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Purification and Characterization of Retinoblastoma like Factor-containing Protein Complexes from *Drosophila melanogaster*

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. Peter Becker betreut und von Herrn Prof. Dr. Dirk Eick vor der Fakultät für Biologie vertreten.

Ehrenwörtliche Versicherung

Ich versichere, dass ich die vorliegende Arbeit selbständig durchgeführt und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe.

München, am 23. Oktober 2006

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Michael Korenjak

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1 Summary

The Retinoblastoma protein (pRb) was the first tumor suppressor protein to be identified. It is the founding member of the so called pRb or pocket protein family, comprising two additional members (p107 and p130) in mammalian cells, and its best characterized function is the regulation of the E2F family of transcription factors. Today, the pRb-E2F network represents one of the best understood pathways implicated in cell cycle regulation and differentiation.

Pocket proteins negatively regulate the transactivation properties of E2F proteins by two mechanisms: First, binding of pocket proteins to E2F masks the E2F transactivation domain and thereby impairs transcriptional activation. Second, pocket proteins interact with several chromatin modifying and chromatin binding proteins and recruit these proteins to E2F target genes, where they help to establish a repressive chromatin conformation.

In this work, advantage was taken of the relative simplicity of the *Drosophila melanogaster* pRb-E2F network to purify and functionally characterize native pRb repressor complexes.

Two related multisubunit complexes that only differ in their pocket protein subunit (RBF1 or RBF2) have been purified from *Drosophila* embryo nuclear extract. These complexes contain several novel pocket protein-associated polypeptides and localize to transcriptionally silent regions on *Drosophila* polytene chromosomes. Moreover, they specifically associate with deacetylated histone tails, which are a hallmark of transcriptionally silent chromatin. In cycling *Drosophila* S2 cells, the purified complexes redundantly repress the expression of a certain class of E2F target genes implicated in differentiation and development, whereas they do not control the expression of cell cycle-regulated E2F targets. Interestingly, the isolated complexes seem to be highly conserved between different organisms. Genes encoding the *Caenorhabditis elegans* homologs of the complex subunits act within the same genetic pathway involved in vulval cell fate determination and they functionally cooperate in different developmental processes. Furthermore, a complex with striking homology to the *Drosophila* complexes also exists in human cells.

In the light of the specific repression of developmentally regulated E2F target genes in cycling *Drosophila* cells, it is conceivable that the complexes prevent the uncontrolled expression of genes important during differentiation. Since the *C. elegans* homologs of the complex subunits are also involved in cell fate determination, this might be a highly conserved feature of the isolated complexes.

2 Introduction

2.1 The pRb-E2F network in mammals

Today, the pRb-E2F pathway represents one of the best understood transcriptional networks in eukaryotes. Its detailed characterization began 20 years ago with the identification of a gene, both copies of which are mutated or deleted in Retinoblastoma, a rare childhood tumor of the eye (Friend et al. 1986). The protein product of this gene, the Retinoblastoma protein (pRb) was the first tumor suppressor protein to be identified. At the same time, a cellular activity associating with and regulating the Adenovirus E2 promoter was identified and named E2 promoter binding factor (E2F) (Kovesdi et al. 1986; Kovesdi et al. 1987). Induction of the E2 promoter is mediated by the Ad-E1A protein, which is a potent oncoprotein, involved in cellular transformation. In untransformed cell lines, E2F can form complexes with cellular proteins, which are disrupted upon addition of the Ad-E1A protein, and this results in the stimulation of E2F-dependent transcription from the E2 promoter (Bagchi et al. 1990). Interestingly, the Ad-E1A protein, and the oncoproteins of other DNA tumor viruses can also interact with pRb (DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989), providing a link between the cellular E2F and pRb proteins. This connection was confirmed by the finding of a physical association between pRb and E2F. Moreover, this interaction is disrupted by viral oncoproteins (Bagchi et al. 1991; Bandara and La Thangue 1991; Chellappan et al. 1991; Chittenden et al. 1991).

These discoveries resulted in the model that pRb negatively regulates the E2F transcription factor. Upon viral transformation, this repression can be overcome by the binding of viral oncoproteins to pRb, which leads to its dissociation from E2F. Subsequently, E2F-dependent transcription promotes cell proliferation due to the activation of E2F target genes involved in cell cycle progression (Dyson 1998). This mechanism represents a crucial step for cellular transformation by oncoviruses. Under non-transforming, physiological conditions, association between E2F and pRb depends on the phosphorylation status of pRb, which is regulated by cyclin/cyclin dependent kinase (cdk) complexes in a cell cycle specific manner (Dyson 1998).

These pioneering studies have established E2F and pRb proteins as master regulators of cell cycle progression.

2.1.1 The E2F transcription factor family

E2F transcription factors act as heterodimeric proteins, consisting of an E2F and a DP (differentiation regulated transcription factor, **D**RTF **P**rotein) subunit (Girling et al. 1993; Helin et al. 1993b; Krek et al. 1993; Bandara et al. 1994). Moreover, the structural features of E2F-DP heterodimers bound to DNA support the preferential heterodimer formation (Zheng et al. 1999), even though homodimers have also been described (Huber et al. 1993). The E2F transcription factor family comprises eight E2F (E2F1-8) and two DP proteins (DP1+2).

E2F1-6 can associate with each of the two DP proteins, resulting in a highly complex network of transcription factors (Trimarchi and Lees 2002). In contrast, two recently identified, atypical E2F proteins (E2F7 and E2F8) do not heterodimerize with DP (Table 2.I). These factors possess a tandem pair of DNA binding domains, which interact with E2F binding sites on DNA (de Bruin et al. 2003; Di Stefano et al. 2003; Logan et al. 2004; Christensen et al. 2005; Logan et al. 2005; Maiti et al. 2005).

E2F1-5 can transactivate reporter genes in transient transfection assays, but they differ in their occupancy of target genes during the cell cycle. E2F1-3 are associated with their target promoters in late G1 and S phase, when the genes are actively transcribed (Takahashi et al. 2000; Wells et al. 2000), and are referred to as "activator E2Fs". In contrast, E2F4 and E2F5 occupy their target genes in quiescent cells and in early G1 when the genes are not expressed, and have been classified as "repressor E2Fs". The transactivation properties of E2F1-5 are repressed by their association with members of the pRb protein family (Dyson 1998).

Unlike the other E2F factors, E2F6-8 do not activate reporter genes in transient transfection experiments. Rather, E2F6-8 repress transcription in a pocket protein-independent manner (Table 2.I) (Morkel et al. 1997; Cartwright et al. 1998; Trimarchi et al. 1998; de Bruin et al. 2003; Di Stefano et al. 2003; Logan et al. 2004; Christensen et al. 2005; Logan et al. 2005; Maiti et al. 2005).

Table 2.I: Features of mammalian E2F proteins

	TAD	DBD*	DP dimerization	pRb family interaction
E2F1	+	1	+	E2F1/pRb
E2F2	+	1	+	E2F2/pRb
E2F3	+	1	+	E2F3/pRb
E2F4	+	1	+	E2F4/pRb, E2F4/p107, E2F4/p130
E2F5	+	1	+	E2F5/p130
E2F6	-	1	+	-
E2F7	-	2	-	-
E2F8	-	2	-	-

TAD, transactivation domain; DBD, DNA binding domain.

* Number of DNA binding domains within the E2F protein.

The exact mechanism of how E2F proteins activate transcription is not fully resolved, but several modes of action can be hypothesized: (1) E2F1 interacts with the TBP and TFIIH subunits of the general transcription machinery and loss of this interaction results in a decrease of E2F-dependent transcription (Hagemeier et al. 1993; Emili and Ingles 1995; Pearson and Greenblatt 1997; Vandel and Kouzarides 1999). (2) E2Fs are known to associate with proteins that have histone acetyltransferase (HAT) activity. Furthermore, these HATs cooperate with E2F in transcriptional activation and acetylate histones on E2F target promoters in an E2F dependent manner (Trouche and Kouzarides 1996; Lang et al. 2001; Taubert et al. 2004). Moreover, acetylation of E2F itself by the same enzymes increases its DNA binding affinity and activation properties (Martinez-Balbas et al. 2000; Marzio et al. 2000). (3) Another possible mechanism of transcriptional activation by E2F factors might involve their ability to bend DNA, thereby creating a more favorable conformation for the interaction of transcription factors with the basal transcription machinery (Cress and Nevins 1996). These putative mechanisms are not mutually exclusive and might therefore also cooperate during E2F transactivation.

2.1.2 E2F target genes

The characterization of the E2F binding site in the Adenovirus E2 promoter subsequently led to the identification of similar sequences in the promoters of cellular genes and revealed their regulation by E2F (Blake and Azizkhan 1989; Thalmeier et al. 1989). Since E2F has been shown to promote S phase entry of quiescent cells (Johnson et al. 1993), the search for novel E2F target genes was originally focused on well known regulators of G1/S phase progression. Therefore, many of the E2F target genes identified to date are important for regulated entry and progression through the S phase of the cell cycle.

Within the last couple of years, however, several labs have used microarray based techniques to identify novel genes regulated by members of the E2F and pRb protein families (Ishida et al. 2001; Muller et al. 2001; Ren et al. 2002; Weinmann et al. 2002). As expected, a large number of E2F target genes identified in these studies are important for cell cycle progression. In addition, many novel E2F-regulated genes are implicated in DNA repair, apoptosis, differentiation and development, suggesting that E2F and pRb protein function is not restricted to cell cycle control (Stevaux and Dyson 2002).

2.1.3 The pocket protein family

In mammalian cells, pRb and the highly related proteins p107 and p130 comprise the so called pocket protein family. This family is characterized by a conserved region, composed of two conserved domains (A and B) separated by a spacer. These motifs give rise to a pocket like structure, called the pocket domain. The pocket domain appears to be the main interaction module of these proteins and is highly conserved between all family members. Within this domain, the LxCxE binding cleft represents the critical interaction module for proteins containing the so called LxCxE (Leu-x-Cys-x-Glu) motif, including viral oncoproteins and cellular transcription factors (Kim and Cho 1997; Lee et al. 1998). However, the pocket region also mediates the interaction with proteins that lack an LxCxE motif, including the E2F family of transcription factors. In addition to the pocket domain, the pRb C-terminus appears to be involved in stabilizing the association with E2Fs (Hiebert et al. 1992; Qian et al. 1992; Qin et al. 1992).

Several lines of evidence suggest that p107 and p130 are more closely related to each other than to pRb. First, p107 and p130 contain an extended spacer region in the pocket domain, which is involved in the binding of cyclin E/cdk2 and cyclin A/cdk2 (Devoto et al. 1992; Lees et al. 1992; Li et al. 1993). The precise function of the stable association between these proteins is still not fully resolved, but it has been shown that when p107 is bound, it inhibits

the target phosphorylation by cyclin E/cdk2 and cyclin A/cdk2 and is involved in growth suppression (Zhu et al. 1995a; Zhu et al. 1995b). Second, p107 and p130 interact exclusively with “repressor E2Fs”, whereas pRb can associate with both “activator” and “repressor E2Fs” (see Table 2.I).

As a consequence, p107 and p130 repress E2F-dependent transcription together with E2F4 and E2F5 during the G0 and early G1 phases of the cell cycle. pRb, however, also represses transactivation by E2F1-3 in late G1, just before these proteins are activated (see 2.1.1 and Fig. 2.1). During the G1 phase, mitogenic signals result in the sequential activation of cyclin D/cdk4 or 6 and cyclin E/cdk2 complexes. Phosphorylation of pRb by these complexes in late G1 reduces its affinity for E2F, which results in pRb dissociation. Uncomplexed E2F1-3 can subsequently activate target genes that are important for the G1/S transition and cell cycle progression (Fig. 2.1).

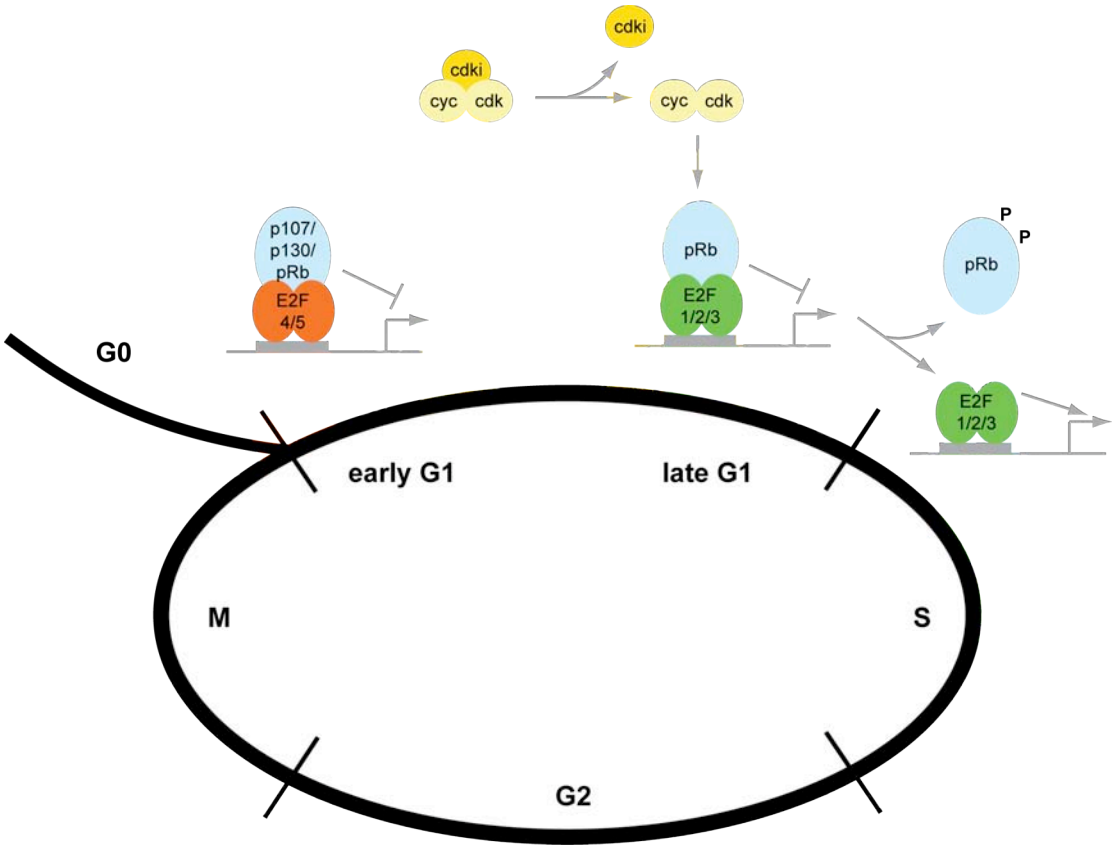


Figure 2.1: Cell cycle regulation by pocket proteins

Cyclin/cdk complexes can phosphorylate pocket proteins, leading to their dissociation from E2F factors. cyc, cyclin; cdk, cyclin dependent kinase; cdki, cdk-inhibitor; P, Phosphate group.

Abbreviations for cell cycle phases: G0, quiescence phase; G1/G2, gap phases; S, DNA synthesis phase; M, mitosis. See text for details.

2.1.4 Mechanisms of pocket protein mediated transcriptional repression

Pocket proteins repress transcription by two different mechanisms: First, binding of pocket proteins to E2F masks the E2F transactivation domain and thereby impairs transcriptional activation (Flemington et al. 1993; Helin et al. 1993a). Second, pocket proteins interact with several chromatin modifying and chromatin binding proteins and recruit these proteins to E2F target genes, where they help to establish a repressive chromatin conformation. This mechanism is also referred to as "active repression".

The degree of chromatin condensation, especially in the promoter region of a gene, is a critical determinant for the activity status of the gene. Depending on the chromatin conformation, important regulatory sequences can be accessible for or excluded from transcription factor binding. Chromatin modifications play an essential role in the establishment of both, active and repressive chromatin conformations, either by creating a binding platform for other proteins or by directly changing chromatin structure.

Enzymes capable of changing chromatin structure can be classified into two groups: Covalent histone modifiers and ATP-dependent chromatin remodelers. The most common covalent histone modifications comprise acetylation, methylation, phosphorylation and ubiquitylation. Acetylation and methylation of histone tails are well characterized with regard to their effect on the transcription of genes. Whereas hyperacetylated histones are a hallmark of actively transcribed genes, hypoacetylation of histones is associated with transcriptional repression. Histone methylation, however, can be a mark for either active or inactive chromatin, depending on the amino acid that is modified. For example, methylation of lysine (K) residues 9 and 27 on histone H3 and K20 on H4 are hallmarks of transcriptionally silent chromatin, whereas H3K4-methylation is associated with active transcription. Besides, several additional lysine (K) and arginine (R) residues within histones are methylated and the impact of these modifications on transcription is currently subject to intensive studies.

The second class of chromatin modifying enzymes, ATP-dependent chromatin remodelers, utilize the energy of ATP hydrolysis to directly move histone octamers relative to the DNA, which is wrapped around them. ATP-dependent chromatin remodeling can be involved in transcriptional repression as well as activation (Saha et al. 2006).

Physical and functional Interactions between pocket proteins and several chromatin modifying enzymes have been proven. Best characterized is the interaction of pocket proteins with histone deacetylases (HDACs). Pocket proteins and HDACs physically interact and cooperatively repress the expression of cell cycle-regulated E2F target genes in

cotransfection experiments. Furthermore, HDAC activity is required for this repression (Brehm et al. 1998; Ferreira et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998).

Moreover, pRb interacts with the SUV39H1 histone methyltransferase (HMT), which methylates K9 on histone H3, and with Heterochromatin Protein 1 (HP1). Interestingly, HP1 specifically recognizes and binds the methylation mark set by SUV39H1 and appears to be a key player in the establishment of transcriptionally silent chromatin (Bannister et al. 2001; Lachner et al. 2001). pRb and SUV39H1 cooperate in the repression of E2F target genes (Nielsen et al. 2001; Vandel et al. 2001) and knockout of the HMT results in the upregulation of endogenous E2F/pocket protein targets (Nielsen et al. 2001). H3K9-methylation at the cyclin E promoter is strongly reduced in $pRb^{-/-}$ cells, further supporting the functional interplay between pRb and SUV39H1. Strikingly, also the association of HP1 with the cyclin E promoter disappears upon loss of pRb (Nielsen et al. 2001).

The assembly of the methyltransferase PRMT5 into an E2F4 repressor complex, which is bound to the cyclin E promoter, shows that also HMTs modifying arginine residues might be involved in the repression of E2F target genes (Fabbrizio et al. 2002). Recently, also PRMT2 has been shown to interact with pRb and repress E2F-regulated reporter genes in a pRb-dependent manner (Yoshimoto et al. 2006).

In overexpression experiments, pRb interacts with the polycomb group (PcG) protein HPC2 (Dahiya et al. 2001). PcG proteins are negative regulators of transcription, important for the maintenance of homeotic (Hox) gene expression patterns. To do so, they act within multisubunit protein complexes with chromatin modifying and binding properties (Bantignies and Cavalli 2006). Like pRb, HPC2 is a negative regulator of cell proliferation (Satijn et al. 1997). pRb and HPC2 cooperate to mediate growth arrest, probably due to the cooperative repression of certain cell cycle-regulated E2F target genes (Dahiya et al. 2001).

Furthermore, pocket proteins interact with BRG1 and hBRM, two members of the SWI2/SNF2 family of ATP-dependent chromatin remodeling enzymes (Dunaief et al. 1994; Strober et al. 1996). Like pRb, expression of BRG1 can induce a so called “flat cell” phenotype in certain cell lines (Dunaief et al. 1994). Flat cells share some of the hallmarks of senescent and differentiated cells. Cellular differentiation and senescence depend on permanent cell cycle withdrawal, which includes the stable repression of E2F-regulated cell cycle genes. pRb is thought to be critical for this repression. Accordingly, the pRb-BRG1 interaction appears to be involved in the repression of cell cycle regulated E2F target genes (Zhang et al. 2000). However, a recent study has shown that BRG1 might cooperate with pRb indirectly through upregulation of the cdk inhibitor p21 (Kang et al. 2004). Consequently, the inactivation of

cyclin/cdk complexes results in the accumulation of hypophosphorylated (active) pRb and cell growth arrest.

RbAp46 and RbAp48, two highly homologous human proteins, provide additional support for pRb-mediated transcriptional repression by recruitment of chromatin modifying and binding proteins. RbAp46 and RbAp48 have originally been identified as p**Rb**-**A**ssociated **p**roteins (Qian et al. 1993; Qian and Lee 1995). Moreover, they are histone-binding proteins (Verreault et al. 1998) and components of several chromatin modifying and binding complexes (Henikoff 2003), suggesting that they might represent a bridging factor between pRb and chromatin modifying/binding complexes.

2.1.5 The role of pocket proteins and E2F proteins in development

The recent use of ChIP-on-chip approaches for the identification of novel E2F target genes has revealed that E2F and pocket proteins are also bound to the promoter regions of genes implicated in processes such as differentiation and development (see 2.1.2). These findings are in agreement with developmental defects observed in mice deficient for different E2F or pocket proteins, which seem not to be attributable to defects in cell proliferation.

Mice deficient for pRb die early during embryogenesis with tissue-specific developmental defects. Even though initiation of differentiation occurs, end-stage differentiation of erythrocytes is strongly impaired (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). Moreover, expression of certain late neuronal differentiation markers is decreased in $pRb^{-/-}$ mice, suggesting that proper neuronal differentiation is also affected (Lee et al. 1994).

Knockout of p107 gives rise to animals without obvious abnormalities. However, the parallel inactivation of pRb results in the same developmental deficiencies as observed for $pRb^{-/-}$ embryos, but the double-knockout animals show these defects earlier and, thus, also die earlier (Lee et al. 1996). Therefore, p107 can, to a limited extent, substitute for loss of pRb, suggesting that it can function in the same developmental processes.

Mice deficient for E2F1 suffer from testicular atrophy and this phenotype is enhanced in $E2F1^{-/-}$ $E2F3^{+/-}$ animals, suggesting that, as seen for p107 and pRb, E2F3 can partially substitute for loss of E2F1 (Yamasaki et al. 1996; Cloud et al. 2002). Furthermore, knockout of E2F3 results in cardiac dysfunction, which is a defect specifically observed upon E2F3 loss (Cloud et al. 2002).

Inactivation of E2F4 results in defects in late stages of erythrocyte differentiation and craniofacial defects (Humbert et al. 2000; Rempel et al. 2000). Strikingly, the erythrocyte differentiation phenotype is very similar to the defects observed upon loss of pRb, suggesting

a tight cooperation of these proteins. Deficiency for E2F5 results in hydrocephalus formation due to a dysfunction of the choroid plexus epithelium in the secretion of cerebrospinal fluid (Lindeman et al. 1998). A mouse knockout model for E2F6 revealed defective spermatogenesis and homeotic transformations of the axial skeleton (Storre et al. 2002). A similar skeletal transformation phenotype is known from polycomb mutant mice. Moreover, E2F6 has been shown to be assembled into complexes containing PcG proteins, supporting the idea that the similar phenotypes observed upon knockout of the genes might be attributable to the loss of function of the protein complexes (Trimarchi et al. 2001; Ogawa et al. 2002; Attwooll et al. 2005).

Interestingly, loss of the “repressor E2F” family members (E2F4-6) does not affect cell cycle kinetics of embryonic fibroblasts, but only developmental defects in the knockout animals are observed. Recent work, however, has shown that roles of the proteins in cell cycle regulation might have been missed in the single-knockout cell lines due to functional redundancy of E2F family members (Gaubatz et al. 2000; Giangrande et al. 2004). Nevertheless, the studies of knockout animals show that the developmental role of repressive E2F proteins is more critical than has been suggested from overexpression studies in cell lines, which mainly implicated them in proliferation control.

2.2 The pRb-E2F network in flies and worms

To a large extent, the pRb-E2F network is conserved between mammals and simpler model organisms like flies and worms (Table 2.II). *Drosophila melanogaster* and *C. elegans* have contributed greatly to our understanding of fundamental developmental processes and are, therefore, ideally suited to study pRb-E2F function in a developmental context. In addition, the lower level of redundancy within the pocket protein and E2F families facilitates analysis.

Table 2.II: pRb-E2F network components in humans, flies and worms

	Human	*Drosophila*	*C. elegans*
Pocket proteins	pRb, p107, p130	RBF1, RBF2	LIN-35
E2F proteins	E2F1, E2F2, E2F3a, E2F3b, E2F4, E2F5, E2F6, E2F7, E2F8	dE2F1, dE2F2	EFL-1, EFL-2
DP proteins	DP1, DP2	dDP	DPL-1

2.2.1 The *Drosophila melanogaster* pRb-E2F network

In the fruitfly *Drosophila melanogaster*, the pRb-E2F network is well characterized and the functional hierarchy is well conserved compared to the mammalian system. The fly possesses two Retinoblastoma like factors (RBF1 and RBF2), two E2F proteins (dE2F1 and dE2F2) and one DP protein (dDP), in addition to G1 cyclins, cdk´s and cdk inhibitors (Sutcliffe et al. 2003).

2.2.1.1 Transcriptional regulation by dE2F and RBF proteins

The domain structure of dE2F proteins is highly conserved, including DNA-binding, DP-dimerization and pocket protein-binding domains. Within these regions the homology to their mammalian counterparts is most pronounced (Dynlacht et al. 1994; Ohtani and Nevins 1994; Sawado et al. 1998). Moreover, association with E2F binding sites requires the cooperation of dE2F and dDP proteins (Dynlacht et al. 1994; Sawado et al. 1998). Reporter gene assays in *Drosophila* cell lines show that dE2F1 acts as a potent activator of transcription (Dynlacht et al. 1994; Ohtani and Nevins 1994) and is negatively regulated by RBF1 (Du et al. 1996a). In the same type of assay, dE2F2 mediates transcriptional repression (Sawado et al. 1998; Stevaux et al. 2002). Accordingly, dE2F1 is referred to as the "activator E2F" and dE2F2 as the "repressor E2F".

The *Drosophila* pocket proteins RBF1 and RBF2 share higher sequence similarity with p107 and p130 than with pRb. The organization of the pocket domain and especially of the spacer region, however, argues for RBF1 being more closely related to pRb, and RBF2 to p107 and p130 (Du et al. 1996a; Stevaux et al. 2002). This is supported by the analysis of interactions between RBF and dE2F proteins (Fig. 2.2): RBF1 can associate with both, dE2F1 and dE2F2,

(Stevaux et al. 2002) and, therefore, resembles mammalian pRb, which also binds to both, activating and repressing E2Fs. In contrast, RBF2 interacts exclusively with the repressive dE2F2, a property that it shares with p107 and p130. Accordingly, RBF2 cooperates with dE2F2 in transcriptional repression but fails to block activation by dE2F1 (Stevaux et al. 2002).

