoptotic effects of dmp53 overexpression whereas expression of a dominant-negative Chk2 rescues this phenotype [56]. Overexpression of Grapes, the *Drosophila* homologue of Chk1, however, does not influence the dmp53 phenotype. Although Chk2 phosphorylation of dmp53 is not regulating an interaction with a MDM2 protein, it is nevertheless important for dmp53 activation. Coexpression of Chk2 and dmp53 in transient transfection assays potentiates transactivation, but does not lead to a detectable increase of dmp53 levels arguing that phosphorylation by Chk2 does not dramatically increase dmp53 stability. These data suggest that Chk2-mediated phosphorylation of p53 might influence properties such as DNA binding activity or interaction with cofactors. Of the cofactors that target p53 to apoptotic genes only the ASPP proteins seem to have a clear homologue in *Drosophila* (CG18375). Whether or not the *Drosophila* ASPP protein interacts with dmp53 remains to be established.

6. Concluding remarks

The study of tumour suppressor proteins in *Drosophila* offers a number of advantages: First, and most obvious, *Drosophila* is much more amenable to genetic analysis than mammalian model organisms. As a consequence, most of what we have learned to date about *Drosophila* tumour suppressors stems from genetic studies. Second, owing to the lower complexity of the *Drosophila* genome, the protein families are generally smaller than in the human system. The fly has only two pRb-related factors and only a single p53-related protein. This alleviates to some extent the problems of redundancy and aids geneticist and biochemist alike. The study of pRb- and p53-related proteins in the fly has revealed remarkable similarities to the human proteins, but has also uncovered some important differences. The RBF network appears in most respects to be a simplified version of the pRb network with all essential elements in place (Fig. 2). However, it should be noted that some intriguing differences exist. For example, the *Drosophila* cyclin D/CDK4 complex, in contrast to the human cyclin D/CDK4/6 complexes, is not absolutely essential for progression through G1 [23–25]. It has been suggested that rather than acting in a sequential fashion and cyclin E/CDK2 activity being dependent on prior cyclin D/CDK4/6 activation, *Drosophila* cyclin D/CDK4 and cyclin E/CDK2 act as independent RBF kinases [24]. In any case, the overall similarity between the two pathways and the relative simplicity of the RBF network promises to greatly facilitate the molecular analysis of principles underlying pocket protein function.

As in humans, dmp53 helps to protect the organism from the effects of genotoxic stress by stimulating apoptosis and it does so by binding to and activating the transcription of proapoptotic genes. In this case, however, the similarities do not extend much further: dmp53 is missing an important function of its human counterpart, namely the ability to induce cell cycle arrest to allow repair of sustained damage. In addition, important regulators of p53 activity such as MDM2 and ARF are absent, but it is not clear if this is related to the lack of a cell cycle arrest function.

The absence of MDM2 and ARF creates another conceptual problem as these proteins constitute an important link between the pRb and the p53 pathways in human cells: Loss of pRb function can result in p53-dependent (and p53-independent, see below) apoptosis: Inactivation of pRb leads to increased E2F activity and increased transcription of the E2F target gene ARF. Increased ARF levels inhibit the MDM2 ubiquitin ligase leading to stabilisation of p53 and induction of apoptosis (Fig. 2). This connection between the two pathways represents an effective protection of the cell against oncogenes which inactivate pRb function as loss of functional pRb is countered by increased p53 activity and apoptosis. Accordingly, successful DNA tumour viruses have evolved to target both pRb and p53 simultaneously. Due to the absence of MDM2 and ARF in the fly an equivalent link between RBF1/RBF2 and dmp53 is missing. Nevertheless, inactivation of RBF1 or overexpression of dE2F1/dDP results in apoptosis, but we do not know by what mechanism [15,22]. It is conceivable that this apoptosis is dmp53-dependent and that an alternative connection between RBF1 and dmp53 exists. It is also possible that this is a dmp53-independent process and that dE2F directly regulates proapoptotic genes. In human cells, p53-independent apoptosis following pRb inactivation has been attributed to the upregulation of E2F target genes such as the p53 family member p73 and the apoptotic protease-activating factor 1 (APAF1) [57–59]. *Drosophila* has a APAF1-related protein (Dark), but lacks a p73 homologue.

Clearly, research into fly tumour suppressor proteins has so far produced many more questions than answers. It has become clear, however, that *Drosophila* has matured into an important tool for scientists aiming to understand the molecular details of tumour suppressor function.

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