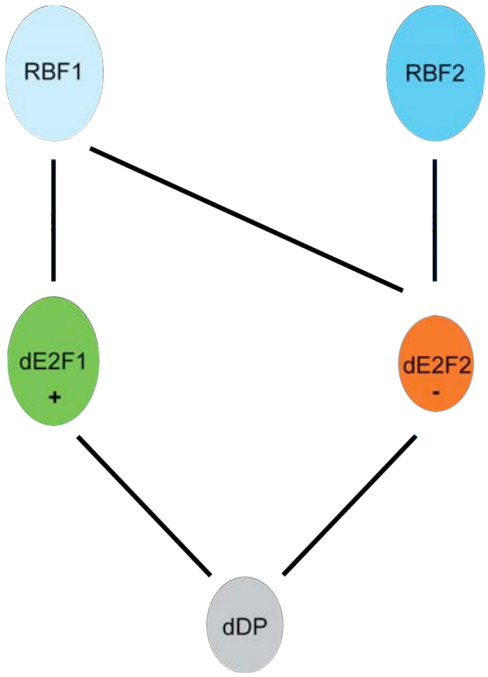


Figure 2.2: The *Drosophila* pRb-E2F network
Interactions between proteins are indicated by black lines. +, "activator E2F"; -, "repressor E2F". See text for details.

2.2.1.2 RBF and chromatin regulation

Like in mammalian cells, repression of dE2F-dependent transcription by RBF proteins might involve chromatin modifying and binding proteins. *de2f1* and genes encoding subunits of the Brahma (Brm) chromatin remodeling complex interact genetically during eye development and have opposing functions (Staehling-Hampton et al. 1999). Moreover, in the fly Brm complex genes act as negative regulators of S phase onset, and RBF1 and Brm interact physically in embryos (Brumby et al. 2002). This is especially interesting in the light of the pRb-hBRM interaction in mammalian cells (see 2.1.4). However, a genetic interaction between *rbf1* and Brm complex genes could not be shown (Brumby et al. 2002).

RBF1 has also been shown to associate with the histone deacetylase dRPD3 as well as with CAF1p55, the *Drosophila* homolog of mammalian RbAp46 and RbAp48 (Taylor-Harding et al. 2004).

However, a cooperation of RBF proteins and chromatin modifying and binding proteins in the regulation of dE2F target gene expression still remains to be established.

2.2.1.3 Cell cycle control by dE2F and RBF proteins

dE2F1 is essential during early *Drosophila* development and *de2f1* knockout flies die with defects in DNA synthesis and cell proliferation (Duronio et al. 1995). In contrast, *de2f2* knockout flies survive to adulthood, although with a lower frequency than wild type flies (Frolov et al. 2001). Interestingly, simultaneous inactivation of *de2f1* and *de2f2* partially rescues the *de2f1* mutant phenotype (Frolov et al. 2001). This suggests that dE2F1 and dE2F2 have antagonistic functions *in vivo* and that the severe phenotype in *de2f1* mutant animals is brought about by the unchecked activity of dE2F2. The fact that flies lacking both dE2F factors still make it through a large part of development demonstrates that dE2F activity is not absolutely essential for correct DNA synthesis and proliferation.

Directed overexpression of dE2F1/dDP in the *Drosophila* eye allows differentiated cells to re-enter the cell cycle (Du et al. 1996b). Conversely, the simultaneous overexpression of RBF1 can suppress this phenotype (Du et al. 1996b). This demonstrates that, analogous to the situation in mammals, RBF1 is a negative regulator of dE2F1 and cell cycle progression *in vivo*.

2.2.1.4 dE2F target genes

Based on the knowledge obtained from the study of mammalian cells and the cell cycle phenotype observed in *de2f1* knockout flies, the first dE2F target genes that have been identified were genes implicated in DNA synthesis. dE2F factors regulate their expression in reporter gene assays (Ohtani and Nevins 1994; Sawado et al. 1998) and deletion of *de2f1* results in the cessation of expression of these genes in embryos (Duronio et al. 1995).

Using a combination of RNA interference and microarray techniques a plethora of novel dE2F target genes has recently been identified (Dimova et al. 2003). As expected, many of the target genes are involved in S-phase entry, DNA replication, mitosis, cell cycle checkpoint control and DNA repair. However, a second group lacks genes implicated in S-phase entry and DNA replication but contains genes encoding for differentiation factors that are expressed in developmentally regulated and sex-specific patterns (Dimova et al. 2003).

Interestingly, these dE2F targets can be classified into five groups (A – E) according to their regulation by distinct RBF and dE2F proteins. These groups of dE2F target genes range from well characterized S phase-specific genes that are activated by dE2F1 and repressed by

RBF1 (A group genes) through genes that are permanently repressed by dE2F2 and the redundant action of RBF1 and RBF2 (E group genes) (Fig. 2.3). These E group genes encompass dE2F targets that are repressed in a cell cycle independent manner. Interestingly, many E group genes are specifically expressed in the germ line and function during gametogenesis (Dimova et al. 2003).

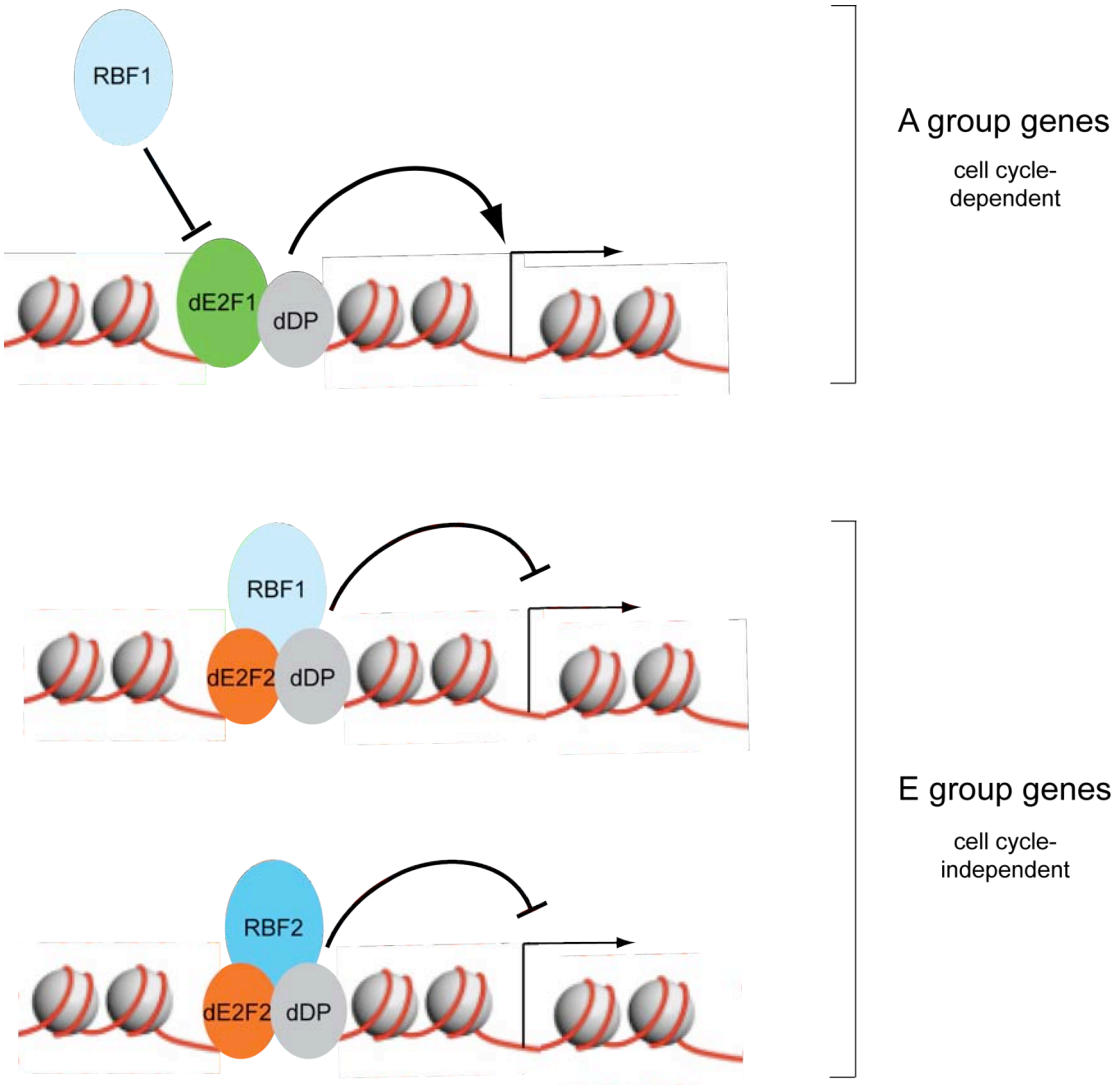


Figure 2.3: Regulation of A and E group genes

A and E group genes are dE2F target genes, the expression of which depends upon distinct dE2F and RBF proteins. See text for details.

2.2.2 The *Caenorhabditis elegans* pRb-E2F network

Growing evidence suggests that the pRb-E2F pathway is also conserved in the nematode worm *C. elegans*. Homologs of the main players of the pathway exist in the worm, including one pocket protein (LIN-35), two E2F factors (EFL-1 and EFL-2) and one DP protein (DPL-1) (Table 2.II). Moreover, genetic studies suggest a regulation of these factors analogous to the mammalian and *Drosophila* systems.

2.2.2.1 EFL-1, DPL-1, LIN-35 and cell cycle regulation

The *C. elegans* homologs of pRb and E2F family proteins show the same domain structure as their mammalian counterparts and also the overall amino acid sequence homology clearly identifies them as the worm homologs of these proteins (Lu and Horvitz 1998; Ceol and Horvitz 2001). EFL-1/E2F displays higher sequence similarity to mammalian repressive than to activating E2Fs and might, therefore, represent the "repressor E2F" in *C. elegans*. In contrast, EFL-2/E2F cannot be classified as “activator” or “repressor E2F” due to its amino acid sequence, despite a clear homology with E2F factors in general. LIN-35/Rb, DPL-1/DP and EFL-1/E2F proteins interact and can form trimeric complexes *in vitro* (Ceol and Horvitz 2001).

In contrast to their *Drosophila* counterparts, animals lacking *lin-35*/Rb, *efl-1*/E2F or *efl-2*/E2F function do not display any obvious cell cycle defects. Only the inactivation of *dpl-1*/DP results in the downregulation of a transgene, which is under the control of the S phase-specific ribonucleotide reductase (rnr) promoter (Boxem and van den Heuvel 2002). Furthermore, *dpl-1*/DP mutation negatively affects the division of specific cell types in the worm, but no general block of cell division or S phase progression occurs (Ceol and Horvitz 2001; Boxem and van den Heuvel 2002). These data indicate that in *C. elegans* DPL-1/DP can act as a positive regulator of transcription and cell cycle progression.

G1/S phase progression during postembryonic cell divisions in *C. elegans* is strictly dependent on CYD-1 and CDK-4, the homologs of mammalian cyclin D and cyclin dependent kinases (cdk) 4/6, respectively. Animals mutated for *cyd-1* or *cdk-4* loose expression of the rnr-reporter gene and intestinal cells which normally undergo endoreplication, arrest with a DNA content of 2n instead of 32n (Boxem and van den Heuvel 2001). The simultaneous inactivation of *lin-35*/Rb restores the reporter gene expression and DNA endoreplication (Boxem and van den Heuvel 2001), suggesting that LIN-35/Rb is a negative regulator of cell cycle progression that acts downstream of CYD-1 and CDK-4. Moreover, RNAi-mediated

knockdown of EFL-1/E2F in a *cyd-1* background can also suppress the cell cycle phenotype, whereas RNAi of EFL-2/E2F does not (Boxem and van den Heuvel 2002).

2.2.2.2 EFL-1, DPL-1, LIN-35 and development

Studies of animals lacking *efl-1*/E2F, *dpl-1*/DP and *lin-35*/Rb have revealed that they act in the same genetic pathway to determine cell fate during vulval differentiation (Lu and Horvitz 1998; Ceol and Horvitz 2001).

During the past two decades, development of the *C. elegans* vulva has emerged as a genetically accessible model system to study the regulatory networks directing cell fate determination (Ferguson and Horvitz 1985; Sternberg and Horvitz 1991; Fay and Han 2000; Wang and Sternberg 2001; Sundaram 2004). Vulval development has been studied in detail and is relatively well understood: Six vulval precursor cells (VPCs) have the intrinsic potential to give rise to the worm´s “egg-laying” organ. Wildtype animals have one single vulva, which develops from three VPCs, whereas the remaining VPCs give rise to the hypodermal syncytium.

The current view is that the decision which VPCs will form the vulva arises from the relative distance of VPCs from a so called anchor cell. The anchor cell transmits a signal (LIN-3/EGF) to the VPCs to start the vulval differentiation program. The three VPCs in closest proximity to the anchor cell receive the strongest signal and can override an antagonizing pathway in the VPCs that blocks differentiation, whereas this threshold level is not overcome in the more distal cells (Fig. 2.4 A).

Investigation of *C. elegans* mutants lacking a vulva altogether (the *vulvaless* phenotype) or sprouting additional vulvae (the *multivulva* phenotype) has led to the identification of numerous genes which cooperate to bring about a precisely orchestrated series of cell division and differentiation events. Several mutant alleles causing a *vulvaless* phenotype were found to encode components of a Ras/MAP kinase signaling cascade. *Multivulva* phenotypes are the result of a combination of at least two mutant alleles, which belong to different classes of so called synthetic multivulva (synMuv) genes. To date three synMuv classes – A, B and C – encompassing more than 30 genes have been identified (Fay and Han 2000; Thomas et al. 2003; Ceol and Horvitz 2004; Ceol et al. 2006). *C. elegans efl-1*/E2F, *dpl-1*/DP and *lin-35*/Rb belong to the class B synMuv genes. According to the prevailing model, synMuv genes appear to counteract Ras/MAP kinase signaling (Figure 2.4 A). Failure to do so results in an abnormally high number of precursor cells adopting a vulval fate and consequently in animals with more than one vulva.

Two recent reports, however, question this idea of an antagonizing pathway in the VPCs. They rather suggest that the synMuv pathway acts in the surrounding hypodermal tissue by inhibiting the expression of LIN-3/EGF (Fig. 2.4 B) (Myers and Greenwald 2005; Cui et al. 2006). According to this model, inactivation of synMuv genes in the hypodermis results in the deregulation of LIN-3/EGF expression, which adds to the LIN-3/EGF signal from the anchor cell, and additional VPCs can adopt a vulval fate.

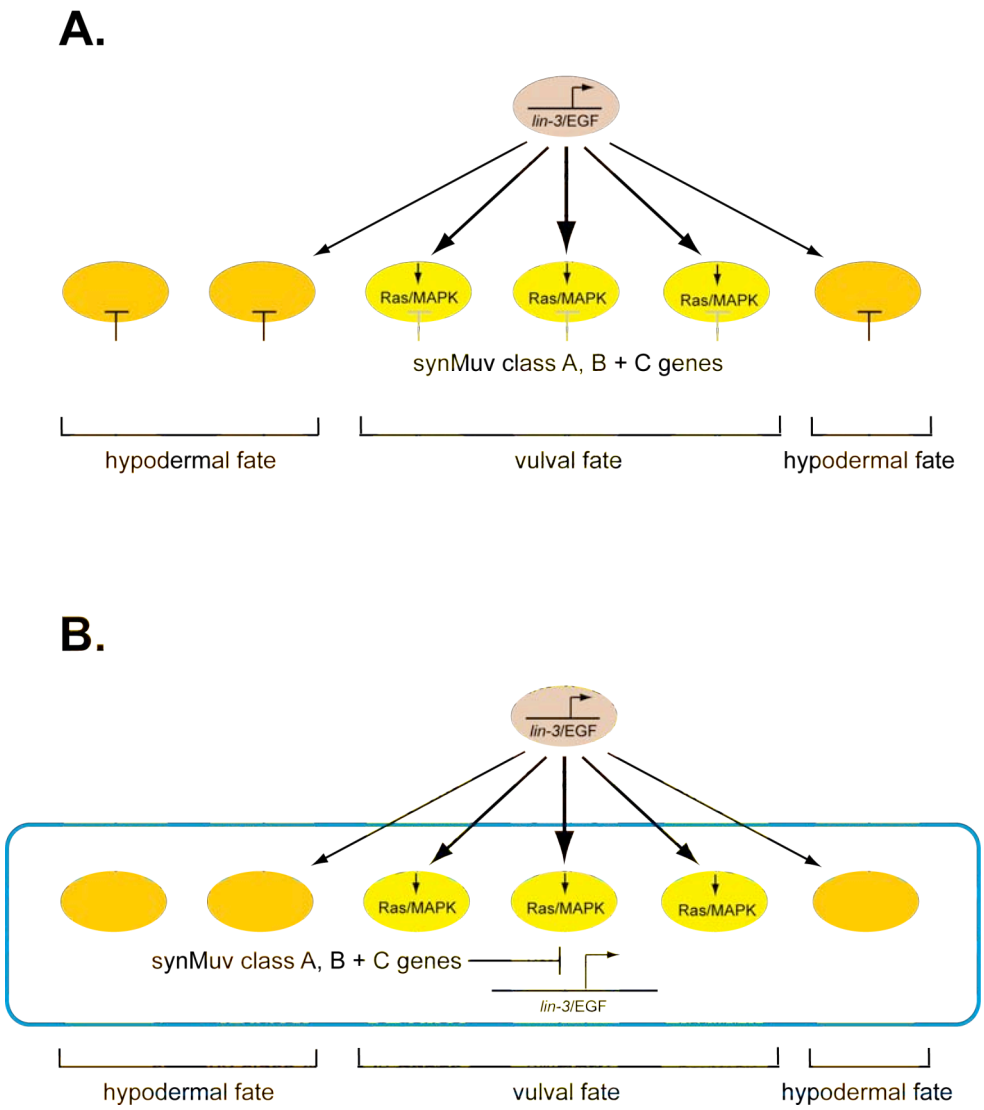


Figure 2.4: Models for vulval development in *C. elegans*

(**A**) Vulval development is initiated through LIN-3/EGF signaling from the anchor cell to the vulval precursor cells, which results in the activation of a Ras/MAP kinase signaling pathway. This pathway is antagonized by the action of class A, B and C synMuv genes. See text for details. Brown ellipse, anchor cell; yellow + orange ellipses, vulval precursor cells; Ras/MAPK, Ras/MAP kinase signaling pathway.

(**B**) Vulval development is initiated through LIN-3/EGF signaling from the anchor cell to the vulval precursor cells, which results in the activation of a Ras/MAP kinase signaling pathway. SynMuv genes repress the expression of LIN-3/EGF in the surrounding hypodermal syncytium. See text for details. Brown ellipse, anchor cell; yellow + orange ellipses, vulval precursor cells; blue rectangle; hypodermal syncytium; Ras/MAPK, Ras/MAP kinase signaling pathway.

In addition to its role during vulva development, LIN-35/Rb has recently been shown to function also in pharynx and larval development (Fay et al. 2003; Cui et al. 2004; Fay et al. 2004). The mechanism underlying LIN-35/Rb function during pharynx development is still unclear. Interestingly, also other class B synMuv genes (such as *efl-1*/E2F*)* but no class A synMuv genes are important for pharynx development (Fay et al. 2004). This suggests that *lin-35*/Rb cooperates within different genetic networks in different developmental contexts.

2.2.2.3 LIN-35 and chromatin regulation

The mechanism of transcriptional repression by LIN-35/Rb has not been studied as extensively as in the mammalian system. Nevertheless, several lines of evidence argue that the basic mechanisms are conserved in *C. elegans*. First, the high degree of conservation of the domain structure of LIN-35/Rb, EFL-1/E2F and EFL-2/E2F is a strong hint that masking of the transactivation domain of the transcription factors is also utilized in the worm. Second, biochemical and genetic studies show that LIN-35/Rb interacts with chromatin modifying and binding proteins: LIN-35/Rb physically interacts with the histone deacetylase HDA-1 and the histone binding protein LIN-53, the *C. elegans* homolog of mammalian RbAp46 and RbAp48 and *Drosophila* CAF1p55, *in vitro* (Lu and Horvitz 1998).

Moreover, *lin-35*/Rb genetically interacts with numerous chromatin modifying and binding proteins in different developmental processes. *lin-35*/Rb acts in the same genetic pathway (synMuv pathway) than genes encoding subunits of the nucleosome remodeling and deacetylase (NuRD) complex, including the enzymatically active subunits CHD-3/Mi-2, CHD-4/Mi-2 and HDA-1/HDAC (Solari and Ahringer 2000; von Zelewsky et al. 2000). Furthermore, many of the proteins encoded by genes from the synMuv pathway have been shown to associate with each other in different organisms, suggesting that they might also form complexes in *C. elegans*.

The gene encoding the *C. elegans* homolog of the histone binding protein HP1, which in mammalian cells has been shown to be implicated in transcriptional repression by pRb (Nielsen et al. 2001), also acts in vulval development and genetically interacts with different class A and B synMuv genes (Couteau et al. 2002). Moreover*, lin-35*/Rb and *hpl-2*/HP1 cooperate during larval development in the worm (Couteau et al. 2002).

Furthermore, *lin-35*/Rb functionally cooperates with genes encoding subunits of the SWI/SNF chromatin remodeling complex (Cui et al. 2004).

These studies strongly suggest that “active repression” by pocket proteins might also occur in the worm, and that it might be mediated by the same enzymes than in mammals and *Drosophila*.

2.3 Objectives

2.3.1 Pocket protein containing complexes in mammals

The major part of research regarding pocket protein function has been performed in mammalian model systems, including cell lines and knockout animals. The findings from these studies have, undoubtedly, greatly contributed to our understanding of the regulation of distinct physiological processes by the pRb-E2F network. However, a number of open questions are difficult to address in the mammalian system. These include the purification and functional characterization of endogenous pocket protein-containing complexes. Experimental approaches, including biochemical purification of protein complexes from mammalian cells, are almost exclusively performed using cell lines that have been transformed by viruses or mutational inactivation of the pRb-E2F pathway. Their fast growth facilitates the accumulation of sufficient starting material. These cell lines, however, harbor a major disadvantage for the purification of pocket protein complexes: Several viral oncoproteins have been shown disrupt the interaction of pocket proteins with some of their binding partners. Moreover, the transforming potential of oncoviruses has, at least partly, been attributed to this function. Even though a plethora of pocket protein interaction partners has been identified (Morris and Dyson 2001), it seems very likely that some critical interacting proteins have been missed due to the use of transformed cell lines. In addition, in mammalian cells the functional analysis of protein complexes comprising members of the pRb-E2F network is further complicated by the high level of redundancy among pRb and E2F family members.

2.3.2 *Drosophila melanogaster* as a model system to analyze pocket protein-containing complexes

The high degree of conservation of the pRb-E2F network together with its lower level of complexity make *Drosophila* an excellent model organism to study pocket protein-containing complexes (Sutcliffe et al. 2003). The streamlined pRb-E2F network in the fly gives rise to a limited number of distinct pocket protein-containing complexes, which facilitates their analysis. In contrast to mammalian cells, *Drosophila* embryos are ideally suited for large scale

biochemical approaches due to the availability of sufficient starting material, and their untransformed status makes the purification of native pocket protein-containing complexes possible. Moreover, E2F target genes are well characterized in the fly with regard to their dependence on distinct pocket proteins and E2F factors (Dimova et al. 2003).
Taken together, *Drosophila* happens to be an ideal model system for the isolation and functional characterization of native pocket protein-containing complexes.

The aim of this PhD thesis was the isolation and functional characterization of pocket protein-containing complexes from the fruitfly *Drosophila melanogaster*. Furthermore, this study sought to determine the contribution of such complexes to the role of pocket proteins in transcriptional regulation.

3 Material and methods

3.1 Material

3.1.1 Chemicals, enzymes, chromatographic and radioactive material

Unless otherwise stated, all common material and chemicals were ordered from Amersham/Pharmacia (Freiburg), E. Merck (Darmstadt), NEN/Perkin Elmer (Rodgau), Pierce (Bonn), Promega (Mannheim), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Radioactive material was ordered from Amersham.

3.1.1.1 Enzymes

Restriction endonucleases	New England Biolabs, Fermentas, Promega, Roche
Klenow enzyme	New England Biolabs
Shrimp alkaline phosphatase	New England Biolabs
T4 polynucleotide kinase (PNK)	Promega
T4 DNA ligase	New England Biolabs
Taq DNA polymerase	Promega
RNasin	Promega

3.1.1.2 Chromatographic material

Q Sepharose FF resin	Amersham
Biorex 70 Resin	BioRad
Q Sepharose HP column	Amersham
SP Sepharose HP column	Amersham
Hydroxyl apatite resin	BioRad
Gelfiltration column (Superose 6)	Amersham
Chromatography systems (ÄKTA, FPLC & HPLC)	Amersham

3.1.1.3 Affinity purification material

Glutathione Sepharose 4B	Amersham
Protein A Sepharose 4 FF	Amersham
Protein G Sepharose 4 FF	Amersham
M2 Agarose (Flag-beads)	Sigma

3.1.1.4 Blotting material

Whatman 3MM paper	Whatman
Hybond-P membrane	Amersham

3.1.1.5 Dialysis and filtration material

Dialysis membranes	Spectra Por
Filtration units	Merck

3.1.2 Standard solutions

Stock solutions and buffers were prepared according to standard protocols. Protease Inhibitors, either Complete® EDTA-free (Roche), or a mix of Leupeptin, Pepstatin, Aprotinin (all 1 µg/ml) and PMSF (0.2 to 1 mM), and the reducing agent DTT (1 mM) were freshly added. The most common solutions are listed below.

Phosphate Buffered Saline (PBS)	140 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH adjusted to 7.4 with HCl
TBE-buffer	90 mM Tris 90 mM Boric acid 2 mM EDTA
TE buffer	10 mM Tris-HCl pH 7.6 1 mM EDTA
Stacking buffer (4x)	0.5 M Tris-HCl 0.4% SDS, pH 6.8 with HCl
Resolving buffer (4x)	1.5 M Tris-HCl 0.4% SDS, pH 8.8 with HCl

SDS-PAGE running buffer	192 mM glycine 25 mM Tris 0,1% (w/v) SDS

Additional buffers are described in the individual method sections.

3.1.3 Antibodies

Unless otherwise stated dilutions are for Western Blot.

αRBF1 (DX3)	Monoclonal antibody (mouse) against RBF1. Kind gift from N. Dyson, used 1:50 and 1:4 for polytene stainings
αRBF1 (DX5)	Monoclonal antibody (mouse) against RBF1. Kind gift from N. Dyson, used 1:10 and 1:2 for polytene stainings
αRBF2 (DR6)	Monoclonal antibody (mouse) against RBF2. Kind gift from N. Dyson (Stevaux et al. 2002), used 1:10
αdDP (YUN6)	Monoclonal antibody (mouse) against dDP. Kind gift from N. Dyson, used 1:50
αdE2F1 (HAO2)	Monoclonal antibody (mouse) against dE2F1. Kind gift from N. Dyson, used 1:10
αdE2F1	Polyclonal antibody (guinea pig) against dE2F1. Kind gift from N. Dyson, used 1:5000
αdE2F2	Polyclonal antibody (rabbit) against dE2F2. Kind gift from N. Dyson (Frolov et al. 2001), used 1:2000 and 1:100 for polytene stainings
αCAF1p55	Polyclonal antibody (rabbit) against CAF1p55. Kind gift from B. Turner, used 1:1000
αMip40 / #3585	Polyclonal antibody (rabbit) against Mip40. Kind gift from M. Botchan (Beall et al. 2002), used 1:5000
αdMyb / #3587	Polyclonal antibody (rabbit) against dMyb. Kind gift from M. Botchan (Beall et al. 2002), used 1:3000
αMip120 / #3672	Polyclonal antibody (rabbit) against Mip120. Kind gift from M. Botchan (Beall et al. 2002), used 1:5000 and 1:100 for polytene stainings
αMip130 / #3527	Polyclonal antibody (rabbit) against Mip130/TWIT. Kind gift from M. Botchan (Beall et al. 2002), used 1:5000 and 1:250 for polytene stainings
αAly	Polyclonal antibody (rabbit) against dAly. Kind gift from H. White-Cooper (White-Cooper et al. 2000), used 1:12000

αdTwilight 2.1	Polyclonal antibody (guinea pig) against Mip130/TWIT. Kind gift from H. White-Cooper, used 1:1000 and 1:250 for polytene stainings
αdTwilight 2.2	Polyclonal antibody (guinea pig) against Mip130/TWIT. Kind gift from H. White-Cooper, used 1:500
αpol II / H5	Commercial antibody (mouse) against the phosphoserine 2 version of RNA-Polymerase II (Covance), used 1:150 for polytene stainings
αPc	Polyclonal antibody (rabbit) against Polycomb. Kind gift from R. Paro, used 1:100 for polytene stainings
αAcetyl Lysine / ab76	Commercial sheep polyclonal antibody against the tetra-acetylated N-terminal tail of histone H4 (aa 1-18) (Abcam), used 1:100 for polytene stainings
αhMip40 / ab12109	Commercial peptide antibody (rabbit) against hMip40 (Abcam), used 1:300
αhMip120 / ab12294	Commercial peptide antibody (rabbit) against hMip120 (Abcam), used 1:250
αhLin-54	Polyclonal antibody (rabbit) against hMip120. Kind gift from S. Gaubatz, used 1:500
αB-Myb / ab12296	Commercial peptide antibody (rabbit) against B-Myb (Abcam), used 1:500
αB-Myb / #5	Monoclonal antibody (mouse) against B-Myb. Kind gift from R. Watson, used 1:10
αhTWIT	Peptide antibody (rabbit) against hMip130/TWIT. Purified antibody was diluted 1:300
αhLin-9	Polyclonal antibody (rabbit) against hMip130/TWIT. Kind gift from S. Gaubatz (Gagrica et al. 2004), used 1:1000
αRbAp46/48 / 15G12	Monoclonal antibody (mouse) against RbAp46 and RbAp48. Kind gift from A. Verreault (Qian and Lee 1995), used 1:1000
αRbAp48 / 11G10	Monoclonal mouse antibody against RbAp48. Kind gift from A. Verreault (Qian and Lee 1995), used 1:500 in TBS-T
αpRb / G3-245	Commercial monoclonal antibody (mouse) against pRb (Pharmingen), used 1:1000
αpRb / C-15	Commercial polyclonal antibody (rabbit) against pRb (Santa Cruz), used 1:500
αp107 / C-18	Commercial polyclonal antibody (rabbit) against p107 (Santa Cruz), used 1:500
αp130 / C-20	Commercial polyclonal antibody (rabbit) against p130 (Santa Cruz), used 1:500

αE2F4 / C-20	Commercial polyclonal antibody (rabbit) against E2F4 (Santa Cruz), used 1:500
αE2F4 / C-108	Commercial polyclonal antibody (rabbit) against E2F4 (Santa Cruz), used 1:500
αMyc / 9E10	Mouse monoclonal antibody (Sigma) produced using a Hybridoma cell line, used 1:5
αHA / 12CA5	Commercial mouse monoclonal antibody (Roche), used 1:4000
αFlag / M2	Commercial monoclonal mouse antibody (Sigma), used 1:1000
α-rabbit HRP	Commercial secondary antibody for Western Blot (Amersham), used 1:10000
α-mouse HRP	Commercial secondary antibody for Western Blot (Amersham), used 1:5000
α-guinea pig HRP	Commercial secondary antibody for Western Blot (Dianova), used 1:5000
α-mouse Cy2	Commercial secondary antibody for Immunofluorescence (Jackson ImmunoResearch), used 1:300 for polytene stainings
α-rabbit Cy2	Commercial secondary antibody for Immunofluorescence (Jackson ImmunoResearch), used 1:300 for polytene stainings
α-guinea pig Cy2	Commercial secondary antibody for Immunofluorescence (Jackson ImmunoResearch), used 1:300 for polytene stainings
α-mouse Cy3	Commercial secondary antibody for Immunofluorescence (Jackson ImmunoResearch), used 1:400 for polytene stainings
α-rabbit Cy3	Commercial secondary antibody for Immunofluorescence (Jackson ImmunoResearch), used 1:400 for polytene stainings
α-sheep Cy3	Commercial secondary antibody for Immunofluorescence (Jackson ImmunoResearch), used 1:300 for polytene stainings

3.1.4 Plasmids

Table 3.I: Plasmids

Primers used for cloning are listed in 3.1.5 (Table 3.II)

Plasmid name	Description	Generated by
pSPORT1-Sfi-hp120sf	cDNA encoding for a short isoform of hp120	RZPD
IRAKp961B1163Q2	cDNA encoding for hTwt	RZPD
pCMVSport6-hp40	cDNA encoding for hp40	
pcDNA3.1	mammalian expression vector	Invitrogen
pcDNA3.1-hp40	full length human Mip40 cDNA in mammalian expression vector, PCR-cloned via BamHI and XhoI, primer: hp40-for, hp40-rev	M. Korenjak
pcDNA3.1-hp40F	full length human Mip40 cDNA in mammalian expression vector with C-terminal Flag-tag, PCR-cloned via BamHI and XhoI, primer: hp40-for, hp40-revflag	M. Korenjak
pcDNA3.1-hp120	cDNA encoding for a short isoform of human Mip120 in mammalian expression vector, PCR-cloned via BamHI and XhoI, primer: hp120-for, hp120-rev	M. Korenjak
pcDNA3.1-hp120F	cDNA encoding for a short isoform of human Mip120 in mammalian expression vector with C-terminal Flag-tag, PCR-cloned via BamHI and XhoI, primer: hp120-for, hp120-revflag	M.Korenjak
pcDNA3.1-hTwt	full length human Mip130/TWIT cDNA in mammalian expression vector, PCR-cloned via	M. Korenjak

	BamHI and XhoI, primer: hTwt-for, hTwt-rev	
pcDNA3.1-hTwtF	full length human Mip130/TWIT cDNA in mammalian expression vector with C-terminal Flag-tag, PCR-cloned via BamHI and XhoI, primer: hTwt-for, hTwt-revflag	M. Korenjak
pcDNA3.1-RbAp48	full length human RbAp48 cDNA in mammalian expression vector, cloned via EcoRI	M. Korenjak
pcDNA3.1-HA-B-Myb	full length mouse B-Myb cDNA in mammalian expression vector with N-terminal HA-tag	R. Watson
pcDNA3.1-myc-pRb	full length human pRb cDNA in mammalian expression vector with N-terminal myc-tag	N. Dyson
pcDNA3.1-myc-p107	full length human p107 cDNA in mammalian expression vector with N-terminal myc-tag	N. Dyson
pcDNA3.1-myc-p130	full length human p130 cDNA in mammalian expression vector with N-terminal myc-tag, cloned via BamHI and XbaI	N. Dyson
pcDNA3.1-flag-ΔN-lin9	human Mip130/TWIT cDNA encoding the C-terminus (aa 296-542) of the protein in mammalian expression vector with N-terminal Flag-tag, cloned via HindIII (Flag-tag) / BamHI (Insert) and XhoI	S. Gaubatz
pcDNA3.1-flag-ΔC-lin9	human Mip130/TWIT cDNA encoding the N-terminus (aa	S. Gaubatz

	1-301) of the protein in mammalian expression vector with N-terminal Flag-tag, cloned via HindIII (Flag-tag) / BamHI (Insert) and XbaI	
pGEM3Z-RbAp48	full length human RbAp48 cDNA, cloned via EcoRI	T. Kouzarides
pGEX2T-RbAp48	human RbAp48 cDNA cloned for expression as a GST-fusion protein	T. Kouzarides
pBS-RbAp46	human RbAp46 cDNA	T. Kouzarides

3.1.5 Oligonucleotides

Table 3.II: Oligonucleotides

Oligoname	Sequence	Description
hTwt-for	5´-GGG GAT CCC AAG ATG GCG GAG CT-3´	forward primer with BamHI restriction site used to clone hMip130/TWIT into pcDNA3.1
hTwt-rev	5´-GGC TCG AGT CAG TCT CTG TTG GTG-3´	reverse primer with XhoI restriction site used to clone hMip130/TWIT into pcDNA3.1
hTwt-revflag	5´-GGC TCG AGC TAC TTG TCA TCG TCG TCC TTG TAG TCG TCT CTG TTG GTG TT-3´	reverse primer with XhoI restriction site and Flag-tag sequence used to clone hMip130/TWIT-F into pcDNA3.1
hp120-for	5´-GGG GAT CCG ATC ATG GAG GTG GT-3´	forward primer with BamHI restriction site used to clone hMip120sf into pcDNA3.1
hp120-rev	5´-GGC TCG AGT TAG CAA TTC ATG GCA-3´	reverse primer with XhoI restriction site used to clone hMip120sf into pcDNA3.1
hp120-revflag	5´-GGC TCG AGC TAC TTG TCA TCG TCG TCC TTG	reverse primer with XhoI restriction site and Flag-tag

	TAG TCG CAA TTC ATG GCA CA-3´	sequence used to clone hMip120sf-F into pcDNA3.1
hp40-for	5´-GGG AAG CTT GGA TCC ACC ATG TTC CCT GTG AAG-3´	forward primer with HindIII and BamHI restriction sites used to clone hMip40 into pcDNA3.1
hp40-rev	5´-GGG TCT AGA CTC GAG TCA CTG TCG TTC GTA CAT C-3´	reverse primer with XbaI and XhoI restriction sites used to clone hMip40 into pcDNA3.1
hp40-revflag	5´-GGG CTC GAG TCA CTT GTC ATC GTC GTC CTT GTA GTC CTG TCG TTC-3´	reverse primer with XhoI restriction site and Flag-tag sequence used to clone hMip40-F into pcDNA3.1

3.1.6 Bacteria, flies and cells

3.1.6.1 Bacteria

E.coli strains DH5α (Invitrogen), SURE (Stratagene) and XL1Blue (Stratagene) were used for DNA plasmid amplifications.

3.1.6.2 Flies

Drosophila melanogaster yw flies used for embryo nuclear extract preparation and polytene stainings are described in Flybase (http://flybase.bio.indiana.edu).

3.1.6.3 Cell lines and tissue culture media

HEK 293 cells: Human embryonic kidney cell line, adenovirally transformed human tumor cell line (ATCC® Number: CRL-1573)

MOLT-4 cells: Human T lymphoblast cell line derived from a patient with acute lymphoblastic leukemia (ATCC® Number: CRL-1582)

Commercially available media and solutions were used for human tissue culture:

DMEM + GlutaMAX-I (Invitrogen), for HEK 293 cells

RPMI1640 + GlutaMAX-I (Invitrogen), for MOLT-4 cells

Fetal bovine serum (FCS, Sigma)

Penicillin/Streptomycin stock solution (Pen/Strep, 10000 U/ml penicillin, 10 mg/ml streptomycin, C. C. Pro)

3.2 Methods

3.2.1 Mammalian tissue culture

3.2.1.1 General cell culture conditions

HEK 293 and MOLT-4 cells were cultured in DMEM/GlutaMAX and RPMI1640/GlutaMAX, respectively. Before use, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% FCS (DMEM) / 15% FCS (RPMI) were added to the media. The cell lines were cultured in tissue culture flasks or stirring bottles in an incubator at 37°C and 5% CO_2. Tissue culture work was done under sterile conditions. When HEK 293 cells reached confluency they were detached from the flask by pipetting, diluted and seeded in fresh flasks. MOLT-4 cells were diluted with fresh medium to 0.5 x 10^6 cells/ml when they reached a density of 1 – 1.3 x 10^6 cells/ml. The cell number was determined using a hemacytometer.

3.2.1.2 Freezing and thawing of cells

For freezing, cells were resuspended at a density of 5 x 10^6 cells/ml in cold freezing medium and aliquoted in 1 ml cryo-tubes. They were frozen for 30 minutes at -20°C, followed by 48-72 hrs at -80°C, before they were stored in liquid nitrogen.

Freezing medium: DMEM or RPMI
15% FCS
5% Dimethyl Sulfoxide (DMSO)

For thawing, the frozen cells were slowly resuspended in 5 ml (MOLT-4) or 10 ml (HEK 293) of cold tissue culture medium. After 24 hrs, the cells were diluted (MOLT-4) or the medium was exchanged (HEK 293).

3.2.1.3 Transfection of mammalian cells

Transfection with Poly(ethylenimine) (PEI):

HEK 293 cells were transfected with expression plasmids using the polycation PEI. Mixing PEI with DNA results in the formation of PEI/DNA complexes which leads to DNA

condensation and the formation of compact colloids. The aggregates on the plasma membrane surface are then taken up by the cell via endocytosis (Godbey et al. 1999).
HEK 293 cells were seeded in 6-well-plates at a density of 200000 – 300000 cells/well and cultured for 24 hrs before transfection, in order to let them adhere. 2 μg of DNA were diluted in 100 μl of DMEM without serum and 6 μl of PEI (1mg/ml) were added for one transfection. After vortexing, the mixture was incubated for 5 min at room temperature. In the meantime, 500 μl of medium were removed from every well. After incubation, 600 μl of DMEM with serum were added to each reaction, mixed and dropped onto the cells. The cells were cultured for 48 hrs and then harvested.

Transfection by electroporation:

MOLT-4 cells were transfected by electroporation. During electroporation the cell membrane gets reversibly perforated by a surge, which allows the DNA to enter the cell.
MOLT-4 cells were washed with sterile PBS once and resuspended in RPMI without serum at a density of 2 x 10^7 cells/ml. 0.5 ml of the cell suspension were mixed with 10 μg of plasmid DNA (0.1 μg/μl) in an electroporation cuvette (BioRad Gene Pulser Cuvette, 0.4 cm electrode) and electroporated using the following conditions:
high capacity
960 μF
200 V

After electroporation the cells were transferred to tissue culture flasks containing 5ml of pre-warmed RPMI containing 20% serum. Transfected MOLT-4 cells were harvested after 24-48hrs.

3.2.1.4 Generation of a MOLT-4 cell line stably expressing hMip40-F

Before transfection, the plasmid DNA was linearized by restriction enzyme digestion to improve the rate of stable integration into the host genome. The DNA was Ethanol (EtOH)-precipitated, resuspended in TE-Buffer and MOLT-4 cells were transfected by electroporation (see 3.2.1.3).
48 hrs after electroporation, cells were transferred to medium containing 1 mg/ml Geneticin (stock solution: 50 mg/ml, Invitrogen) to select for cells that had stably integrated the expression plasmid. Cell survival was regularly determined by Trypan-Blue staining and living cells were kept at a density of 0.5 – 1 x 10^6 cells/ml. Expression of the protein of interest from

the stably integrated expression plasmid was controlled by Western Blot and stable cell lines were frozen and stored in liquid nitrogen (see 3.2.1.2).

Western Blot using a hMip40 antibody revealed the presence of two distinct forms of hMip40 in the extract derived from the stable cell line (Fig. 3.1 A). The faster migrating form corresponds to the band detected in the control cell line and represents the endogenous protein (black arrowhead), whereas the Flag-tagged protein runs slightly slower (black arrow). The expression level of hMip40-F is comparable to the endogenous protein. Moreover, hMip40-F is located to the nucleus like the endogenous B-Myb and hMip130/TWIT proteins, as assessed by Western Blot of nuclear and cytoplasmic extract using an αFlag-antibody (Fig. 3.1 B). A specific band (black arrowhead) was detected in the stable cell line, whereas only a cross-reacting band was observed in the control cell line (asterisk).

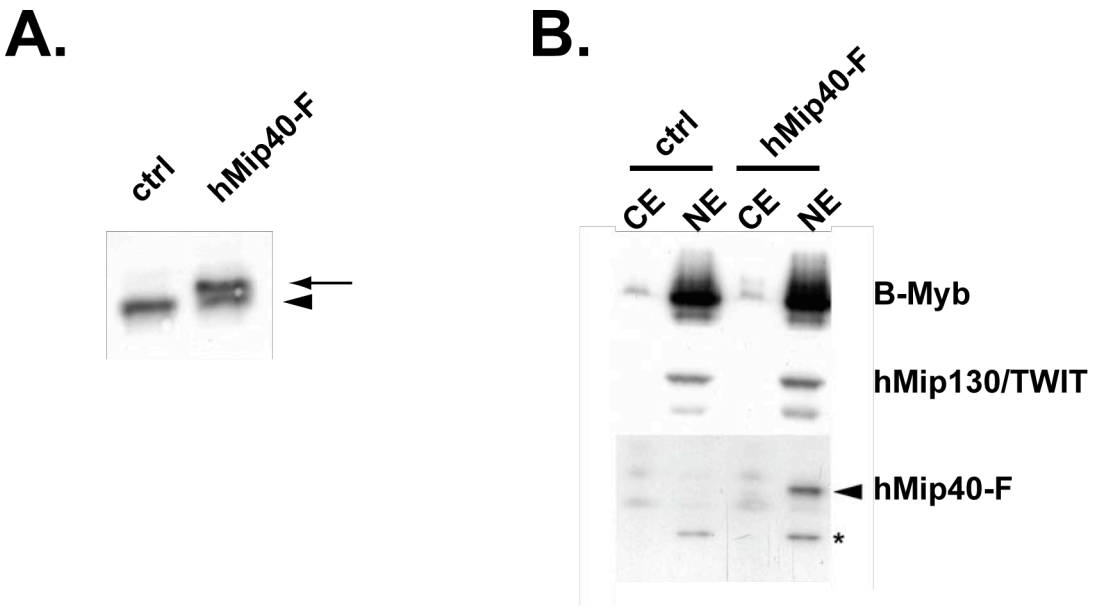


Figure 3.1: Generation of MOLT-4 cells stably expressing hMip40-F

(**A**) Western Blot using an antibody directed against hMip40. Cell lines from which the extracts were derived are denoted on top. Black arrowhead indicates endogenous hMip40, black arrow indicates hMip40-F. (**B**) Western Blot using αB-Myb, αhMip130/TWIT and αFlag antibodies. Cell lines from which the extracts were derived are indicated on top. Black arrowhead indicates hMip40-F, asterisk indicates a cross-reacting polypeptide. ctrl, control; CE, cytoplasmic extract; NE, nuclear extract.

Stable MOLT-4 cell clones were generated from the heterogeneous cell population by limited dilution in 96-well-plates. In order to improve the outgrowth of clones, α-Thioglycerol (α-TG) and Bathocuproinedisulfonic acid (BCS) were added to the medium to final concentrations of 50 μM and 20 nM, respectively (Brielmeier et al. 1998).

3.2.1.5 Serum-arrest of MOLT-4 cells

The removal of growth factors arrests cells in the G_0 phase of the cell cycle, which allows a synchronization of cells in either G_0 (by serum starvation) or S phase (by re-addition of serum).

Exponentially growing MOLT-4 cells were washed with sterile PBS once and synchronized in G_0 by resuspension and subsequent culturing in serum-free medium for 36 hrs (Jayadev et al. 1995).

3.2.2 Analysis of DNA

Standard procedures in molecular biology, including preparation of competent bacteria, transformation of electro- or chemically-competent bacteria with DNA, amplification of plasmid DNA in bacteria, purification, concentration determination, restriction enzyme digestion, ligation of DNA fragments, analysis of DNA on agarose gels and amplification of DNA by polymerase chain reaction (PCR) were performed according to standard protocols.

In addition, plasmid DNA was prepared using plasmid purification kits (Qiagen, Promega) for different amounts of DNA. Isolation of DNA fragments from agarose gels was performed using the Qiagen Gel Extraction kit.

3.2.3 Analysis of proteins

Protein analysis was performed according to standard protocols. In general, proteins were kept on ice (4°C), in the presence of protease inhibitors, either complete® (Roche), or a mix of Leupeptin, Pepstatin, Aprotinin (all 1 µg/ml), PMSF (0.2 to 1 mM) and the reducing agent DTT (1 mM).

3.2.3.1 Preparation of whole cell extract (WCE) from mammalian cells

Cells were harvested, washed once with PBS and resuspended in an appropriate amount of Lysis Buffer by pipetting. The cell suspension was incubated for 15 min on ice and centrifuged at 13000 rpm and 4°C for 15 min in a table-top centrifuge. The supernatant (WCE) was transferred to a fresh tube, frozen in liquid nitrogen and stored at -80°C.

Lysis Buffer: 50 mM Tris pH 8.0
300 mM NaCl
10 mM $MgCl_2$
0.4% NP-40
protease inhibitors
DTT

3.2.3.2 Preparation of nuclear extract (NE) from mammalian cells (MOLT-4)

All steps were performed on **ice** or at **4°C**.

Nuclear extract preparation 1:

Cells were harvested by centrifugation at 4630 g for 15 min in a Heraeus Cryofuge 6000i and the pellet was washed once with PBS and transferred to 50 ml Falcon tubes. After centrifugation at 2850g for 10 min the packed cell volume (PCV) was noted. The pellet was resuspended in 5 x PCV of Buffer A by vortexing on high for 2 sec and subsequently incubated on ice for 20 min. Following a centrifugation step at 2850 g for 10 min, the pellet was resuspended in 2 x PCV of Buffer A and the cells were disrupted by applying 14 strokes using a dounce homogenizer (pestle B). Disruption of the cells was microscopically controlled. The dounced cell suspension was first centrifuged at 1000 g for 10 min, followed by centrifugation at 4000 g for 10 min. The supernatants were combined and represented the cytoplasmic fraction. The nuclear pellet was resuspended in 3 ml Lysis Buffer / 1 x 10^9 cells by pipetting and incubated on ice for 15 min. Following centrifugation in a Sorvall RC5C, SS-34 rotor at 13000 rpm for 15 min, the supernatant (nuclear extract) was diluted 1:2 with Dilution Buffer, frozen in liquid nitrogen and stored at -80°C.

Buffer A: 10 mM HEPES pH 7.9
10 mM KCl
1.5 mM $MgCl_2$
protease inhibitors
DTT

Lysis Buffer: 50 mM Tris pH 8.0
300 mM NaCl
10 mM $MgCl_2$
0.4% NP-40
protease inhibitors
DTT

Dilution Buffer: 50 mM Tris pH 8.0
protease inhibitors
DTT

Nuclear extract preparation 2:

Cells were harvested by centrifugation at 4630 g for 15 min in a Heraeus Cryofuge 6000i and the pellet was washed once with PBS and transferred to 50 ml Falcon tubes. After centrifugation at 2850 g for 10 min the packed cell volume (PCV) was noted. The pellet was resuspended in 5 x PCV of Buffer A by vortexing on high for 2 sec and subsequently incubated on ice for 20 min. Following a centrifugation step at 2850 g for 10 min, the pellet was resuspended in 2 x PCV of Buffer A and the cells were disrupted by applying 14 strokes using a dounce homogenizer (pestle B). The dounced cell suspension was centrifuged at 1450 g for 10 min and to the supernatant (cytoplasmic fraction) 0.11 vol of Buffer B were added. The nuclear pellet was frozen in liquid nitrogen and stored at -80°C or immediately continued for nuclear extract preparation.

0.9 vol of Buffer C were added to the nuclei while stirring and the suspension was dounced 20 times with a type B glass homogenizer. After stirring for an additional 30 min on ice, a centrifugation step in a Sorvall RC5C, SS-34 rotor at 15000 rpm for 30 min was carried out. Over a period of about 15 min, 0.33 g of finely ground ammonium sulfate per ml supernatant (nuclear extract) and 8 µl 5 M KOH per g ammonium sulfate were added while stirring in the coldroom on ice. After stirring for an additional hr, the suspension was centrifuged in an SS-34 rotor at 15000 rpm for 30 min. The pellet was resuspended in 1 ml of Buffer D-20 per 1 l of cells harvested and dialyzed twice for 2 hrs against 0.5 l of Buffer D-125. The nuclear extract was frozen in liquid nitrogen and stored at -80°C.

Buffer A:
10 mM HEPES pH 7.6
10 mM KCl
1.5 mM $MgCl_2$
pH 7.9 with 5 M KOH
protease inhibitors
DTT

Buffer B:
300 mM HEPES pH 7.6
1.4 M KCl
30 mM $MgCl_2$
protease inhibitors
DTT

Buffer C:
20 mM HEPES pH 7.6
420 mM NaCl
1.5 mM $MgCl_2$
0.2 mM EDTA
25% Glycerol
pH 7.9 with 5 M NaOH
protease inhibitors
DTT

Buffer D-X:
20 mM HEPES pH 7.6
X mM KCl
2 mM $MgCl_2$
0.2 mM EDTA
20% Glycerol
pH 7.9 with 5 M KOH
protease inhibitors
DTT

3.2.3.3 Determination of protein concentration

Protein concentration was determined using the colorimetric assay described by Bradford (Bradford 1976). The concentration of purified proteins was also estimated according to protein standards with a known concentration (e.g. BSA) in SDS-PAGE followed by Coomassie blue staining.

3.2.3.4 Trichloroacetic acid (TCA) precipitation of proteins

TCA was added to the protein sample at a final concentration of 20%, mixed and incubated for 10 min on ice. After spinning the sample at 13000 rpm and 4°C for 10 min, the supernatant was removed and the pellet washed twice with 500 µl of cold acetone by centrifuging it at 13.000 rpm and 4°C for 5 min. The protein pellet was dried and resuspended in 1 x SDS-PAGE loading buffer and 1/40 volume of 1 M Tris pH 8.0.

3.2.3.5 *In vitro* translation

In vitro translation was carried out using the TNT rabbit reticulocyte lysate system (Promega) and performed according to the manufacturer´s instructions.

3.2.3.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Pouring and electrophoresis of SDS-polyacrylamide gels was performed using the Novex system (pre-assembled gel cassettes). Resolving and stacking gels were prepared according to standard protocols using ready-to-use polyacrylamide solutions from Roth (Rotigel, 30%, 49:1) (see 3.1.2 for buffers). For electrophoresis, protein samples were mixed with SDS-PAGE sample buffer, heat-denatured for 5 min at 95°C and directly loaded onto the gel. Proteins were separated at 200V until the dye front had reached the end of the gel. The molecular weight of proteins was estimated by running pre-stained or non-stained marker proteins (Peqlab, peqgold protein marker) in parallel. Following electrophoresis, proteins were stained with either Coomassie Brilliant Blue, Silver or subjected to Western blotting.

3.2.3.7 Coomassie Blue staining of protein gels

Polyacrylamide gels were fixed for at least 30 min in fixation solution (50% methanol / 10% acetic acid) and stained for 60 min to overnight on a slowly rocking platform with Coomassie staining solution (0.025% Coomassie Blue R in 10% Acetic acid). To visualize proteins, gels

were destained in 10% acetic acid. After documentation, the gels were dried onto a Whatman paper at 80°C for 1 hr on a gel dryer (BioRad).

In order to analyze proteins by Mass Spectrometry, gels were stained with a Colloidal Coomassie staining kit (Merck). Briefly, gels were fixed for at least 2 hrs in fixation solution (50% methanol / 10% acetic acid) and incubated overnight in staining solution. Destaining of the gels was performed using ddH_2O. After documentation, the bands were excised with a scalpel and stored in 0.2 ml PCR tubes with 150 µl of ddH_2O at -20°C. Mass Spectrometry analysis of the proteins by MALDI-TOF or nano-spray-LC-MS/MS was carried out in a core facility (http://proteinanalytik.web.med.uni-muenchen.de/index.php/home/).

Staining solution: 10 ml Stainer A
2.5 ml Stainer B
10 ml Methanol
27.5 ml ddH_2O

3.2.3.8 Silver staining of protein gels

The staining of protein gels with silver nitrate solution was carried out according to the protocol of Blum. The gel was fixed in 50% ethanol / 10% acetic acid for at least 2 hrs and washed three times in 30% ethanol (20 min each), incubated for 1 min in 0.02% $Na_2S_2O_3$ (sodium thiosulfate), washed three times with water (ddH_2O, 20 sec each) and stained with 0.2% $AgNO_3$ solution for 1 hr. Afterwards, the gel was washed with water (three times, 20 sec each) and developed using developing solution (3% Na_2CO_3, 0.05% H_2CO, 0.0004% $Na_2S_2O_3$) until the desired proteins were visible (typically, after 5 to 10 min). After a short wash in water (1 min) the reaction was stopped by incubating the gel in 0.5% glycine stop solution (more than 5 min). After a final water wash (>30 min), the gel was documented and dried onto a Whatman paper at 80°C for 1 hr on a gel dryer (BioRad).

3.2.3.9 Western Blotting

Proteins were separated by SDS-PAGE and transferred to PVDF membranes using the BioRad “Wet Blot system”. The gel was placed onto a membrane and sandwiched between gel-sized Whatman paper soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The proteins were then transferred onto the membrane for 1.5 hrs (400 mA constant) at room temperature. The transfer reaction was cooled by the addition of an ice block into the transfer chamber. After transfer, the PVDF membranes were incubated for 1 hr in blocking solution (PBS/0.1% Tween-20/4% dried milk) in order to reduce the non-specific

background. Membranes were sealed in a plastic bag and incubated overnight on a horizontal shaker in the coldroom with an appropriate dilution of the primary antibody directed against the protein of interest. PVDF membranes were washed three times in PBS/0.1% Tween-20 (10 min each) and incubated for one additional hr with horseradish peroxidase-coupled secondary antibody at room temperature. After three washes (10 min each, in PBS/0.1% Tween-20) antigen-antibody complexes were detected using the Enhanced Chemi-Luminescence Kit (ECL, Amersham) and autoradiography according to the manufacturer´s instructions.

3.2.3.10 Chromatographic purification of dE2F2 complexes from *Drosophila* embryo nuclear extract (TRAX)

Chromatographic purification was performed using Chromatography Systems from Pharmacia (ÄKTA: FPLC & HPLC). Members of the *Drosophila* RBF-dE2F network were monitored during the purification procedure by Western Blot.

50 – 100 ml *Drosophila* embryo nuclear extract (0 – 12 hr TRAX) (10 – 15 μg/μl) in Buffer Q-100 was loaded onto a 70 ml Q Sepharose FF column, washed with Buffer Q-100 and eluted stepwise with 450 and 1000 mM KCl. The 450 mM fraction, which contained the proteins of interest, was dialyzed against Buffer HEMG-100 and loaded onto a 25 ml Biorex 70 column. The column was washed with Buffer HEMG-100 and bound proteins were eluted with 250, 500 and 1000 mM KCl. RBF, dE2F and dDP proteins were detected in the 250 mM elution and dialyzed against Buffer Q-100. The proteins were loaded onto a 5 ml Q Sepharose HP column, washed with 3 column volumes (CV) of Buffer Q-100 and eluted with a gradient from 100 to 500 mM KCl over 25 CV (fraction size: 2.5 ml). dE2F2 eluted in one peak at a salt concentration of about 400 mM KCl. 2 ml of the peak fraction were directly loaded onto a 0.8 ml Hydroxyl apatite column, the column was washed with 2 CV of Buffer Q-400/10mM Phosphate and eluted with a Phosphate gradient from 10 to 500 mM over 25 CV (fraction size: 500 μl). 200 μl of the dE2F2 peak fraction were subsequently separated on a Superose 6 gelfiltration column using Buffer EX-300 (fraction size: 500 μl). The eluted fractions were precipitated either by TCA-precipitation or by using StrataClean Resin (Stratagene), resuspended in 1 x SDS-PAGE loading buffer and analyzed by Western Blot, Coomassie or Silver staining.

Buffer Q-X: 20 mM Tris pH 8.0
1 mM $MgCl_2$
10% Glycerol
X mM KCl
PMSF
DTT

Buffer HEMG-X: 25 mM HEPES pH 7.6
12.5 mM $MgCl_2$
0.1 mM EDTA
10% Glycerol
X mM KCl
PMSF
DTT

HyAp Buffer A: 20 mM Tris pH 8.0
1 mM $MgCl_2$
10% Glycerol
400 mM KCl
PMSF
DTT

HyAp Buffer B: 20 mM Tris pH 8.0
1 mM $MgCl_2$
10% Glycerol
400 mM KCl
500 mM K_2HPO_4/KH_2PO_4
PMSF
DTT

Buffer EX-300: 10 mM HEPES pH 7.6
300 mM KCl
1.5 mM $MgCl_2$
0.5 mM EGTA
10% Glycerol
10 mM β-Glycerophosphate
PMSF
DTT

3.2.3.11 Chromatographic purification of a complex homologous to the dE2F2 complexes from MOLT-4 nuclear extract

Chromatographic purification was performed using Chromatography Systems from Pharmacia (ÄKTA: FPLC & HPLC). The proteins of interest were monitored during the purification procedure by Western Blot.

2 g of MOLT-4 nuclear extract in Lysis Buffer (150 mM NaCl, see 3.2.3.2) was loaded onto a 70 ml Q Sepharose FF column, washed with Buffer Q-150 and eluted stepwise with 300, 450 and 1000 mM KCl. The 300 mM fraction, which contained the proteins of interest, was diluted to 125 mM KCl with Buffer Q-0, loaded onto a 25 ml Biorex 70 column and washed with Buffer HEMG-125. Bound proteins were eluted with 250, 500 and 1000 mM KCl. The 250 mM elution was diluted with Buffer HEMG-0 to 150 mM KCl, loaded onto a 5 ml Q Sepharose HP column, washed with 3 CV of Buffer Q-150 and eluted with a gradient from 150 to 500 mM

KCl over 25 CV (fraction size: 2.5 ml). The proteins of interest eluted in one peak and the corresponding fractions were combined and diluted to 125 mM KCl with Buffer Q-0. They were loaded onto a 1 ml SP Sepharose HP column, washed with 2 CV of Buffer SP-125 and eluted with a gradient from 125 to 500 mM KCl over 20 CV (fraction size: 500 µl). The complex eluted at a salt concentration of about 250 mM KCl. The pooled peak fractions were directly loaded onto a 0.8 ml Hydroxyl apatite column, washed with 2 CV of Buffer SP-250/10 mM Phosphate and eluted with a Phosphate gradient from 10 to 300 mM over 20 CV and from 300 to 500 mM over 2 CV (fraction size: 500 µl). 250 µl of the peak fraction of the complex were subsequently separated on a Superose 6 gelfiltration column using Buffer EX-300 (fraction size: 500 µl). The eluted fractions were precipitated either by TCA-precipitation or by using StrataClean Resin (Stratagene), resuspended in 1 x SDS-PAGE loading buffer and analyzed by Western Blot, Coomassie or Silver staining.

Buffer Q-X: 20 mM Tris pH 8.0
1 mM $MgCl_2$
10% Glycerol
X mM KCl
PMSF
DTT

Buffer HEMG-X: 25 mM HEPES pH 7.6
12.5 mM $MgCl_2$
0.1 mM EDTA
10% Glycerol
X mM KCl
PMSF
DTT

HyAp Buffer A: 20 mM HEPES pH 7.6
250 mM KCl
10% Glycerol
PMSF
DTT

HyAp Buffer B: 20 mM HEPES pH 7.6
250 mM KCl
10% Glycerol
500 mM K_2HPO_4/KH_2PO_4
PMSF
DTT

Buffer EX-300: 10 mM HEPES pH 7.6
300 mM KCl
1.5 mM $MgCl_2$
0.5 mM EGTA
10% Glycerol
10 mM β-Glycerophosphate
PMSF
DTT

Buffer SP-X: 20 mM HEPES pH 7.6
X mM KCl
10% Glycerol
PMSF
DTT

3.2.3.12 Histone tail-peptide pulldown

Histone tail-peptides were either synthesized and coupled to a column matrix by Peptide Specialty Laboratories or biotinylated peptides were purchased from Upstate and bound to Dynabeads® M-280 Streptavidin (Dynal).

Drosophila:

Pulldown experiments were done in siliconized reaction tubes. First, beads were equilibrated with Binding Buffer and then blocked with Binding Buffer + 0.2 mg/ml BSA for 20 min at 4°C on a rotating wheel. 50 µl of TRAX or partially purified fractions (Q Sepharose HP) were diluted to 500 µl with Binding Buffer and incubated with 15 µl of a 1:1 bead slurry for 3 hrs at 4°C on a rotating wheel. After extensively washing the beads once with Binding Buffer and three times with Washing Buffer, they were resuspended in 1 x SDS-PAGE loading buffer and analyzed by Western Blot.

Binding Buffer:	Washing Buffer:
20 mM Tris pH 8.0	20 mM Tris pH 8.0
1 mM $MgCl_2$	1 mM $MgCl_2$
100 mM KCl	300 mM KCl
0.2% NP-40	0.2% NP-40
10% Glycerol	10% Glycerol
protease inhibitors	protease inhibitors
DTT	DTT

Human cell lines:

Pulldown experiments from MOLT-4 NE were performed essentially as described for *Drosophila* extracts but buffers differed in their stringency.

Binding Buffer:	Washing Buffer:
20 mM HEPES pH 7.9	20 mM HEPES pH 7.9
100 mM KCl	150 mM KCl
0.2 mM EDTA	0.2 mM EDTA
0.05% NP-40	0.05% NP-40
10% Glycerol	10% Glycerol
protease inhibitors	protease inhibitors
DTT	DTT

Histone tail-peptides:	
H3 (aa 1 – 20) wt	H3 (21 – 34) wt
H3 (1 – 20) K9me2	H3 (21 – 34) K27me2

H3 (1 – 20) K4me2

H4 (1 – 20) wt	H4 (9 – 35) wt
H4 (1 – 20) K5,8,12,16-Ac	H4 (9 – 35) K20me2

H3 (1 – 20)-, H3 (21 – 34)- and H4 (9 – 35)-peptides were kindly provided by A. Imhof.

3.2.3.13 GST-pulldown assays

Glutathione-Sepharose 4B beads were equilibrated in Buffer EX-250/0.05% NP-40 and loaded with recombinant, *E. coli*-expressed GST and GST-pRb (379 – 928). Binding of approximately 0.75 mg protein/ml bead volume occurred by rotating overnight at 4°C and subsequently the beads were washed twice with Buffer EX-250/0.05% NP-40.

GST-pulldown assay of in vitro translated proteins:

GST fusion proteins on beads were pre-incubated with 1 mg/ml BSA in Buffer Z´ for 5 min at room temperature. *In vitro* translated test proteins in 200 µl of Buffer Z´ were incubated with equal amounts of GST or GST-pRb (20 µl of a 1:1 bead slurry each) for 1 hr at room temperature on a rotating wheel. The beads were washed three times in 1.5 ml of Buffer NETN, resuspended in 1 x SDS-PAGE loading buffer and analyzed by autoradiography.

Buffer Z´: 25 mM HEPES-KOH pH 7.5
12.5 mM $MgCl_2$
150 mM KCl
0.1% NP-40
20 µM $ZnSO_4$
20% Glycerol
protease inhibitors
DTT

Buffer NETN: 20 mM Tris pH 8.0
150 mM NaCl
1 mM EDTA
0.5% NP-40
protease inhibitors
DTT

GST-pulldown assay from partially purified MOLT-4 NE:

GST fusion proteins on beads were pre-incubated with 1 mg/ml BSA in Buffer Q-150/0.05% NP-40 for 5 min at room temperature. 50 µl of the Q Sepharose HP fraction containing the human homolog of the dE2F2 complexes (assessed by Western Blot) were diluted to 200 µl with Buffer Q-150/0.05% NP-40 and rotated with equal amounts of GST or GST-pRb (20 µl of a 1:1 bead slurry each) for 3 hrs at 4°C. The beads were washed three times in 500 µl of

Buffer Q-300/0.05% NP-40, resuspended in 1 x SDS-PAGE loading buffer and analyzed by Western Blot.

3.2.3.14 αFlag-Co-immunoprecipitation

αFlag M2 beads were equilibrated in Buffer D-125/0.05% NP-40 and 10 µl of a 1:1 bead slurry were added to 400 µg of either transiently transfected HEK 293 WCE or stably transfected MOLT-4 NE. The extract with the beads was incubated in Buffer D-125/10% Glycerol/0.05% NP-40 (final concentration) for 3 hrs at 4°C on a rotating wheel and the beads were washed once with Buffer D-125/10% Glycerol/0.05% NP-40 and three times with Buffer D-300/10% Glycerol/0.05% NP-40. After washing one more time with Buffer D-125/0.05% NP-40, bound proteins were eluted using Flag-peptide in the same buffer (final concentration: 0.4 mg /ml). The beads were diluted 1:1 with elution buffer and the elution was carried out for 2 hrs on ice by regular mixing of the slurry. An additional elution was performed overnight at 4°C on a rotating wheel. The eluted material was subsequently analyzed by Western Blot or Mass Spectrometry.

For Mass Spec analysis, 8 mg of MOLT-4 NE were precipitated in 4 aliquots, the eluted material was combined and precipitated using StrataClean Resin (Stratagene).

3.2.3.15 Binding and covalent coupling of antibodies to Protein G beads

Unless otherwise indicated all steps were carried out at 4°C.

First, the pH of the antibody preparation was adjusted to 8.0 by adding 1/10 volume of 1 M Tris pH 8.0. The Protein G beads were incubated with the respective antibody for 1 hr on a rotating wheel (beads bind approximately 10 – 20 mg of antibody per ml of wet beads). The antibody bead slurry was then loaded onto a plastic column, the flowthrough was collected and reloaded twice onto the column. After washing the beads with 10 CV of 100 mM Tris pH 8.0 and subsequently with 10 CV of 10 mM Tris pH 8.0, the efficiency of the binding was checked by Western Blot.

For coupling, the beads were washed twice with 10 volumes of 0.2 M sodium borate pH 9.0 and resuspended in 10 volumes of 0.2 M sodium borate pH 9.0 (the equivalent of 10 µl of beads was removed to control for the efficiency of coupling). Dimethylpimelimidate (solid) was added to a final concentration of 20 mM and incubated for 30 min at **room temperature** on a

rotating wheel (coupling efficiency control, see above). The coupling reaction was stopped by washing the beads once in 0.2 M ethanolamine pH 8.0 and rotating them for additional 2 hrs at **room temperature** in 0.2 M ethanolamine.

The beads were then washed with PBS, followed by a wash with 4 – 5 CV of 100 mM glycine pH 3.0 in a column to remove any antibodies that were not covalently bound to the protein G beads. After washing the beads with 10 CV of PBS (coupling efficiency control, see above), they were resuspended in PBS/0.02% sodium azide.

The efficiency of coupling was tested by boiling the removed bead samples and Coomassie staining of the eluted heavy chain.

3.2.3.16 Co-immunoprecipitation

Protein G beads were equilibrated in Buffer Q-125/0.05% NP-40. Protein extracts were either incubated with ethidium bromide (final concentration: 50 μg/ml) for 30 min on ice, centrifuged at 13000 rpm for 5 min and the supernatant was used for immunoprecipitation (IP), or directly used for IP.

Extracts were incubated with the respective antibody for 3 hrs at 4°C on a rotating wheel and after addition of 30 μl of protein G beads for an additional hour. Alternatively, extracts were rotated with 30 μl of antibody coupled protein G beads for 3 hrs at 4°C. The beads were washed three times with Buffer Q-300/0.05% NP-40 (optional: 50 μg/ml ethidium bromide), resuspended in 1 x SDS-PAGE loading buffer and analyzed by Western Blot.

3.2.3.17 Immunofluorescence of polytene chromosomes

Drosophila salivary glands were dissected from 3rd instar larvae in 0.7% NaCl solution and incubated for 10 min in fixing solution (45% acetic acid / 1.85% formaldehyde) on a siliconized coverslip. The coverslip was taken up by a poly-lysine-treated slide and the glands were broken by regularly dotting the coverslip with the back end of a paintbrush in a spiral movement. Polytene chromosomes were spread by squeezing the slide and coverslip with the thumb. The polytene chromosome quality was checked by phase contrast microscopy and acceptable slides were frozen in liquid nitrogen. The coverslip was removed from the frozen slide with a razor blade and the slide was washed in PBS for 5 min and in PBS/0.1% Triton-X-100 for 10 min.

For blocking, the slide was incubated in PBS/0.1% Triton-X-100/1% BSA. Subsequently, the slide was placed in a humid chamber and the squashed polytene chromosomes were covered

overnight at 4°C with 20 µl of the primary antibody dilution and a fresh coverslip. All antibodies were diluted in PBS/0.1% Triton-X-100/1% BSA.

The next day, the slides were washed three times for 5 min in PBS and twice for 15 min in PBS/0.1% Triton-X-100/1% BSA before the polytene chromosomes were incubated with the appropriately diluted secondary antibody as described before for 1 hr at room temperature. Slides were washed twice for 10 min with PBS/0.1% Triton-X-100/1% BSA and three times for 5 min in PBS, before staining the DNA with Hoechst dye (1:20000 in PBS) for 2 min. The slides were washed twice for 5 min in PBS, mounted with PBS/0.1 M n-propyl-gallate/50% glycerol and stored at 4°C in the dark.

4 Results

4.1 Retinoblastoma complexes in *Drosophila melanogaster*

Sequencing of the *Drosophila* genome has revealed that a plethora of human disease related genes have counterparts in the fly (Adams et al. 2000; Fortini et al. 2000). Further studies on several of them have shown that also the context in which these proteins act in *Drosophila* is often highly conserved compared to the mammalian system. Among these candidates are the fly´s Retinoblastoma like factors, RBF1 and RBF2 (Du et al. 1996a; Stevaux et al. 2002). The identification of RBF1 and RBF2 has enabled us to study the Retinoblastoma tumor suppressor pathway in a relatively simple model organism and gain further insight into its mechanism of action.

4.1.1 High molecular weight RBF complexes exist in *Drosophila* embryos and cell lines

In order to get a first insight regarding the existence and size of RBF complexes, *Drosophila* embryo nuclear extract (TRAX) from embryos of different age (0 – 12 and 0 – 2 hr) and nuclear extract from a *Drosophila* cell line were separated on a Superose 6 gel filtration column (Fig. 4.1). In 0 – 12 hr embryos, peaks of RBF1, RBF2 and dDP were found in high molecular weight fractions reflecting a molecular weight larger than 669 kDa (Fig. 4.1 A, B, C; upper panel), and a minor RBF1 peak was detected with an apparent molecular weight of about 100 kDa, probably reflecting monomeric RBF1 (Fig. 4.1 A; upper panel). The faster migrating form of RBF1, which was detected in the Western Blot of fraction 31 is probably the result of degradation of the protein (Fig. 4.1 A; upper + middle panel). Interestingly, in early embryos RBF1 was present only in its monomeric form (Fig. 4.1 A; middle panel), whereas RBF2 was detected in high (>669 kDa) and low (100 kDa) molecular weight fractions (Fig. 4.1 B; middle panel). dDP eluted in a broad molecular weight range from 100 to >669 kDa in size (Fig. 4.1 C; middle panel). In KC cells however, RBF1 mainly eluted in medium (~500 kDa) and RBF2 in high (>669 kDa) molecular weight fractions (Fig. 4.1 A, B; lower panel). dDP eluted with a similar pattern as in early embryos (Fig. 4.1 C; lower panel).

These results suggest, that RBF and dDP proteins are assembled into high molecular weight complexes, both in *Drosophila* embryos and cells. These complexes differ in their size, depending on the origin (early and late embryos vs. KC cells). Furthermore, RBF2 is

assembled into large complexes early in development, whereas RBF1 exists only in monomeric form in 0 – 2 hr *Drosophila* embryos.

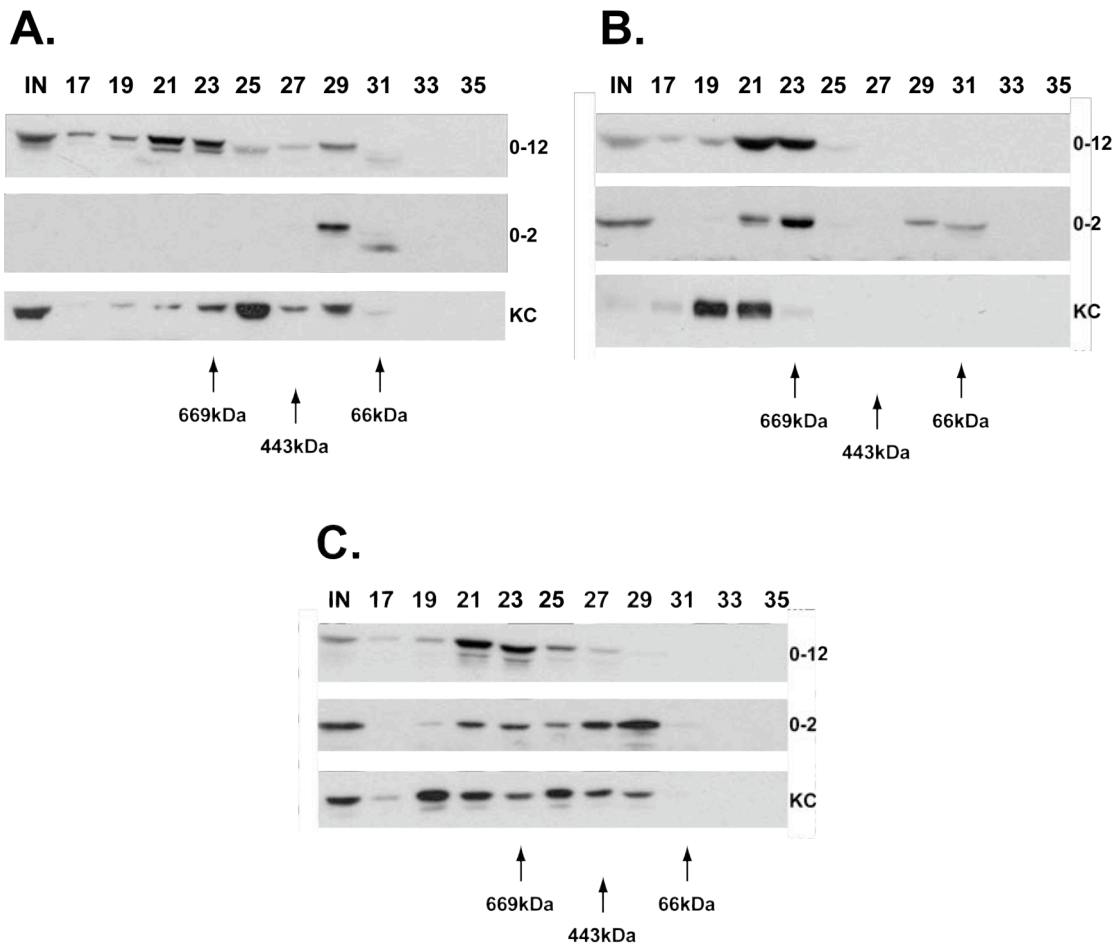


Figure 4.1: RBF and dDP proteins exist in high molecular weight complexes in *Drosophila* embryos and cells

TRAX from 0 – 12 and 0 – 2 hr embryos and KC cell nuclear extract were separated on a Superose 6 gel filtration column. The eluted fractions were tested by Western Blot for the presence of RBF1 (**A**), RBF2 (**B**) and dDP (**C**). Upper panel: 0 – 12 hr, Middle panel: 0 – 2 hr, Lower panel: KC cells. Fraction numbers are denoted on top, size standards on the bottom. IN, input.

4.1.2 0 – 12 hr *Drosophila* embryo nuclear extract contains at least three distinct RBF-containing complexes

In order to separate distinct complexes that could not be distinguished by the gel filtration experiments (see 4.1.1), *Drosophila* embryo nuclear extract (0 – 12 hr) was loaded onto a Q Sepharose HP ion exchange column. Western Blot analysis of the eluted material using antibodies against all members of the RBF-dE2F network identified three protein peaks (Fig. 4.2). Peak I contained only RBF1 and subsequent analysis by gel filtration revealed a

molecular weight of approximately 100 kDa, probably reflecting monomeric RBF1 (Fig. 4.3 A). In peak II, dE2F1 and dDP were identified in addition to RBF1 (Fig. 4.2). Gel filtration analysis of peak II showed that those proteins were assembled into a complex of about 500 kDa in size (Fig. 4.3 B). RBF1, RBF2, dE2F2 and dDP coeluted in peak III (Fig. 4.2) and are part of high molecular weight complexes (>669 kDa), as shown by gel filtration analysis of the corresponding Q Sepharose fractions (Fig. 4.3 C).

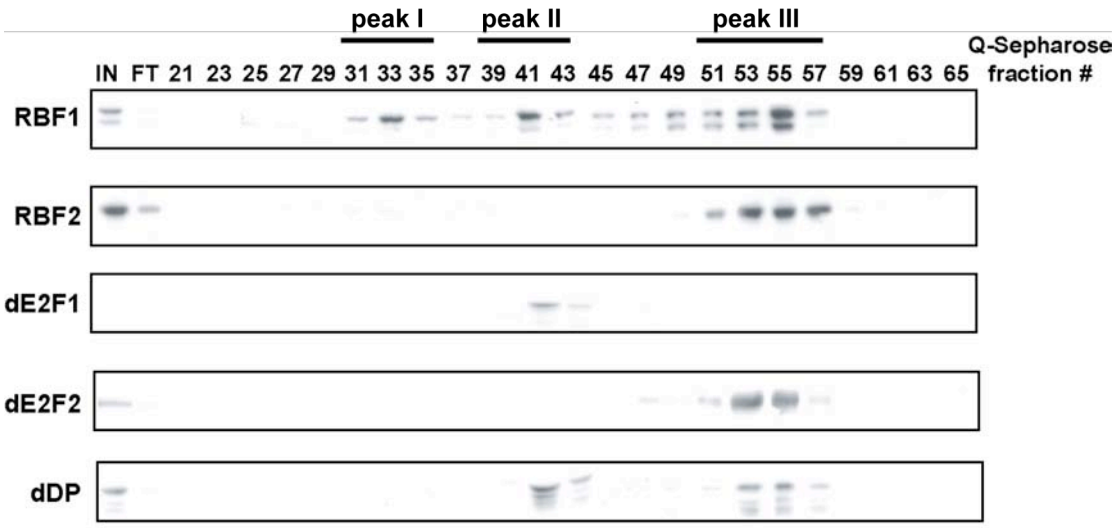


Figure 4.2: RBF complexes in *Drosophila* embryos

Nuclear extract was separated over a Q Sepharose HP ion exchange column. Fractions were analyzed by Western Blot using specific antibodies as shown. Three RBF1 peaks and fraction numbers are indicated on top. IN, input; FT, flowthrough. This figure is taken from (Korenjak et al. 2004).

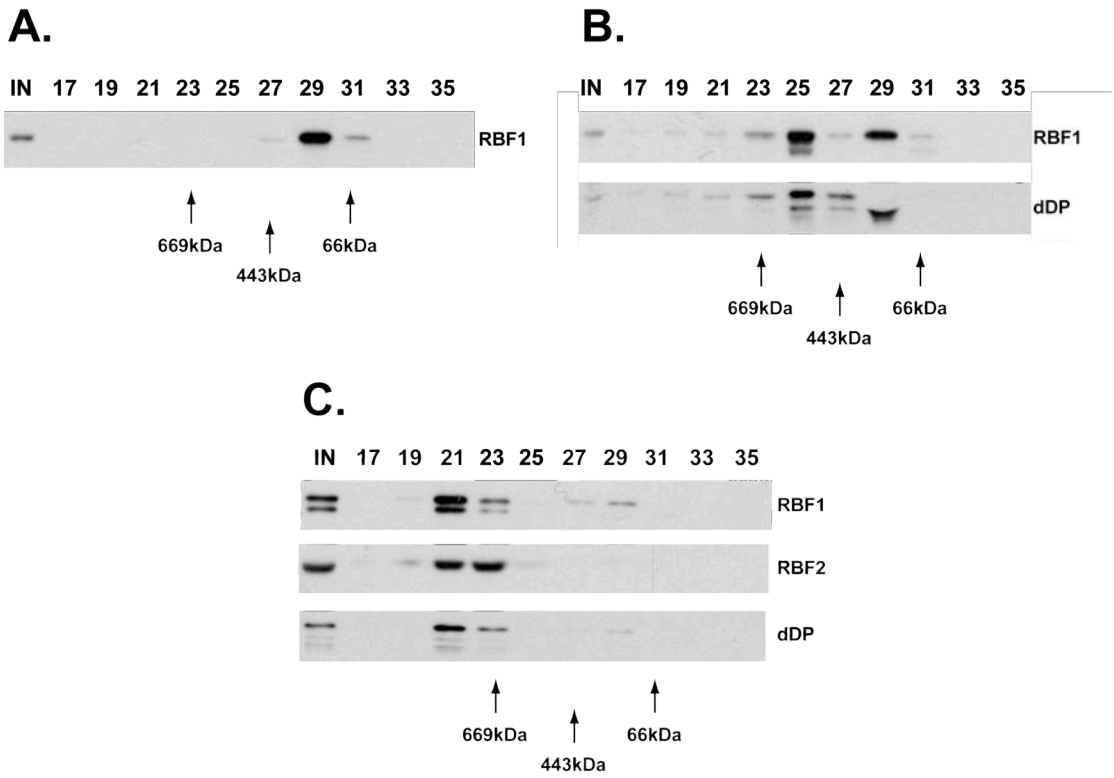


Figure 4.3: At least three distinct RBF complexes exist in *Drosophila* embryos
RBF1 peaks I, II and III (Fig. 4.2) were separated on a Superose 6 gel filtration column. (**A**) RBF1 peak I, (**B**) RBF1 peak II, (**C**) RBF1 peak III. Fractions were analyzed by Western Blot using specific antibodies as shown. Fraction numbers are indicated on top, size standards on the bottom. IN, input.

The elution profile of RBF-dE2F network members (Fig. 4.2) and the fact that RBF1 and RBF2 do not interact with each other (Stevaux et al. 2002) suggest the existence of at least three distinct RBF-containing complexes in *Drosophila* embryos: an approximately 500 kDa complex containing RBF1 and dE2F1 and two complexes of higher molecular weight containing dE2F2 and either RBF1 or RBF2. The identical elution profile of the dE2F2/RBF complexes on the ion exchange column suggests that these complexes are otherwise very similar in their subunit composition.

4.1.3 Purification of dE2F2/RBF complexes

Due to the apparent molecular weight of dE2F2 complexes (>669 kDa) it seemed very likely that they contained additional subunits. Therefore, a purification strategy using conventional chromatography was established to isolate the endogenous complexes from 0 – 12 hr embryo extract (Fig. 4.4 A). The applied scheme included chromatography columns that were tested for the binding and elution of dE2F2/RBF complexes. Throughout the purification procedure RBF1, RBF2, dE2F2 and dDP cofractionated on all columns and eluted with a molecular

weight between 669 kDa and 1.2 MDa from the final Superose 6 gel filtration column (Fig. 4.4 B). Silver staining of the Superose 6 fractions revealed that 7 bands were coeluting perfectly with the Western Blot signals (Fig. 4.4 C). In order to identify the corresponding proteins, the bands were excised and analyzed by Mass Spectrometry. The results confirmed the presence of RBF1, RBF2, dE2F2 and dDP, as expected from the Western Blot experiments. Four additional proteins could be identified: Mip130/TWIT, dMyb, CAF1p55 and Mip40 (Fig. 4.4 C). Interestingly, all four newly identified proteins have previously been shown to be subunits of a dMyb complex (Beall et al. 2002). Mip120, which is also a component of this complex, has not been identified in the Mass Spectrometry analysis. However, Western Blot using a Mip120-specific antibody revealed its presence in Superose 6 fractions also containing the RBF proteins (see Fig. 4.5). Furthermore, Mip120 co-immunoprecipitated with RBF1 and RBF2 (see Fig. 4.6 and 4.11). This strongly suggests that Mip120 is an integral subunit of dE2F2/RBF complexes but has been progressively lost or degraded during the purification procedure.

CAF1p55 is the *Drosophila* homolog of mammalian RbAp46 and RbAp48 that have originally been identified as pRb interacting proteins (Qian et al. 1993; Qian and Lee 1995). This, and the fact that all copurifying proteins were present in stoichiometric amounts in the silver staining (Fig. 4.4 C) – except of one stronger protein band, which comprises two proteins (dE2F2 and CAF1p55) – strongly suggest that these proteins form stable complexes in *Drosophila* embryos.

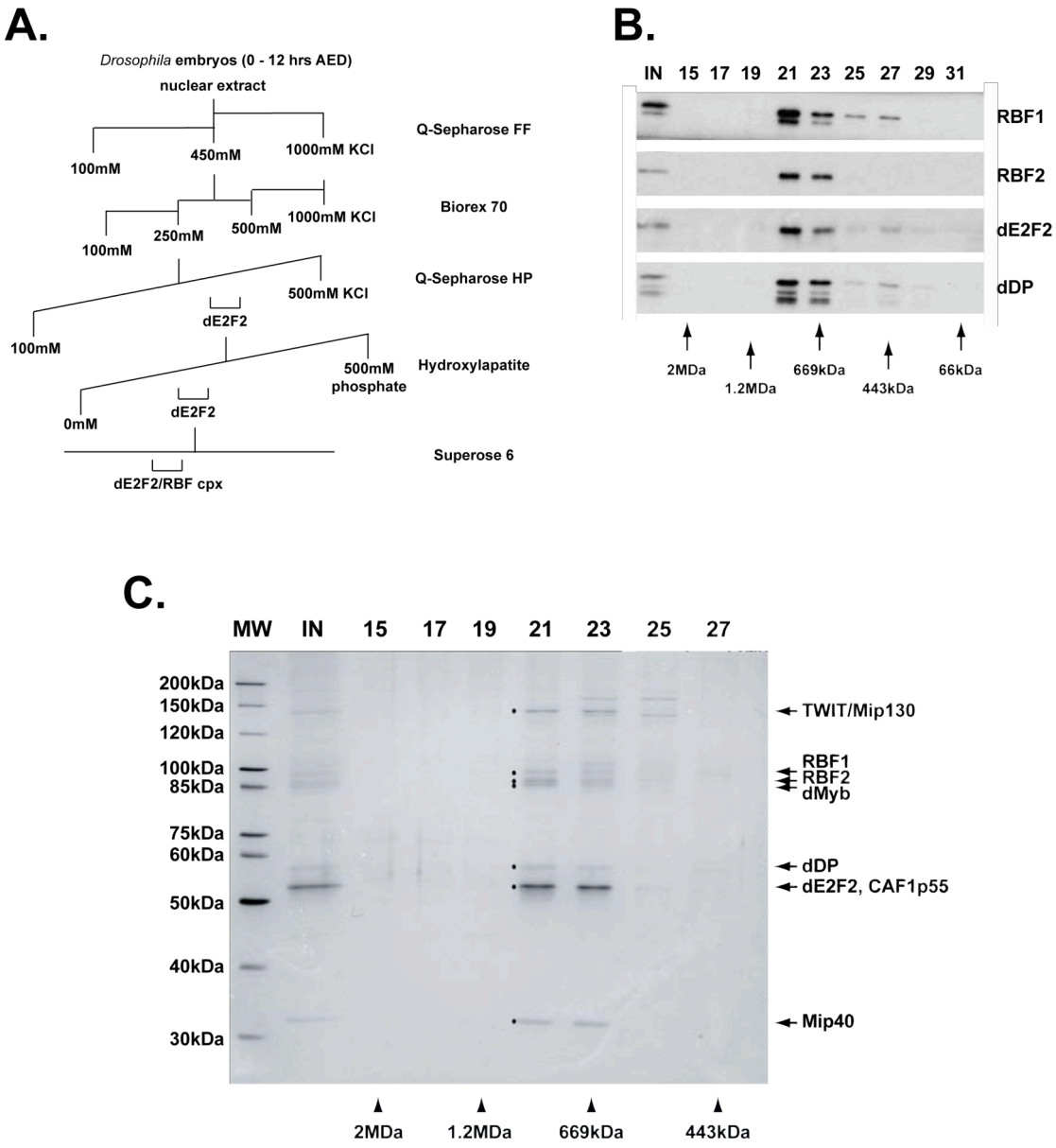


Figure 4.4: Purification of dE2F2/RBF complexes

(**A**) Purification scheme for dE2F2/RBF complexes. (**B**) Western Blot using specific antibodies as indicated after the final Superose 6 gel filtration column of the purification scheme. Fraction numbers are denoted on top, size standards on bottom. IN, input. (**C**) Silver staining of the eluted material after the final gel filtration column. Bands that were excised for Mass Spectrometry analysis are highlighted with black circles and the identified proteins are indicated on the right. Molecular weight markers are indicated on the left. Fraction numbers are denoted on top, size standards on bottom. MW, molecular weight marker; IN, input. This figure is taken from (Korenjak et al. 2004).

The fractions from the final Superose 6 gel filtration column were analyzed by Western Blot for the elution profile of the newly identified proteins. All proteins were present in high molecular weight fractions between 669 kDa and 1.2 MDa, confirming the results obtained by Mass Spectrometry (Fig. 4.5). Moreover, also Mip120, which has not been identified in the

original Mass Spectrometry approach, could readily be detected in the final fractions of the purification.

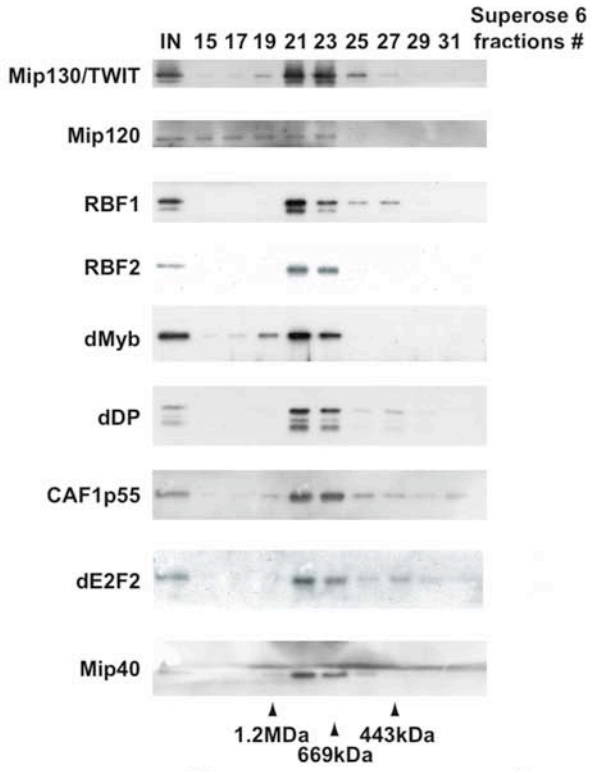


Figure 4.5: Copurification of putative dE2F2/RBF complex subunits

Western Blot of the final Superose 6 gel filtration column from the purification scheme for dE2F2/RBF complexes (Fig. 4.4 A) using the indicated antibodies. Fraction numbers are indicated on top, size standards on bottom. IN, input. This figure is taken from (Korenjak et al. 2004).

4.1.4 Co-immunoprecipitation of putative dE2F2/RBF complex subunits

In spite of the copurification experiments that strongly argue for the association of the identified proteins in dE2F2/RBF1 and dE2F2/RBF2 complexes, the possibility that distinct RBF- and dMyb-containing complexes have been copurified could not be ruled out with certainty. However, the size of the purified complex(es) and the lack of additional bands in the silver staining (Fig. 4.4 C) argue against this possibility.

In order to confirm that the identified proteins were associated in dE2F2/RBF1 and dE2F2/RBF2 complexes, co-immunoprecipitation experiments from Q Sepharose peak III (see Fig. 4.2) were carried out. RBF1 (DX3) and RBF2 (DR6) antibodies covalently coupled to protein G beads were used for precipitation. Western Blot analysis of the immunoprecipitated material revealed an association of Mip130/TWIT, Mip120, CAF1p55, Mip40, dE2F2 and dDP with RBF1 and RBF2 (Fig. 4.6 A, lanes 2 – 3, 6 – 7, 11 – 12) while they were not coprecipitated using a control antibody (Fig. 4.6 A, lanes 4, 8, 10). The RBF1 antibody did not

coprecipitate RBF2 and vice versa. These results strongly support the idea of distinct dE2F2/RBF1 and dE2F2/RBF2 complexes in *Drosophila* embryos that share the same subunit composition and only differ in their RBF subunit (Fig. 4.6 B).

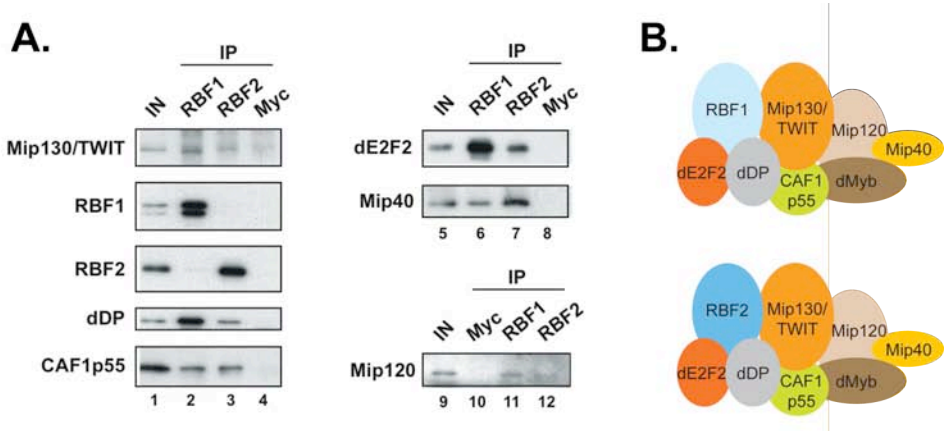


Figure 4.6: Interaction of dE2F2/RBF complex subunits

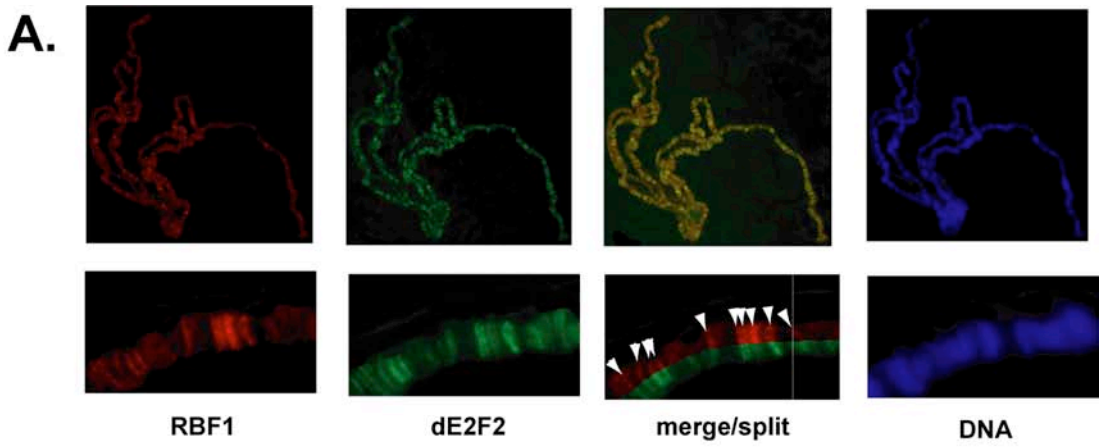
(**A**) RBF1 (DX3), RBF2 (DR6) and Myc (9E10, control) antibodies were covalently coupled to protein G beads and used for immunoprecipitation from Q Sepharose peak III fractions (see Fig. 4.2). Co-immunoprecipitations were performed in the presence of ethidium bromide to minimize DNA-mediated interactions. Western Blot using antibodies specific for the proteins indicated on the left. Antibodies used for immunoprecipitation are indicated on top. IN, input; IP, immunoprecipitation. This figure is taken from (Korenjak et al. 2004). (**B**) Schematic depiction of the two dE2F2/RBF complexes purified from 0 – 12 hr *Drosophila* embryo nuclear extract. The complexes only differ in their RBF subunit (RBF1 or RBF2), otherwise they share the same subunit composition.

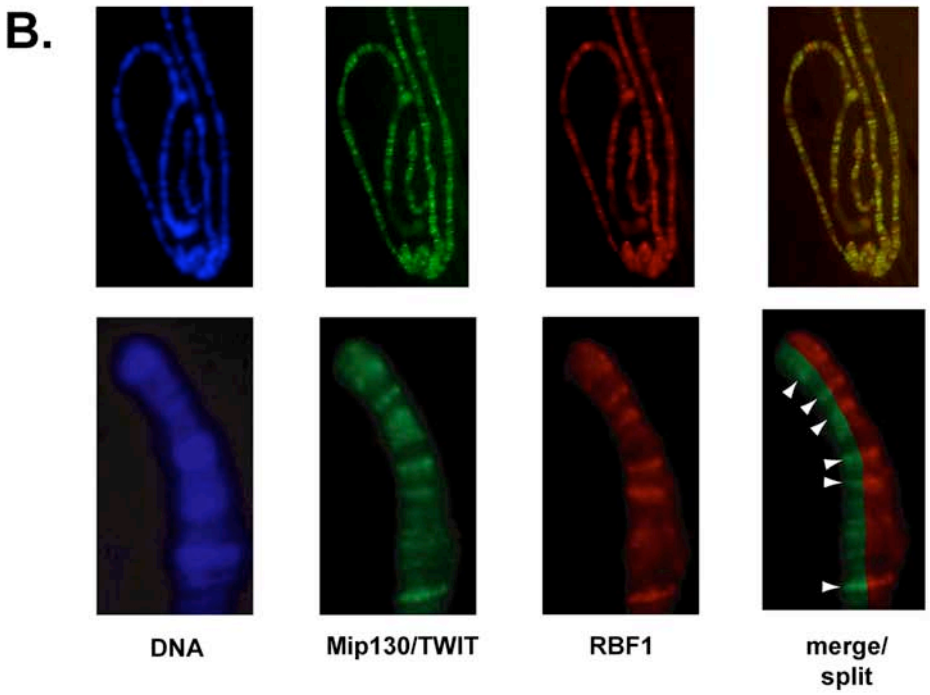
4.1.5 dE2F2/RBF complex subunits colocalize on *Drosophila* polytene chromosomes

Since dE2F2/RBF complexes contain several DNA-binding proteins (dE2F2/dDP, dMyb, Mip120) (Sawado et al. 1998; Beall et al. 2002) and the role of the RBF-dE2F network in transcriptional regulation is well established (Dimova et al. 2003) it seemed plausible to look for chromatin distribution of dE2F2/RBF complexes. *Drosophila* RBF1 and RBF2 proteins are known to be expressed not only in embryos, but also in larvae and adult flies (Stevaux et al. 2002; Keller et al. 2005). Moreover, *Drosophila* third instar larvae offer a unique possibility to study the *in vivo* distribution of chromatin-associated proteins on polytene chromosomes by indirect immunofluorescence.

Therefore, polytene chromosomes were stained with antibodies directed against different dE2F2/RBF complex subunits (Fig. 4.7). Costaining experiments revealed colocalization of

RBF1/dE2F2 (Fig. 4.7 A), RBF1/Mip130 (Fig. 4.7 B), RBF1/Mip120 (Fig. 4.7 C) and Mip130/Mip120 (Fig. 4.7 D). All proteins localized to numerous sites on the chromosomes. These results provide further support for the dE2F2/RBF complex purification and show that the complexes likely exist at different stages of *Drosophila* development.





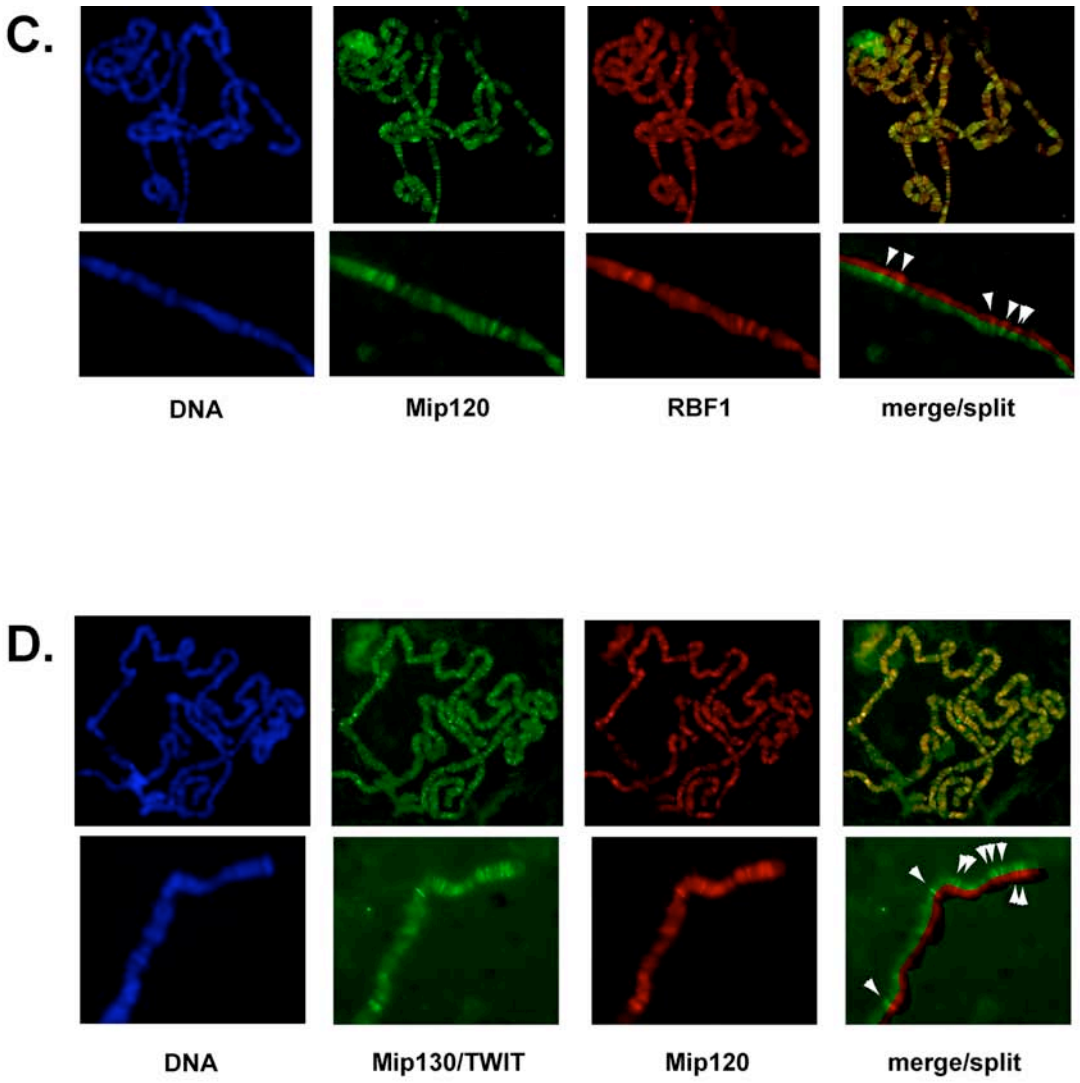


Figure 4.7: dE2F2/RBF complex subunits colocalize on polytene chromosomes

Immunostaining of *Drosophila* polytene chromosomes using antibodies directed against the indicated proteins. (**A**) RBF1/dE2F2, (**B**) RBF1/Mip130, (**C**) RBF1/Mip120 and (**D**) Mip130/Mip120 costainings. White arrowheads in the split image indicate colocalizing bands. DNA was counterstained using Hoechst. This figure is taken from (Korenjak et al. 2004).

4.1.6 dE2F2/RBF complexes localize to transcriptionally silent chromatin

dE2F2/RBF complexes bind to numerous sites on polytene chromosomes (see Fig. 4.7). Given the general role of RBF-containing complexes in transcriptional repression, it was tempting to speculate that dE2F2/RBF complexes would be associated with transcriptionally inactive chromatin.

In order to test this, *Drosophila* polytene chromosomes were costained using a Mip130/TWIT antibody and an antibody specifically recognizing the elongating form of RNA-Polymerase II (RNA-Pol II phosphorylated at Ser2) (Fig. 4.8). Costainings of RNA-Pol II and Polycomb (Pc), a well characterized transcriptional repressor, were performed as a control. As expected, no overlap between RNA-Pol II and Pc was observed (Fig. 4.8 A). Interestingly, the Mip130/RNA-Pol II costaining was also mutually exclusive, suggesting that dE2F2/RBF complexes localize to transcriptionally silent chromatin (Fig. 4.8 B). Furthermore, the parallel staining of Mip130/TWIT and Pc revealed binding of distinct regions of inactive chromatin (Fig. 4.8 C), suggesting that dE2F2/RBF complexes are associated with specific transcriptionally silent regions.

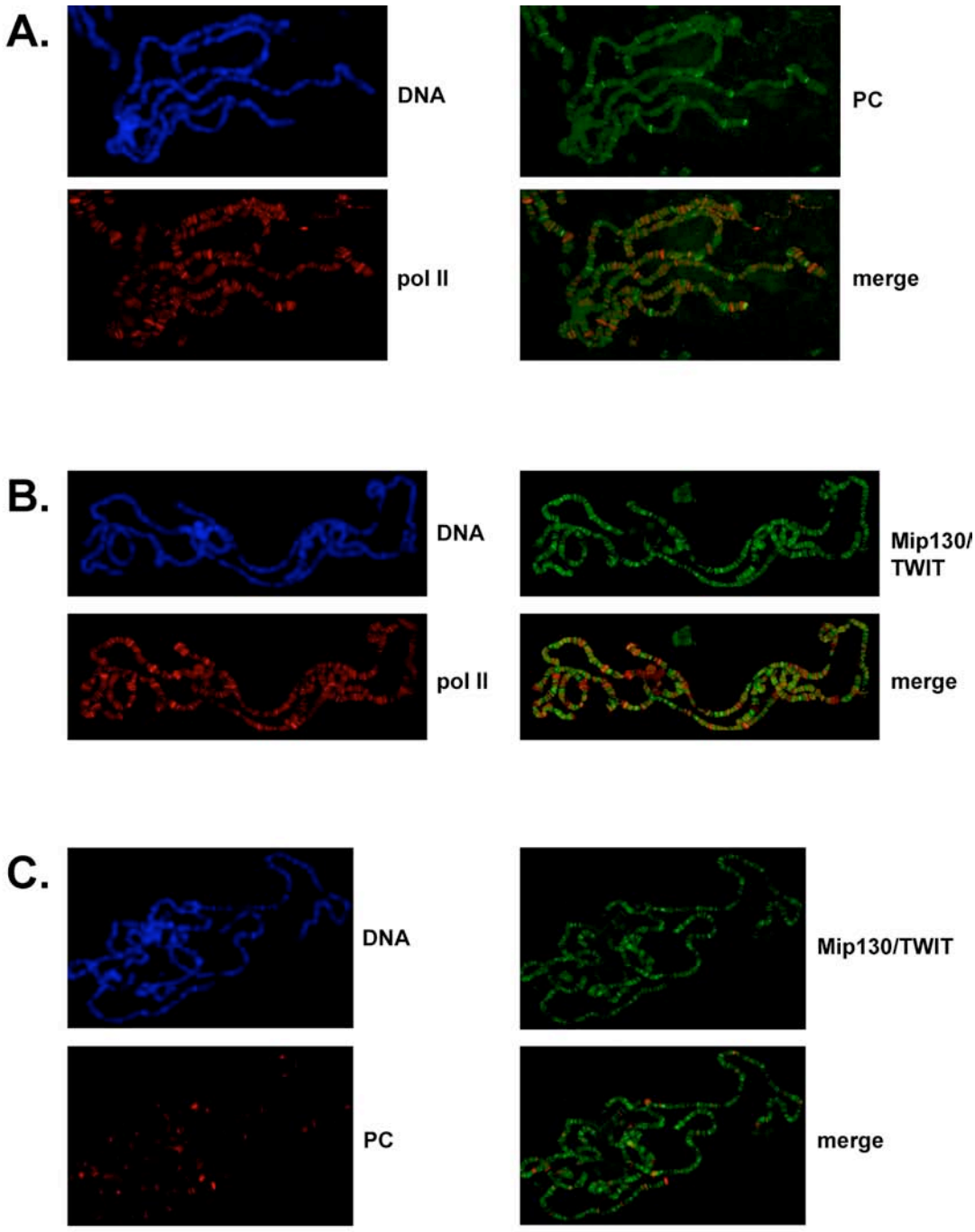


Figure 4.8: dE2F2/RBF complexes bind to transcriptionally inactive chromatin

Immunostaining of *Drosophila* polytene chromosomes using antibodies directed against the indicated proteins. (**A**) Pc/RNA-Pol II, (**B**) Mip130/RNA-Pol II and (**C**) Mip130/Pc costainings. DNA was counterstained using Hoechst. The figure is taken from (Korenjak et al. 2004).

4.1.7 dE2F2/RBF complexes bind to deacetylated histone tails *in vitro*

In a chromatin context, transcriptionally inactive regions have been shown to be enriched in specific histone tail modifications, whereas others are excluded from these regions. These specific patterns of histone modification provide a platform for chromatin binding proteins and, as a consequence, could be important for the formation of higher order chromatin structures typical for silent genomic regions. In general, silent chromatin has a high proportion of hypoacetylated histone tails, whereas histones in active chromatin are hyperacetylated. Furthermore, the methylation of specific lysine (K) residues in histone tails is another well characterized modification affecting the establishment of either active or inactive chromatin. While histone H3K4-methylation is a hallmark of transcriptionally active regions, H3K9-, H3K27- and H4K20-methylation are associated with silent chromatin in metazoans.

To test whether dE2F2/RBF complexes had a specificity for modified histone tails, binding experiments were performed using the partially purified complexes and differentially modified histone tail-peptides coupled to beads. First, the impact of methylation of histone tails on the binding of the complexes was analyzed (Fig. 4.9).

Chromatography fractions containing Heterochromatin Protein 1 (HP1) were used as a positive control for the pulldown experiment. HP1 is known to specifically bind to H3-peptides methylated at K9 (Bannister et al. 2001; Lachner et al. 2001). Accordingly, it was found strongly associated with the H3K9-methylated peptide and to a lesser extent with the unmodified H3-peptide (Fig. 4.9 A, compare lanes 4 + 6). HP1 did not interact with the H3K4-methylated peptide (lane 5). Hence, the binding profile of HP1 reflects its established binding properties, showing that the histone tail-peptide pulldown has worked properly.

Fractions containing monomeric RBF1 were used as a negative control for histone tail-association. Western Blot analysis revealed that monomeric RBF1 did not bind to any of the peptides (Fig. 4.9 B).

When RBF1 was assembled into the dE2F2/RBF1 complex, histone tail-binding was detected, as analyzed by Western Blot of the bound material (Fig. 4.9 C, upper panel). This interaction was further confirmed by Western Blot using an antibody directed against the Mip130/TWIT protein (Fig. 4.9 C, lower panel). However, only binding to unmodified H3 (aa 1 – 20)- and H4 (9 – 35)-peptides was observed, whereas no association to the unmodified H3 (21 – 34)-peptide was detected (compare lanes 2, 4 + 7). This difference in binding, regardless of the methylation status, might be due to the different number of charged amino acids in the peptides. The H3 (21 – 34)-peptide contains the lowest number of charged amino acid residues, suggesting that the observed binding difference is due to a charge effect.

The methylation status of the peptides did not result in a significant change in the binding efficiency of RBF1 and Mip130/TWIT compared to the unmodified peptides (Fig. 4.9 C).
These results suggest that the dimethylation of distinct residues in histone tails, implicated in transcriptional activation/repression, is not important for the binding of dE2F2/RBF complexes *in vitro*. Rather, the overall charge of the histone tails could be involved in the binding of dE2F2/RBF complexes to histones.

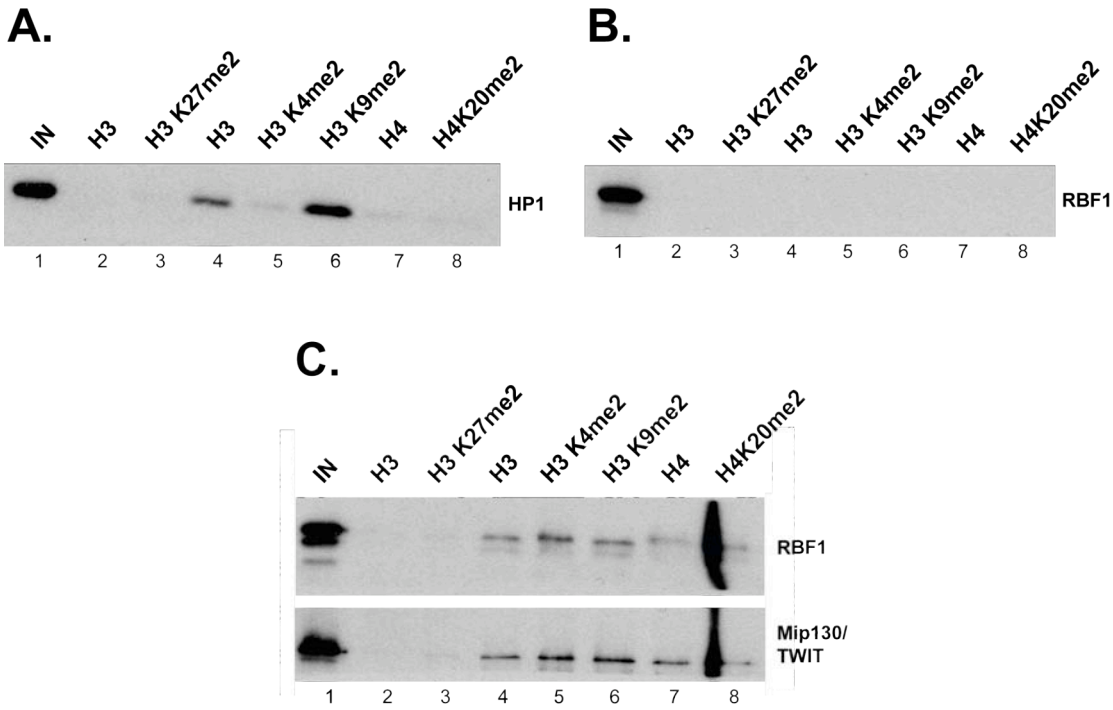


Figure 4.9: Binding of dE2F2/RBF complexes to histone tails *in vitro*
Partially purified fractions containing HP1 (**A**), monomeric RBF1 (**B**) or dE2F2/RBF complexes (**C**) were used for pulldown experiments with differentially modified histone tail-peptides coupled to beads. The bound material was analyzed by Western Blot using antibodies directed against the proteins indicated on the right. Histone tail-peptides are indicated on top. H3 + H3K27me2 (lanes 2 + 3) comprised aa 21 – 34. H3, H3K4me2 + H3K9me2 (lanes 4 – 6) comprised aa 1 – 20. H4 + H4K20me2 (lanes 7 + 8) comprised aa 9 – 35. IN, input.

In contrast to methylation, modification of histone tails by acetylation results in the neutralization of charged amino acid residues. The histone H4 tail is known to be highly modified by acetylation of lysine residues 5, 8, 12 and 16 *in vivo*. Therefore, binding of dE2F2/RBF complexes to unmodified and tetra-acetylated H4 tails was assessed by pulldown experiments (Fig. 4.10). Partially purified fractions containing dE2F2/RBF complexes, dE2F1/RBF1 complex or monomeric RBF1 were used as input material. dE2F2/RBF complexes specifically associated with unmodified H4 tails, whereas no binding to tetra-

acetylated tails was observed, as assessed by Western Blot using antibodies directed against Mip130/TWIT, RBF1 and RBF2 (Fig. 4.10, lanes 10 + 11). The dE2F1/RBF1 complex did neither bind to unmodified nor to tetra-acetylated H4 tails (Fig. 4.10, lanes 6 + 7), whereas monomeric RBF1 showed weak binding to tetra-acetylated H4 tails and did not bind to unmodified tails (Fig. 4.10, lanes 2 + 3).

The specific binding of dE2F2/RBF complexes to deacetylated H4 tails, which are a hallmark of transcriptionally inactive chromatin, is in agreement with a putative role of these complexes in transcriptional repression. With respect to the results presented above, the exact binding mechanism is likely to involve charge effects. However, specific binding of one or more complex subunits to deacetylated H4 tails, which might become sterically impossible upon acetylation, is possible.

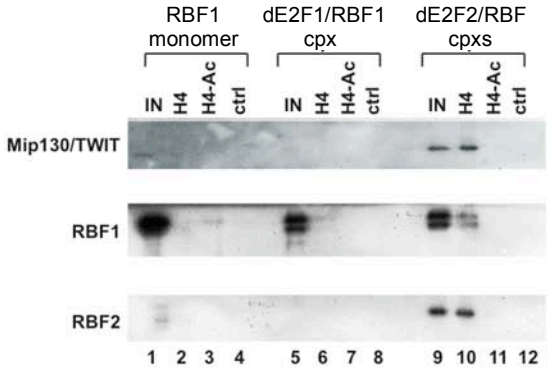


Figure 4.10: dE2F2/RBF complexes specifically bind to deacetylated H4 tails *in vitro*

Partially purified fractions containing dE2F2/RBF complexes, dE2F1/RBF1 complex or monomeric RBF1 were used for pulldown experiments with unmodified or tetra-acetylated histone H4 tail-peptides immobilized on beads. Beads without bound peptide were used as a control (ctrl). The bound material was analyzed by Western Blot using antibodies directed against Mip130/TWIT, RBF1 and RBF2, as indicated. Input material and histone tail-peptides are indicated on top. H4 tail-peptides comprised aa 1 – 20. IN, input; H4-Ac, tetra-acetylated H4 tail; H4, unmodified H4 tail. This figure is taken from (Korenjak et al. 2004).

4.1.8 dE2F2/RBF complexes repress a specific set of dE2F target genes

Several lines of evidence suggest an involvement of dE2F2/RBF complexes in transcriptional repression. The RBF-dE2F transcriptional network and dE2F target genes have been characterized in detail in S2 cells and target genes have been classified into five groups due to their dependence on different RBF and dE2F proteins (Dimova et al. 2003). Among these groups, the so-called A group genes comprise well characterized E2F target genes, implicated in cell cycle progression. These genes are regulated in a cell cycle-dependent

manner and they are activated by dE2F1, whereas RBF1 represses their expression. E group genes, on the other hand, are negatively regulated by dE2F2 and the redundant action of RBF1 and RBF2. The results presented above, showing that the purified dE2F2/RBF complexes share the same subunit composition and only differ in the RBF factor, makes these complexes interesting candidates for the regulation of E group genes. E group genes are stably repressed in somatic, cycling *Drosophila* S2 cells. Many of them are specifically expressed in the germ line.

In collaboration with the group of Nick Dyson (MGH Cancer Center, Boston), attempts were undertaken to identify genes that are regulated by dE2F2/RBF complexes. In order to confirm that the complexes also exist in S2 cells, immunoprecipitation experiments were performed on extracts derived from cells stably expressing Flag-tagged RBF1 or RBF2 (Fig. 4.11). The known subunits of the complexes coprecipitated with RBF1 and RBF2, suggesting that dE2F2/RBF complexes also exist in S2 cells. As expected, dE2F1 coprecipitated only with RBF1, whereas histone H3 and HP1 interacted with neither RBF1 nor RBF2. Co-immunoprecipitations were performed in the absence and presence of ethidium bromide to rule out unspecific interactions mediated by DNA.

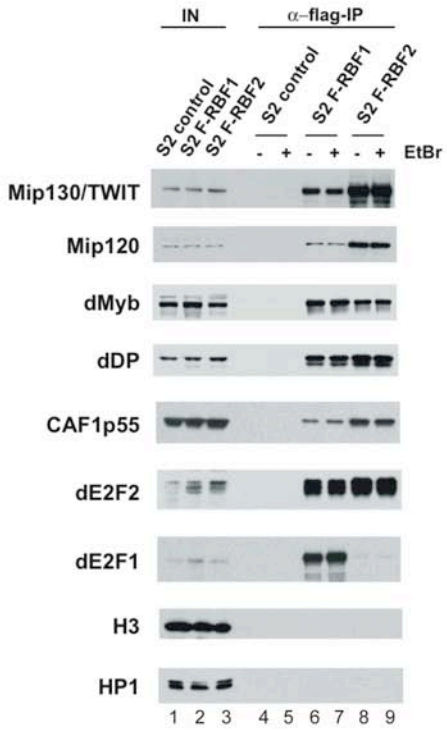


Figure 4.11: dE2F2/RBF complexes exist in *Drosophila* S2 cells

αFlag M2 Agarose beads were used for immunoprecipitation from extracts derived from cells stably expressing Flag-tagged RBF1, RBF2 or a control cell line. Western Blot using antibodies specific for the proteins indicated on the left. Cell extracts from which the precipitation was performed are denoted on top. Immunoprecipitations were carried out in the absence or presence of ethidium bromide (EtBr), as indicated. IN, input; IP, immunoprecipitation. The experiment was carried out by N. Dyson and U. Binne. The figure is taken from (Korenjak et al. 2004).

A possible regulation of A and E group genes by dE2F2/RBF complexes was investigated using RNAi mediated knockdown of individual subunits in S2 cells followed by expression analysis of A and E group genes by Northern Blot (Fig. 4.12 A). Knockdown of Mip130/TWIT, Mip120 or dE2F2 did not affect the expression of selected A group genes (lanes 2, 4, 6). Only downregulation of RBF1 resulted in a strong derepression of these genes (lane 3), which is in agreement with previous work (Dimova et al. 2003). These results suggest that A group genes are not regulated by dE2F2/RBF complexes but by (a) distinct RBF1-containing complex(es). Strikingly, knockdown of Mip130/TWIT and Mip120 led to a massive derepression of E group genes comparable to the known effect of dE2F2 downregulation (compare lanes 2 + 6 to lane 4). RBF1 RNAi did not result in upregulation of E group genes (lane 3). This can be explained by the redundant function of RBF1 and RBF2 on this group of genes (Dimova et al. 2003). Taken together, this suggests that dE2F2/RBF1 and dE2F2/RBF2 complexes regulate E group genes in a redundant manner, whereas they do not affect A group gene expression.

The efficiency of RNAi mediated knockdown was controlled by Western Blot (Fig. 4.12 B). Interestingly, downregulation of Mip130/TWIT resulted in a simultaneous decline of dE2F2 protein levels and vice versa (lanes 2 + 4). Such an effect can sometimes be observed for interacting proteins and could be due to destabilization of a protein as a result of the loss of its binding partner.

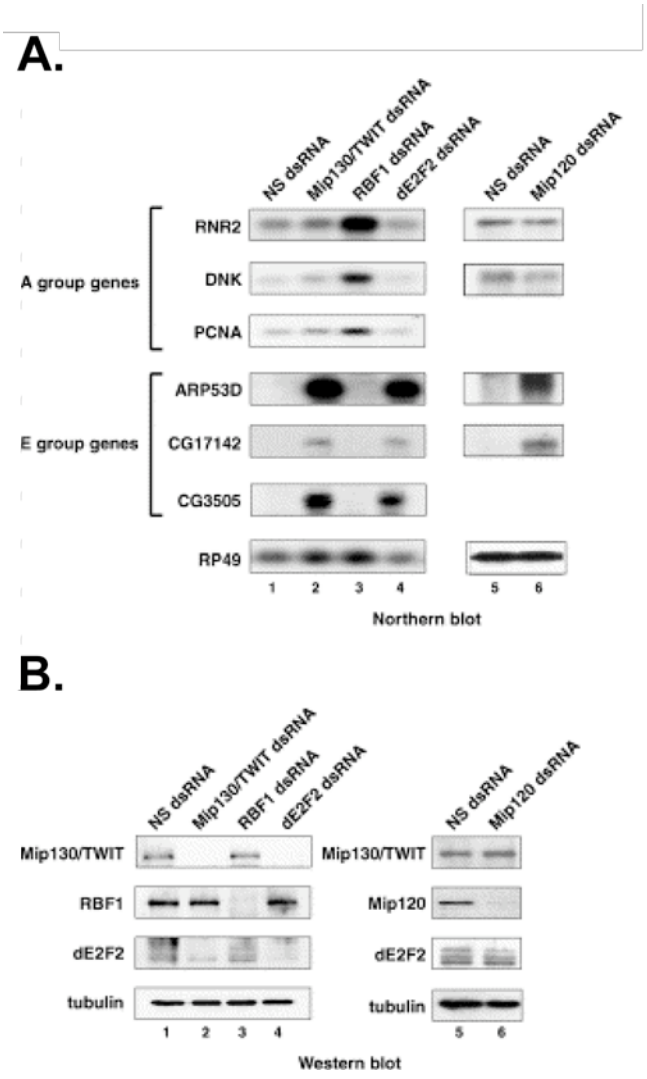


Figure 4.12: Mip130/TWIT and Mip120 specifically repress E group genes

(**A**) Expression of A (*rnr2*, *dnk*, *pcna*) and E group genes (*arp53D, CG17142*, *CG3505*) and one control gene (*rp49*) was monitored by Northern Blot in *Drosophila* S2 cells. dsRNAs used for depletion of the respective proteins are denoted on top, probes used for Northern Blot are indicated on the left. NS dsRNA, nonspecific dsRNA (luciferase) (**B**) Western Blot control for the efficiency of the RNAi mediated knockdown. dsRNAs used for knockdown of the respective proteins are denoted on top, specific antibodies are indicated on the left. The experiment was carried out by N. Dyson and B. Taylor-Harding. The figure is taken from (Korenjak et al. 2004).

In agreement with the role of dE2F2/RBF complexes in the regulation of E group gene repression, also CAF1p55 is essential for the stable repression of these genes in S2 cells (Taylor-Harding et al. 2004). However, RNAi mediated knockdown of dMyb, another subunit of the complexes, did not result in the upregulation of E group genes (Fig. 4.13 A, left panel). Furthermore, the simultaneous downregulation of dMyb and dE2F2 did not enhance the deregulation of E group genes observed by knockdown of dE2F2 alone (Fig. 4.13 A, right panel). Western Blot analysis confirmed the efficiency of RNA interference (Fig. 4.13 B).

These results show that even though dMyb is an integral component of dE2F2/RBF complexes, it is the only subunit that is not required for the transcriptional repression mediated by the complexes. This could be due to an important role of the other complex subunits in keeping the integrity of the complexes, whereas dMyb could be dispensable for this purpose. Alternatively, in contrast to the other complex subunits dMyb might be involved in the activation of E group genes in specific developmental situations, therefore having no effect when these genes are repressed by the other subunits of the complexes.

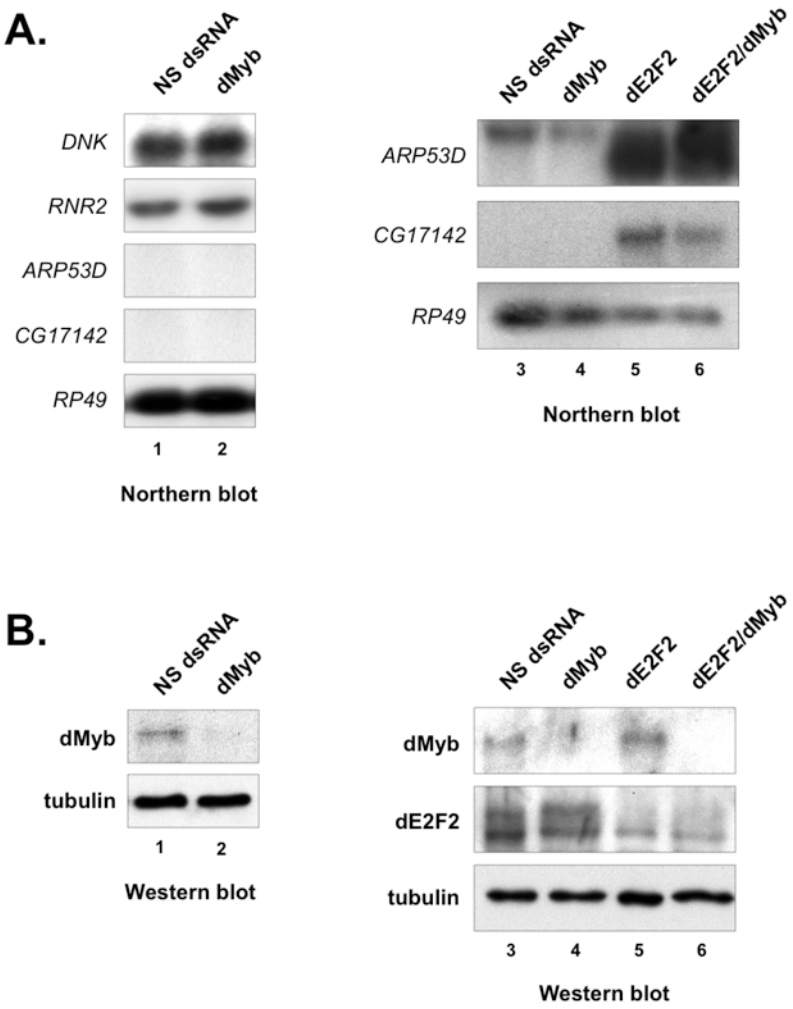


Figure 4.13: dMyb is dispensable for repression of E group genes

(**A**) Expression of different A (*dnk, rnr2*) and E group genes (*arp53D, CG17142*) and one control gene (*rp49*) was monitored by Northern Blot in *Drosophila* S2 cells. dsRNAs used for knockdown of the respective proteins are denoted on top, probes used for Northern Blot are indicated on the left. NS dsRNA, nonspecific dsRNA (luciferase) (**B**) Western Blot control for the efficiency of the RNAi mediated knockdown. dsRNAs used for knockdown of the respective proteins are denoted on top, specific antibodies are indicated on the left. The experiment was carried out by N. Dyson and B. Taylor-Harding. The figure is taken from (Korenjak et al. 2004).

4.1.9 Mip130/TWIT is bound to E group genes *in vivo*

In order to show an association of dE2F2/RBF complexes with promoters of E group genes, Chromatin immunoprecipitation (ChIP) experiments were carried out using antibodies directed against Mip130/TWIT and dE2F2 (Fig. 4.14). Both proteins were specifically found to associate with the promoter regions of *arp53D* and *CG17142*, whereas the *rp49* control gene was not bound (lanes 3 + 4). A control antibody did not precipitate *arp53D* and *CG17142*

(lane 2). Hence, this supports the notion that dE2F2/RBF complexes are physically associated with E group genes in S2 cells.

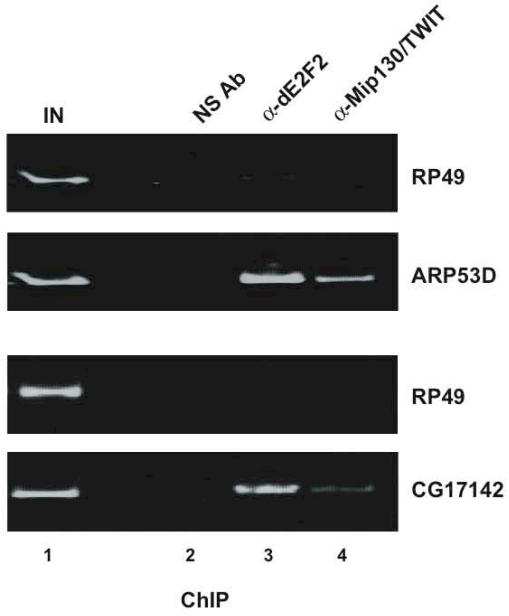


Figure 4.14: Mip130/TWIT and dE2F2 are associated with E group gene promoters
ChIP was performed from *Drosophila* S2 cell extracts using antibodies directed against Mip130/TWIT and dE2F2. The precipitated chromatin was used as a template for PCR reactions using primers for the promoter regions of the E group genes *arp53D* and *CG17142* and the control gene *rp49*. Antibodies for IP are indicated on top, specific primers on the right. IN, input genomic DNA; NS Ab, nonspecific antibody. The experiment was carried out by N. Dyson and B. Taylor-Harding. The figure is taken from (Korenjak et al. 2004).

4.1.10 *Caenorhabditis elegans* homologs of dE2F2/RBF complex subunits interact genetically

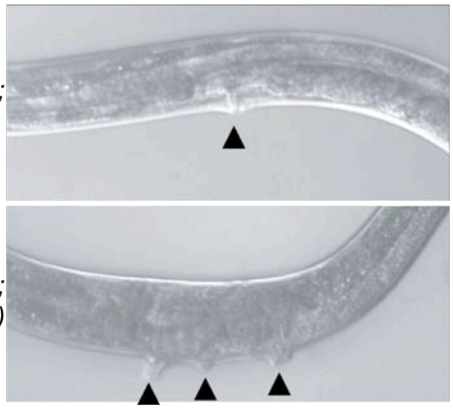
An interesting feature of dE2F2/RBF complexes is the high degree of conservation of their subunits between different species. Homologs of RBF1/2, dE2F2, dDP, Mip130/TWIT and CAF1p55 have been identified in the nematode worm *C. elegans*. Strikingly, all of these proteins act as so called class B synthetic multivulva (synMuv) genes. The synMuv pathway consists of three classes of genes (A, B and C) and is involved in antagonizing vulval differentiation in *C. elegans* (see introduction). SynMuv genes can be identified by a *multivulva* phenotype, which is the result of a combination of at least two mutant alleles, which belong to different classes of synMuv genes.

To test whether in worms the homologs of Mip120 and dMyb were also acting as class B synMuv genes, RNAi experiments were performed to functionally knockdown these subunits

in a class A synMuv mutant background. These experiments were done in collaboration with the group of Nick Dyson (MGH Cancer Center, Boston).

Knockdown of the Mip120 homolog, JC8.6, by feeding RNAi resulted in a highly penetrant *multivulva* phenotype (Fig. 4.15 A). This phenotype was comparable to the ones produced by inactivation of the established class B synMuv genes *lin-9* and *lin-35*, the *C. elegans* homologs of Mip130/TWIT and RBF1/2, respectively (Fig. 4.15 B). Interestingly, another study has also identified JC8.6 as a synMuv gene, supporting the results presented in this work (Owen et al. 2003). Inactivation of the putative dMyb homolog GEI-11 resulted in only few progeny, suggesting that GEI-11 is important for early steps of embryogenesis, and the survivors displayed normal vulvae. However, the similarity between dMyb and GEI-11 is restricted to tandemly arranged SANT domains, which are found in many nuclear proteins with diverse functions (Aasland et al. 1996). It is therefore unclear whether GEI-11 represents a homolog of dMyb.

A.



B.

Genotype	avg. % Muv	+/- S.E.
lin-15A(n767); lin-9(RNAi)	*69.0*	*5.77*
lin-15A(n767); lin-35(RNAi)	*99.7*	*0.33*
lin-15A(n767); JC8.6(RNAi)	*94.3*	*1.77*
lin-15A(n767); odr-10(RNAi)	*0*	*0*

Figure 4.15: The *C. elegans* Mip120 homolog acts as a synMuv class B gene

(**A**) Feeding RNAi using gfp (control) or JC8.6 constructs in lin-15 (n767) synMuv class A mutant background animals. Black arrowheads indicate vulva-like structures. (**B**) Statistic analysis of the frequency of multiple vulva-like structures in lin-15 (n767) mutant animals depleted for lin-9, lin-35, JC8.6 or odr-10 (control) by RNAi. avg. % Muv, average percentage of animals with multiple vulvae from three independent experiments; +/- SE, standard errors. The experiment was carried out by N. Dyson and J. Satterlee. The figure is taken from (Korenjak et al. 2004).

A Mip40 homolog could not be identified in *C. elegans* by a conventional BLAST search. In collaboration with Rein Aasland (University of Bergen, Norway), a sequence profile was created from Mip40 and different vertebrate homologs. A subsequent profile search resulted in the identification of *C. elegans* LIN-37 and a related protein in *C.briggsiae*. The similarity with Mip40 is restricted to three segments (A, B and C) within the proteins and it is not very high, which might explain why it was not picked up in the conventional BLAST search (Fig. 4.16). Strikingly, *lin-37* has previously been shown to act as a class B synMuv gene and its protein product has been identified as a LIN-53 interaction partner (Walhout et al. 2000). LIN-53 is the *C. elegans* homolog of CAF1p55.

Taken together, these results show that the *C. elegans* homologs of dE2F2/RBF complex subunits interact genetically. Furthermore, the *Drosophila* complex purification supports the hypothesis that synMuv gene products might act together in transcriptional repressor complexes (Ceol and Horvitz 2001). It seems likely that complexes similar to dE2F2/RBF also exist in the worm.

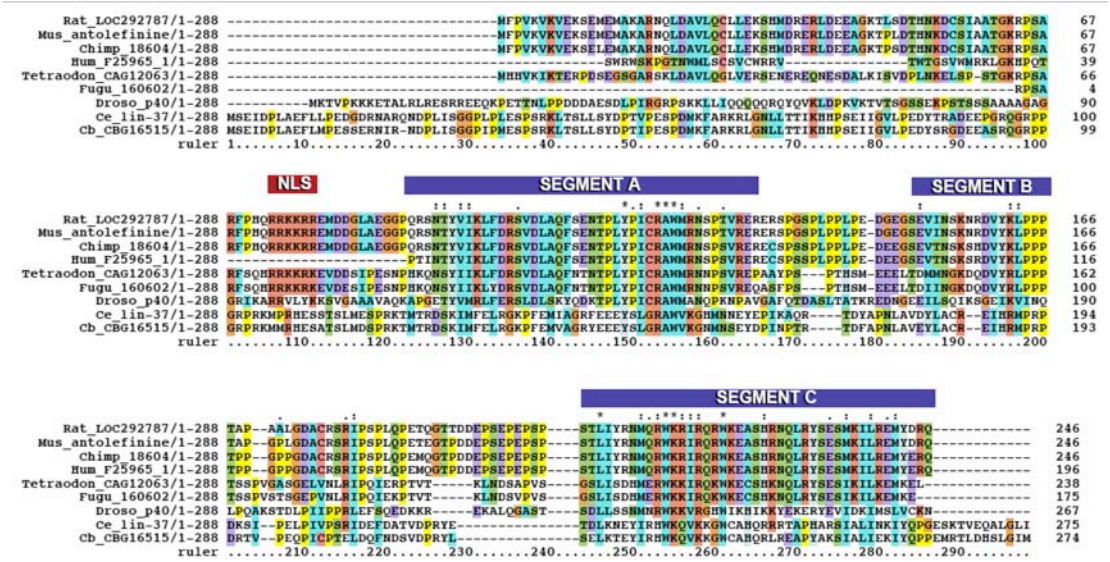


Figure 4.16: LIN-37 is the *C. elegans* homolog of Mip40

Color-coded multiple sequence alignments of Mip40-related and LIN-37 proteins. A putative nuclear localization sequence in the vertebrate sequences (NLS) and three segments of similarity (A, B and C) are shown. The figure is taken from (Korenjak et al. 2004).

4.2 Retinoblastoma complexes in the human system

The conservation of dE2F2/RBF complexes between *Drosophila* and *C. elegans* raised the question whether such complexes also existed in higher organisms. Strikingly, homologs of all subunits exist in the human genome. Whereas some of the human proteins are well characterized transcriptional regulators (pocket proteins, E2F/DP factors, B-Myb, RbAp46 and RbAp48), others are not characterized, and the genes exist only as predicted open reading frames (hMip130/TWIT, hMip120, hMip40).

4.2.1 Generation of antibodies directed against hMip130/TWIT, hMip120 and hMip40

In order to be able to biochemically characterize dE2F2/RBF complex subunit homologs in human cells, peptide antibodies were raised against hMip130/TWIT, hMip120 and hMip40. The specificity of the antibodies was tested by Western Blot using extracts derived from transiently transfected cells and *in vitro* translated proteins. The antibody directed against hMip130/TWIT gave rise to four bands when HEK 293 extract was subjected to Western Blot analysis (Fig. 4.17 A). One of the proteins recognized migrated with a molecular weight of approximately 65 kDa, which is close to the calculated molecular weight of hMip130/TWIT (64 kDa) (lane 1, black arrow). This band increased in intensity when extract derived from HEK 293 cells transfected with a hMip130/TWIT-expression vector was analyzed (lane 2, black arrow). Expression of Flag-tagged hMip130/TWIT resulted in the detection of an additional band, which migrated just above the 65 kDa band (lane 3, black arrowhead). Furthermore, the antibody specifically recognized *in vitro* translated untagged (black arrow) and Flag-tagged (black arrowhead) hMip130/TWIT, whereas an unrelated protein was not detected (lanes 4 – 6).

The specificity of the hMip40 and hMip120 antibodies was assessed in a similar manner. The antibody raised against hMip40 specifically recognized a protein of the expected size in the transfection and *in vitro* translation experiments (Fig. 4.17 B, lanes 2 + 4, black arrowhead). Two cDNAs encoding hMip120 proteins with a molecular weight of 60 kDa (from hereon referred to as hMip120 short form (hMip120sf)) and 90 kDa (from hereon referred to as hMip120 long form (hMip120lf)) have been reported. For these experiments only hMip120sf was available. The hMip120 antibody detected a specific band in the *in vitro* translation experiment (Fig. 4.17 C, lane 4, black arrowhead), but no specific signal was recognized in the transfection experiment (Fig. 4.17 C, lane 2). The corresponding protein product might be

highly unstable, which could be an explanation for the lack of detection of the protein in the transfection experiment. But since nothing is known about the biochemical properties of hMip120 this remains speculation. Moreover, the antibody cross-reacted with several polypeptides from the cell extract (Fig. 4.17 C, lanes 1 + 2).

Taken together, these results suggest that antibodies specifically recognizing hMip130/TWIT and hMip40 have been generated that can be used for detection of these proteins in human cell extracts. The hMip120 antibody, however, could not reliably be used for further studies.

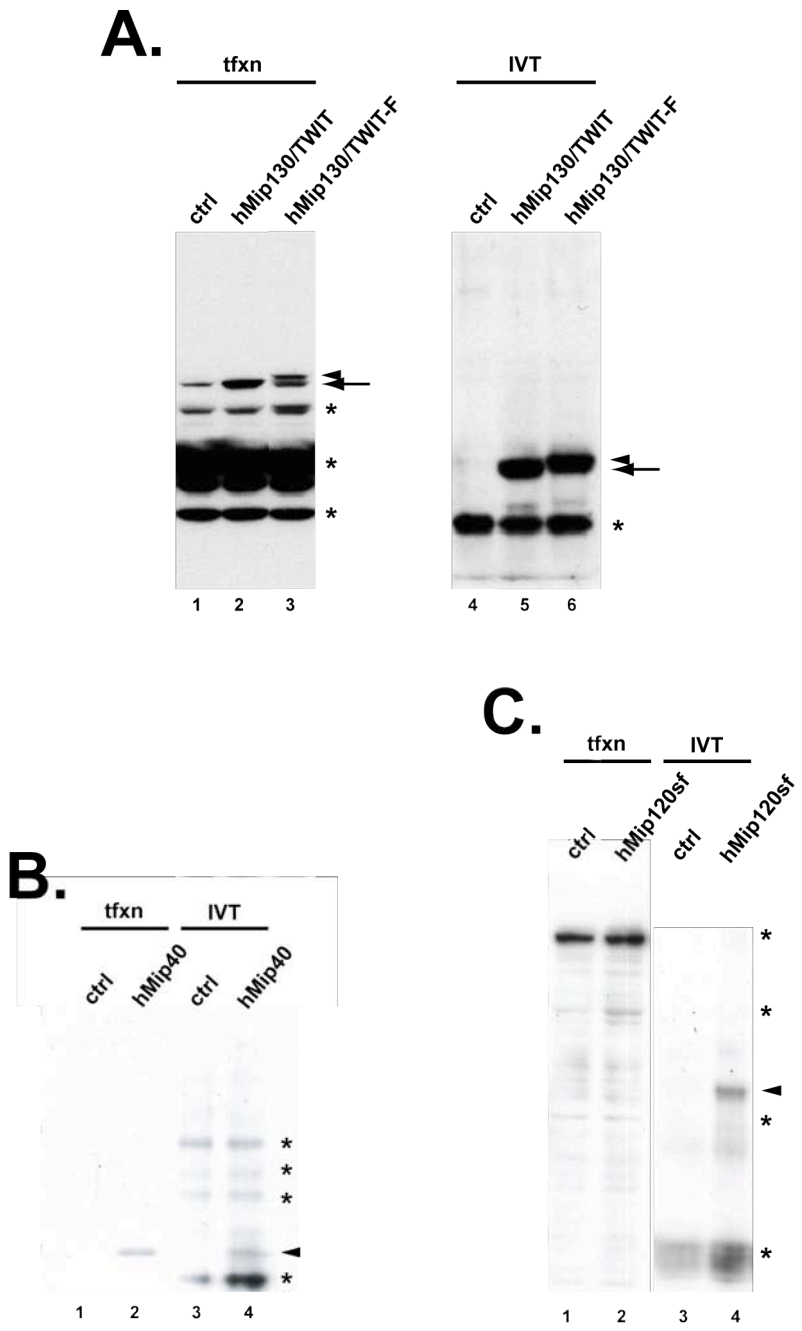


Figure 4.17: Tests for assaying the specificity of antibodies raised against hMip130/TWIT, hMip40 and hMip120

(**A**) Western Blot using the antibody directed against hMip130/TWIT of extracts derived from transiently transfected HEK 293 cells (lanes 1 – 3) or *in vitro* translated proteins (lanes 4 – 6). (**B**) Western Blot using the antibody directed against hMip40 of extracts derived from transiently transfected HEK 293 cells (lanes 1 – 2) or *in vitro* translated proteins (lanes 3 – 4). (**C**) Western Blot using the antibody directed against hMip120 of extracts derived from transiently transfected HEK 293 cells (lanes 1 – 2) or *in vitro* translated proteins (lanes 3 – 4). Transiently expressed or *in vitro* translated proteins are denoted on top. hMip120sf, hMip120 short form; ctrl, control; tfxn, transfection; IVT, *in vitro* translation. Black arrows and arrowheads indicate specific Western Blot signals. Asterisks indicate cross-reacting polypeptides.

4.2.2 Human homologs of dE2F2/RBF complex subunits interact in HEK 293 cells

Interactions between pRb and RbAp46 and RbAp48 (Qian et al. 1993; Qian and Lee 1995), as well as an association of p107 and p130 with B-Myb (Joaquin et al. 2002) have been reported previously. These data provide a first hint that complexes homologous to dE2F2/RBF might also exist in human cells.

To further test whether the human proteins could interact, a Flag-tagged version of hMip40 was overexpressed in HEK 293 cells together with different human homologs of the *Drosophila* complex subunits. Interaction of the cotransfected proteins was tested by immunoprecipitation with an αFlag-antibody and verified by Western Blot. hMip40-F efficiently co-immunoprecipitated B-Myb, hMip130/TWIT and RbAp48 (Fig. 4.18, lanes 4, 8, 12). The multiple bands detected in the hMip40-F Western Blot are probably due to degradation of the protein.

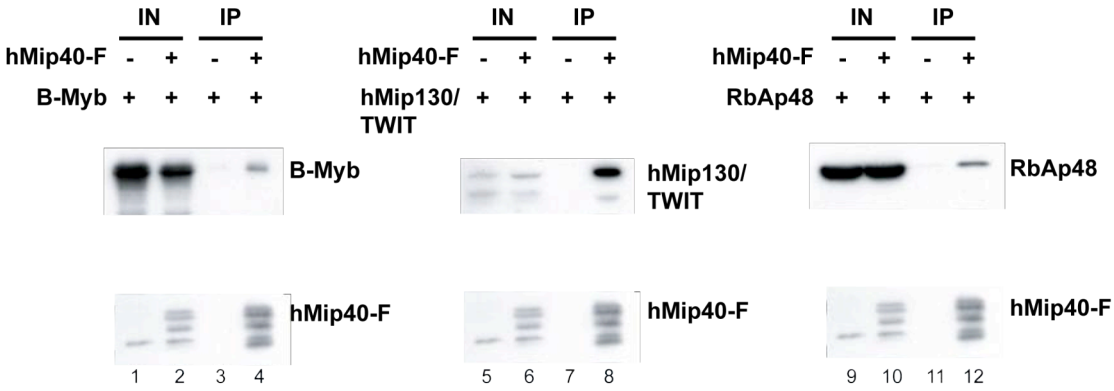


Figure 4.18: B-Myb, hMip130/TWIT and RbAp48 interact with hMip40 in HEK 293 cells

HEK 293 cells were transfected with expression plasmids for hMip40-F and either B-Myb (lanes 1 – 4), hMip130/TWIT (lanes 5 – 8) or RbAp48 (lanes 9 – 12), as indicated. Transfected plasmids are denoted on top, proteins analyzed by Western Blot on the right. hMip40 was detected using an αFlag-antibody. IN, input; IP, immunoprecipitation.

The interactions presented above comprise human homologs of proteins that have originally been identified as subunits of the dMyb-complex (Beall et al. 2002). In the next step, interactions between hMip40-F and the human pocket proteins were investigated using the same co-immunoprecipitation approach. Unexpectedly, none of the pocket proteins was found associated with hMip40, even though the precipitation of the Flag-tagged protein worked efficiently (Fig. 4.19, lanes 4, 8, 12). The pRb signal detected in the precipitated material is due to unspecific binding, since a signal of the same intensity also appeared in the control lane, where no Flag-tagged protein was present.

Several possibilities could account for the lack of interaction between hMip40 and pocket proteins in HEK 293 cells. One reason could be a general lack of this interaction in human cells. Alternatively, the association could be cell type specific. HEK 293 cells are virally transformed and express the potent viral oncoproteins Ad5-E1A and SV40-T, both of which have been shown to be able to bind the pocket domain. This results in the disruption of the interaction between pocket proteins and cellular targets (e.g. E2F/DP). It is also conceivable that the interaction between hMip40 and pocket proteins is not direct and in the cotransfection assay a bridging factor might have been missing or underrepresented compared to the overexpressed proteins.

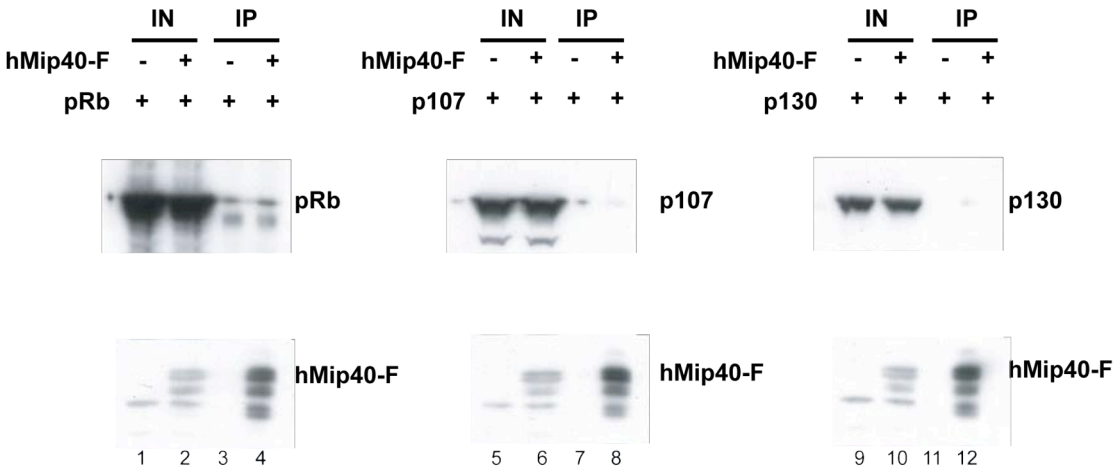


Figure 4.19: Pocket proteins do not interact with hMip40 in HEK 293 cells

HEK 293 cells were transfected with expression plasmids for hMip40-F and either pRb (lanes 1 – 4), p107 (lanes 5 – 8) or p130 (lanes 9 – 12) as indicated. Transfected plasmids are denoted on top, proteins analyzed by Western Blot on the right. hMip40 was detected using an αFlag-antibody. IN, input; IP, immunoprecipitation.

Since RbAp48 interacts with both, hMip40 and pRb, it was expected to be the most likely candidate for executing such a bridging function. In order to address this point, HEK 293 cells were cotransfected with Flag-tagged hMip40, RbAp48 and different pocket proteins. Immunoprecipitation of hMip40 revealed a clear interaction with RbAp48, as seen before, but the pocket proteins did not associate to form trimeric complexes (Fig. 4.20, lanes 4, 8, 12). Even though it cannot be excluded that another protein acts as a bridging factor, these results rather suggest that pocket proteins do not stably interact with hMip40 in HEK 293 cells.

Taken together, these data provide evidence for interactions between human homologs of dMyb-complex subunits, but fail to show an association with pocket proteins.

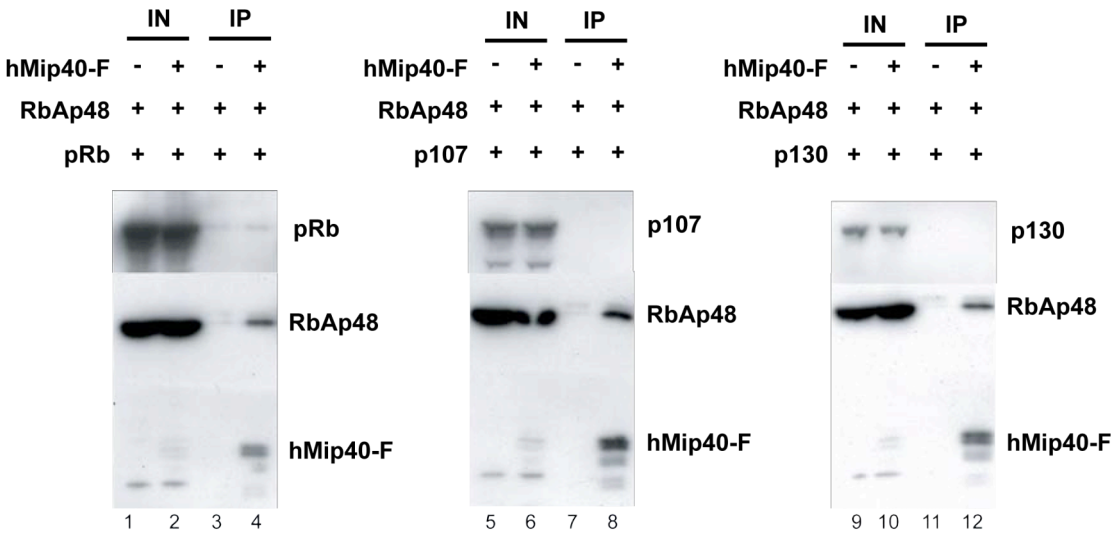


Figure 4.20: Pocket proteins do not form trimeric complexes with hMip40 and RbAp48 in HEK 293 cells

HEK 293 cells were transfected with expression plasmids for hMip40-F, RbAp48 and either pRb (lanes 1 – 4), p107 (lanes 5 – 8) or p130 (lanes 9 – 12) as indicated. Transfected plasmids are denoted on top, proteins analyzed by Western Blot on the right. hMip40 was detected using an αFlag-antibody. IN, input; IP, immunoprecipitation.

4.2.3 Pocket proteins, B-Myb and hMip130/TWIT are assembled into high molecular weight complexes in MOLT-4 cells

Since the use of HEK 293 cells as a system to look for pocket protein interactions holds caveats (see above), MOLT-4 cells were used for further studies of a putative human homolog of dE2F2/RBF complexes. MOLT-4 cells are a human T lymphoblast cell line, which is not virally transformed. It offers the additional advantage of growing in suspension and is, therefore, also more suitable for large scale biochemical approaches.

Separation of MOLT-4 cell nuclear extract on a Superose 6 gel filtration column (Fig. 4.21) revealed that both, B-Myb and hMip130/TWIT eluted with an apparent molecular weight >669 kDa, suggesting that these proteins are subunits of a large protein complex. pRb and p107 showed a broad molecular weight distribution, ranging from 400 kDa to more than 1 MDa. Hence, they might be subunits of distinct protein complexes of different size. The p130 peak with a size of ~500 kDa further confirms the co-immunoprecipitation results from HEK 293 cells, suggesting that p130 is not assembled into the same complex(es) as B-Myb and hMip130/TWIT in MOLT-4 cells.

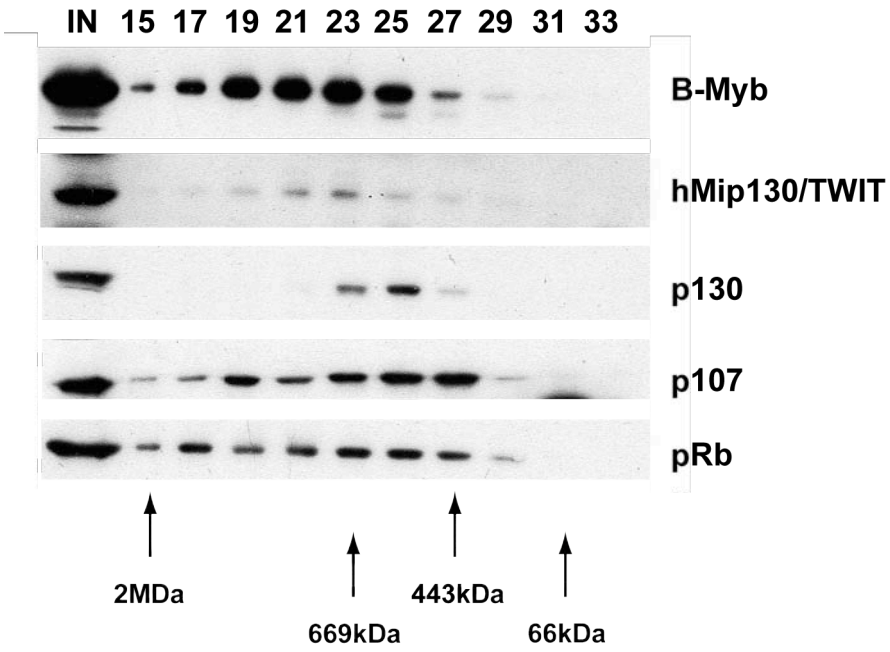


Figure 4.21: B-Myb, hMip130/TWIT and pocket proteins exist in large complexes in MOLT-4 cells
MOLT-4 nuclear extract was loaded onto a Superose 6 gel filtration column. Elution of the indicated proteins was assessed by Western Blot, as indicated on the right. Fraction numbers are denoted on top, size standards on bottom.

4.2.4 Partial purification of endogenous dE2F2/RBF-like complexes from MOLT-4 cells

The presence of B-Myb, hMip130/TWIT and pocket proteins in high molecular weight complexes in MOLT-4 cells offered the possibility to purify the endogenous complex(es) and identify the cofractionating proteins by Western Blot and Mass Spectrometry.

A similar purification protocol to the *Drosophila* one was used for the isolation of the human complex(es) (Fig. 4.22 A). Western Blot on the fractions from the final Superose 6 gel filtration column was performed using antibodies directed against B-Myb, hMip120, hMip130/TWIT, RbAp48 and hMip40 and the mammalian pocket proteins. The hMip120 antibody used for this and the following experiments was kindly provided by S. Gaubatz. B-Myb, hMip120lf, hMip130/TWIT, RbAp48 and hMip40 perfectly coeluted in fractions representing an apparent molecular weight >669 kDa (Fig. 4.22 B). Interestingly, these five proteins represent the human homologs of the subunits of the previously described dMyb complex (Beall et al. 2002). The size of the purified human complex, however, suggests the presence of additional proteins. Alternatively, the complex could exist as a dimer.

All three human pocket proteins were still detectable by Western Blot following the applied purification scheme. However, a clear coelution, as seen for the dMyb complex homologs,

was not observed (Fig. 4.22 B). This does not necessarily rule out pocket proteins as complex subunits, since pRb and p107 were still present in the B-Myb, hMip120lf, hMip130/TWIT, RbAp48 and hMip40 peak fractions. It is conceivable that a minor fraction of pRb or p107, which cannot be resolved with the applied purification procedure, is assembled into the complex. The lack of perfect coelution might also reflect that pocket proteins are no stable subunits of the complex. Alternatively, it is possible that the interaction between pRb and/or p107 and the other proteins was disrupted during the chromatographic purification.

The homogeneity of the purified material was not sufficient for mass spectrometrical analysis of coeluting proteins and the establishment of additional columns in the purification process resulted in the drop of protein levels below the detection limit.

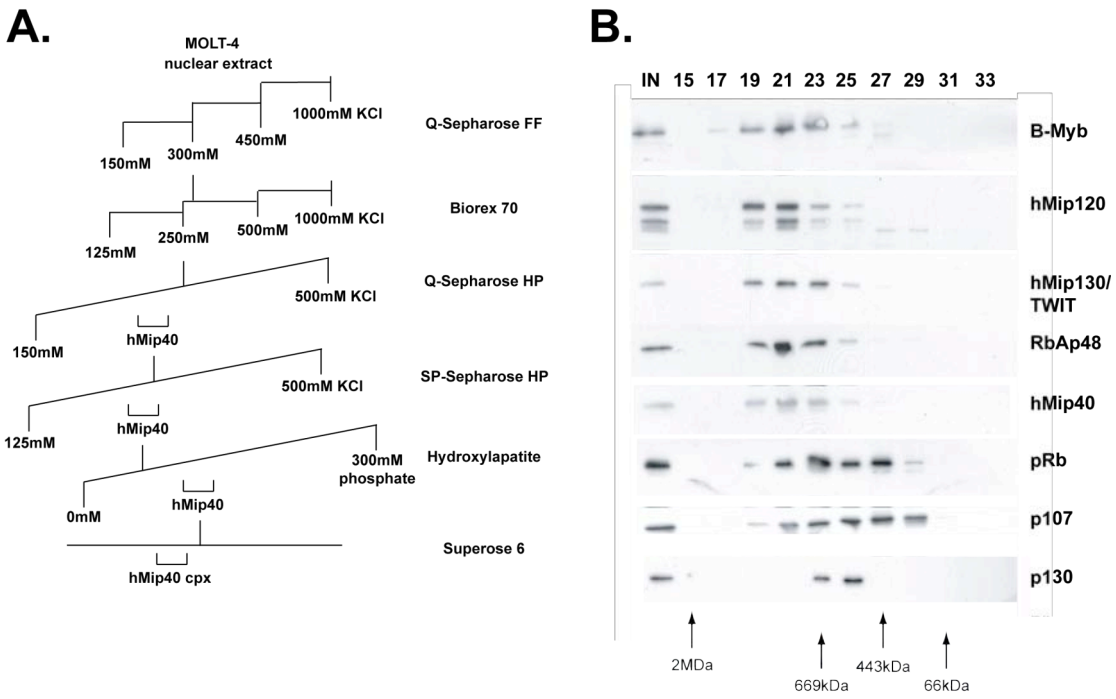


Figure 4.22: Partial purification of an endogenous hMip40-containing complex from MOLT-4 cells
(**A**) 2 g of MOLT-4 nuclear extract were subjected to the depicted purification scheme.
(**B**) Western Blot using antibodies directed against the indicated proteins after the final Superose 6 gel filtration column of the purification procedure. Fraction numbers are denoted on top, size standards on bottom. IN, input.

4.2.5 hMip40 interacts with homologs of all dE2F2/RBF subunits in MOLT-4 cells

The purification of the endogenous complex did not answer the question whether pocket proteins are subunits of the human complex. Therefore, an alternative strategy to resolve this point was established. A MOLT-4 cell line stably expressing a Flag-tagged hMip40 protein was generated as an efficient tool for identifying hMip40-associated proteins (see methods, 3.2.1.4).

Nuclear extracts derived from the hMip40-F and a control cell line were used for immunoprecipitations with an αFlag-antibody. The precipitated material was eluted and resolved by SDS-PAGE. Colloidal Coomassie staining revealed specific bands in the hMip40-F IP, which were analyzed by Mass Spectrometry (Fig. 4.23). Six bands with stoichiometric intensity were detected on the Coomassie stained gel (lane 5, black ellipses), five of which could be identified and turned out to be hMip40, hMip130/TWIT, hMip120lf, B-Myb and p107. One stoichiometric band could not be identified by different mass spectrometical approaches. The stoichiometric appearance of these proteins and the fact that they are human homologs of dE2F2/RBF complex subunits strongly argue for their association in a protein complex. In contrast to the results obtained from the co-immunoprecipitation studies in HEK 293 cells (see 4.2.2), a pocket protein (p107) was identified as a hMip40 interaction partner. Interestingly, neither pRb nor p130 were found in the Mass Spectrometry analysis. Furthermore, E2F/DP proteins and RbAp48 were not identified. This might be explained by the molecular weight of these proteins, which makes them migrate in the size range of the IgG heavy chain. The strong signal of the heavy chain might, therefore, cover these proteins in the Coomassie staining. In summary, these results suggest that a complex similar to dE2F2/RBF, including pocket protein(s), might exist in human cells.

Among the bands that were present with lower or higher stoichiometry (white ellipses) were Tubulin and HSP70. Due to their high abundance in cells and the differences in stoichiometry, they were not considered as putative complex subunits.

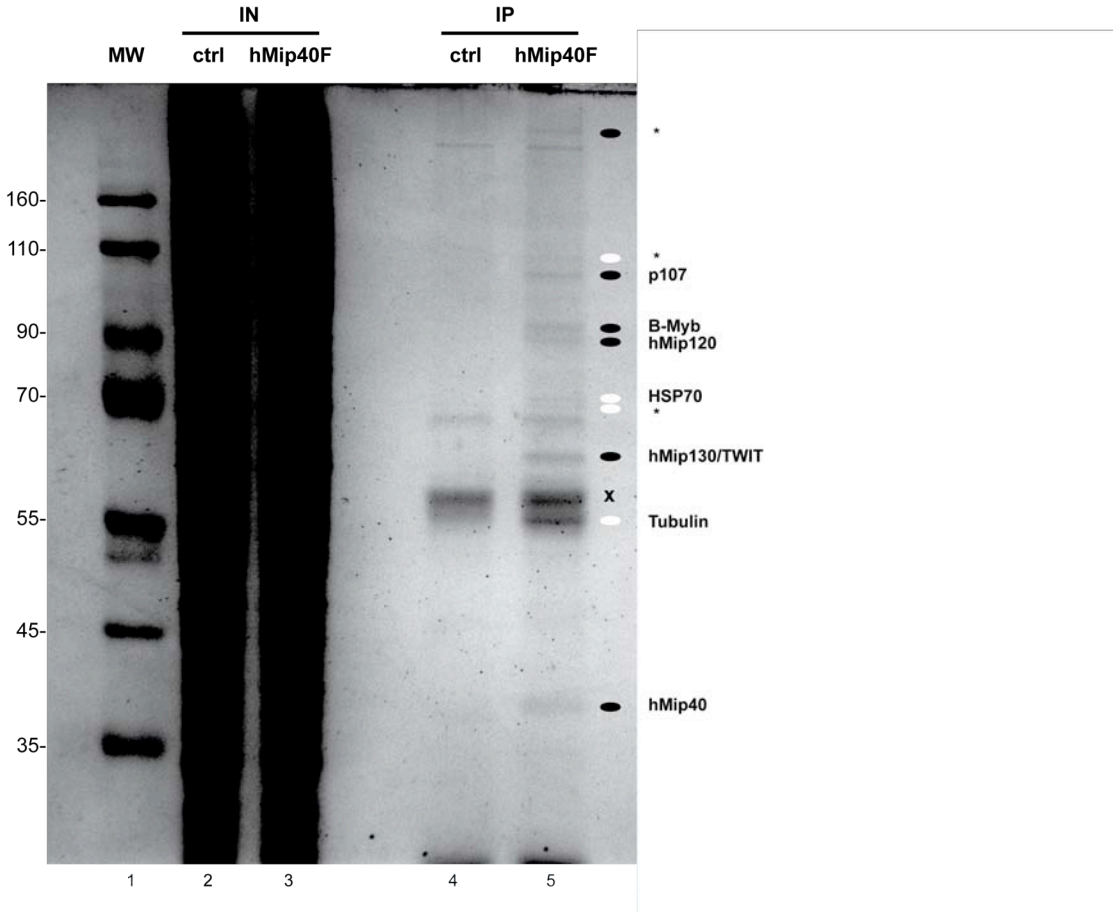


Figure 4.23: Identification of hMip40-associated proteins in MOLT-4 cells

Colloidal Coomassie staining of an αFlag-Immunoprecipitation from nuclear extract of a hMip40-F-expressing (lane 5) or control cell line (lane 4). Precipitated material was eluted with Flag-peptide. Specific bands were excised and analyzed by Mass Spectrometry. Molecular weight markers are indicated on the left, identified polypeptides on the right. Black ellipses, proteins with comparable stoichiometry; white ellipses, proteins with lower or higher stoichiometry; *, not identified; x, IgG heavy chain; MW, molecular weight marker; IN, input; IP, immunoprecipitation; ctrl, control cell line (transfected with empty vector).

The Mass Spectrometry data were further confirmed by Western Blot analysis of the precipitated material using antibodies directed against human homologs of dE2F2/RBF complex proteins (Fig. 4.24). As expected, all stoichiometric proteins that have been identified by Mass Spectrometry, were also detected by Western Blot. Moreover, by Western Blot also RbAp48 and E2F4 were identified as hMip40 interacting partners. Interestingly, E2F4 and E2F5 constitute the repressive E2F factors in the human system, which are the functional homologs of dE2F2 in the fly. In contrast to p107, no clear coprecipitation of pRb and p130 with hMip40 could be detected, which is in contrast to *Drosophila* embryos and cell lines where both Retinoblastoma like factors (RBF1 + RBF2) are assembled into the complexes.

Taken together, the presented data strongly support the idea that a complex homologous to dE2F2/RBF also exists in human cells.

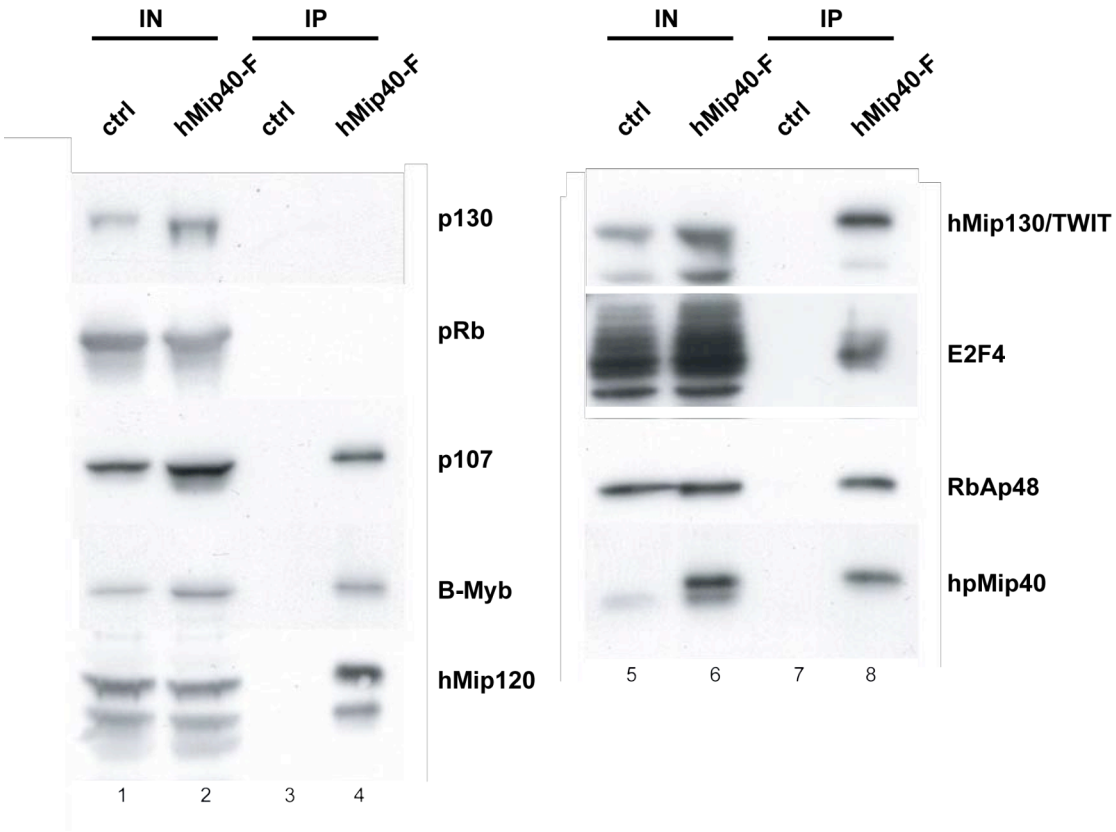


Figure 4.24: Human homologs of dE2F2/RBF complex subunits interact with hMip40
Western Blot of an αFlag-immunoprecipitation from nuclear extract derived from a hMip40-F-expressing (lanes 4, 8) or control cell line (lanes 3, 7) using the antibodies indicated on the right. The precipitated material was eluted with Flag-peptide. IN, input; IP, immunoprecipitation; ctrl, control cell line.

4.2.6 Chromatographic purification disrupts the interaction of p107/E2F4 with hMip40 and associated proteins

The fact that hMip40 coprecipitates the human homologs of all dE2F2/RBF subunits strongly argues for the presence of a similar complex in human cells. On the other hand, purification of the endogenous complex revealed a lack of exact coelution of p107 with the other proteins (see Fig. 4.22 B). The most likely explanation for this discrepancy would be the disruption of this interaction during the purification procedure.

To test this hypothesis, nuclear extract derived from MOLT-4 cells stably expressing hMip40-F was separated on the Q Sepharose HP ion exchange column. B-Myb, hMip130/TWIT and hMip40 perfectly coeluted in one peak, whereas two p107/E2F4 peaks were observed, both

of which were clearly shifted compared to the hMip40 profile (Fig. 4.25). Interestingly, hMip40 eluted in one sharp peak, argueing against hMip40 being independently associated with hMip130/B-Myb and p107/E2F4. The data rather suggest a disruption of the p107/E2F4 – hMip40/hMip130/B-Myb interaction on the column. Nevertheless, the idea of two distinct hMip40-containing complexes could not be ruled out completely, since a certain degree of overlap between hMip40 and p107/E2F4 was still detectable.

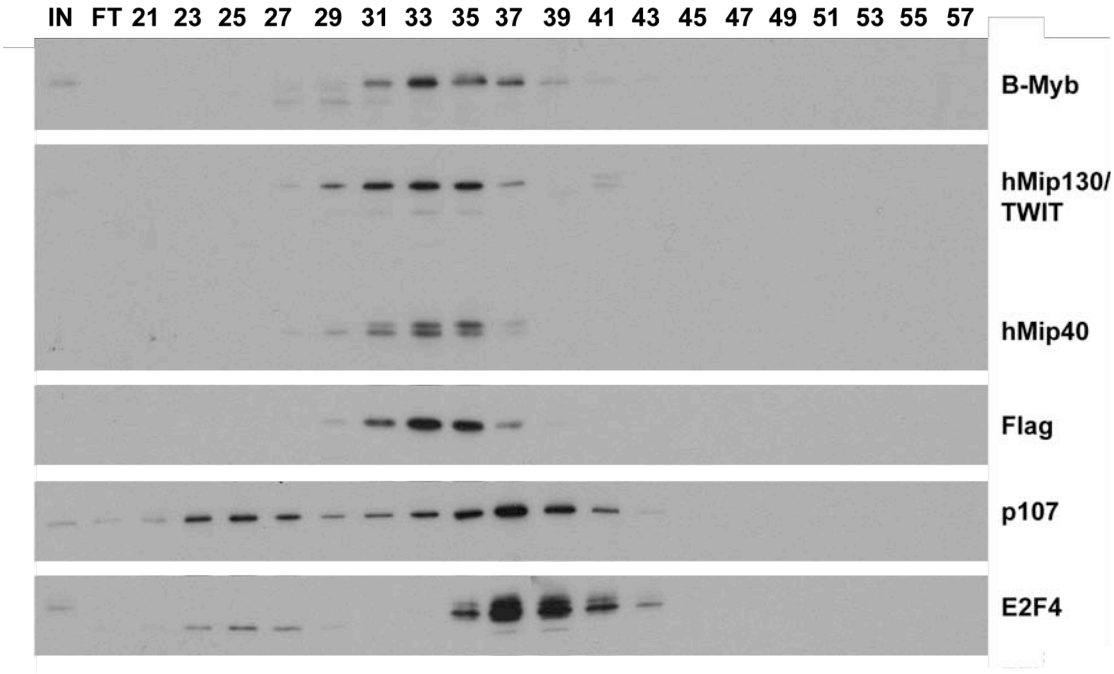


Figure 4.25: hMip40/hMip130/B-Myb and p107/E2F4 do not coelute on an ion exchange column
Nuclear extract derived from the MOLT-4 hMip40-F cell line was loaded onto a Q Sepharose HP column and the bound material was eluted with a shallow salt gradient. The eluted material was analyzed by Western Blot using the indicated antibodies. The doublet in the hMip40 Western represents endogenous and Flag-tagged hMip40. Fraction numbers are denoted on top. IN, input; FT, flowthrough.

To investigate a possible disruption of the hMip40 – p107/E2F4 interaction following chromatographic separation of nuclear extract on the Q Sepharose column, the hMip40 (#33) and the p107/E2F4 peak fractions (#25 + #37) were immunoprecipitated with an αFlag-antibody (Fig. 4.26). hMip40, hMip130/TWIT and B-Myb were found to associate in #33 as well as #37 (lanes 5 + 8). Strikingly, neither p107 nor E2F4 specifically coprecipitated with hMip40 (lane 8). The intensity of the p107 signal detected in the immunoprecipitation is comparable to the p107 signal in the control-IP, where no hMip40-F was present (compare lanes 2 + 8).

The fact that hMip40 and p107/E2F4 associate in MOLT-4 nuclear extract (see 4.2.5) but this interaction is lost after separation of the extract on the Q Sepharose HP column, strongly argue for the disruption of this association during the purification process. This might also explain the discrepancies observed in the elution profile of p107 compared to the other complex components, which has been observed during the purification of the endogenous complex (see 4.2.4).

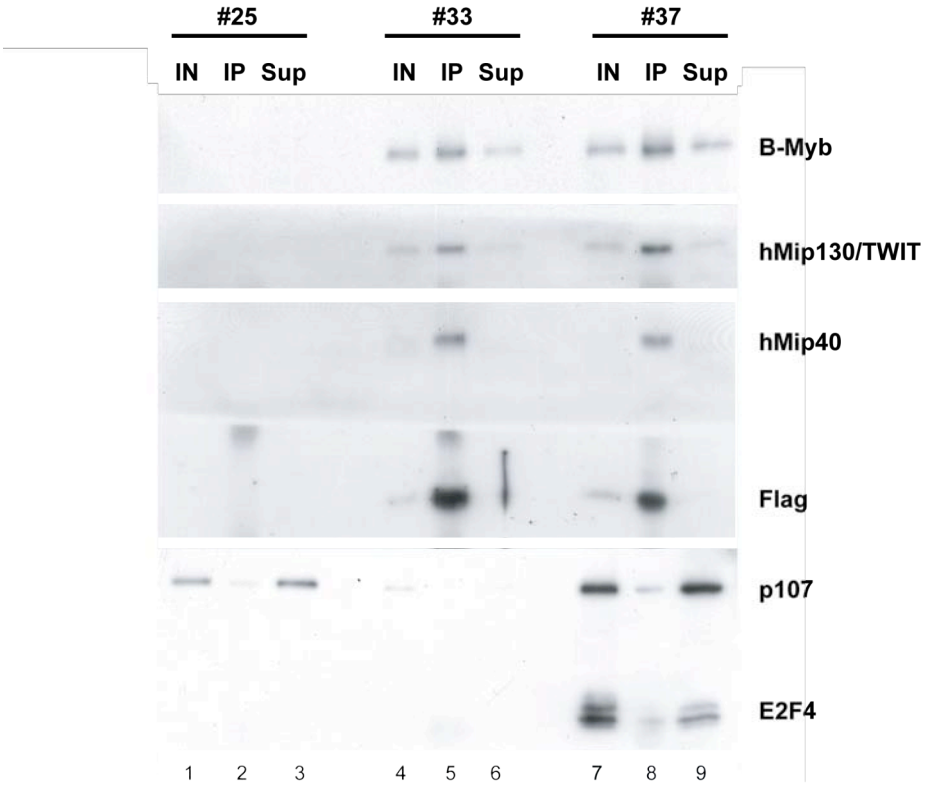


Figure 4.26: The purification procedure disrupts the hMip40 – p107/E2F4 interaction

Partially purified fractions from Fig. 4.25 were used as input material for immunoprecipitation using M2 Agarose (Flag-beads). Equal amounts of hMip40-F in the input material from fractions 33 and 37 were estimated from the intensity of the signals in the elution profile in Fig. 4.25. The precipitated material was analyzed by Western Blot using the indicated antibodies. Input fractions for the IP are denoted on top. IN, input; IP, immunoprecipitation; Sup, supernatant; #, fraction.

4.2.7 The human B-Myb subcomplex specifically binds to deacetylated histone H4 tails

Drosophila dE2F2/RBF complexes are implicated in transcriptional repression of a special class of dE2F target genes and they specifically interact with deacetylated histone H4 tails *in vitro*. Therefore, it was tested whether the human B-Myb subcomplex (B-Myb, hMip120, hMip130/TWIT, RbAp48, hMip40) shared some of these features.

The properties of the partially purified human B-Myb subcomplex regarding its interaction with H4 tails were determined using the same H4 tail-peptide pulldown described for dE2F2/RBF complexes (see 4.1.7). Briefly, the partially purified complex was incubated with tetra-acetylated or unmodified H4 tails bound to beads, and the precipitated material was analyzed by Western Blot. All proteins tested, bound specifically to unmodified H4 tails, whereas no interaction was observed with acetylated tails (Fig. 4.27). Since tetra-acetylated H4 is a mark for transcriptionally active chromatin, whereas deacetylated H4 is a feature of silenced regions, these results are consistent with an involvement of the complex in transcriptional repression. As suggested for the *Drosophila* complexes, binding might be due to the different charge of unmodified versus acetylated H4 tails. Alternatively, the specific binding of one or more complex subunits to deacetylated tails, which might become sterically impossible upon acetylation, is possible.

Interestingly, clear differences were observed in the binding efficiency of the tested proteins (Fig. 4.27, compare lanes 1 + 4 to lanes 3 + 6). A possible explanation for the strong binding of RbAp48 might be the fact that it is a subunit of several chromatin modifying complexes, which might still be present in the partially purified fractions that were used as input material for the experiment. Therefore, the efficient binding observed for RbAp48 might result from the association of (a) different protein complex(es) with deacetylated H4 tails. Alternatively, the RbAp48 subunit might directly interact with the deacetylated H4 tails, whereas the other subunits might only be associated with the tails via RbAp48. Therefore, they might have got progressively lost during the washing steps.

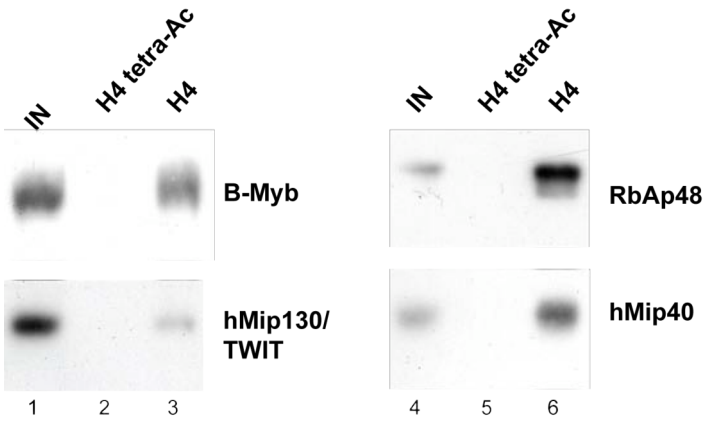


Figure 4.27: Specific interaction of subunits of the human B-Myb subcomplex with deacetylated histone H4 tails

Fractions containing partially purified B-Myb subcomplex (Hydroxylapatite column, see Fig. 4.22 A) were incubated with unmodified or tetra-acetylated H4 tail-peptides immobilized on beads. The bound material was analyzed by Western Blot using antibodies directed against the indicated proteins. Histone tail-peptides are indicated on top. H4 tail-peptides comprised aa 1 – 20. IN, input; H4 tetra-Ac, tetra-acetylated H4 tail; H4, unmodified H4 tail.

Interestingly, binding of the B-Myb subcomplex to unmodified H4 tails occurs independently of the association with p107/E2F4, showing that the subunits of the B-Myb complex are sufficient for this property. Therefore, the ability of each individual subunit to specifically bind to H4 tails was determined using *in vitro* translated proteins (Fig. 4.28). pRb and B-Myb did neither bind to tetra-acetylated nor to unmodified H4 tails (lanes 5 + 6, 8 + 9). Given the binding of B-Myb to unmodified H4 tails in the context of the complex (Fig. 4.27), this suggests that binding of *in vitro* translated proteins to H4 tails is probably not due to complex formation with the proteins from the reticulocyte lysate but is rather an intrinsic property of the proteins. hMip130/TWIT and hMip120sf interacted weakly, but specifically, with unmodified tails (lanes 12, 15). Since only the short form of hMip120 was analyzed for its binding properties, it remains possible that hMip120lf could behave differently. hMip40 bound both, acetylated and unmodified H4 tails, although the association with the unmodified tails was stronger (lanes 20 + 21). The most prominent interaction with unmodified H4 tails was found for RbAp48 (lane 18), suggesting that it might be the critical subunit for the binding of the B-Myb complex to deacetylated H4 tails. Given that also hMip130/TWIT, hMip120sf and hMip40 can interact with unmodified H4 tails, it is conceivable that they also contribute to the histone binding properties of the complex.

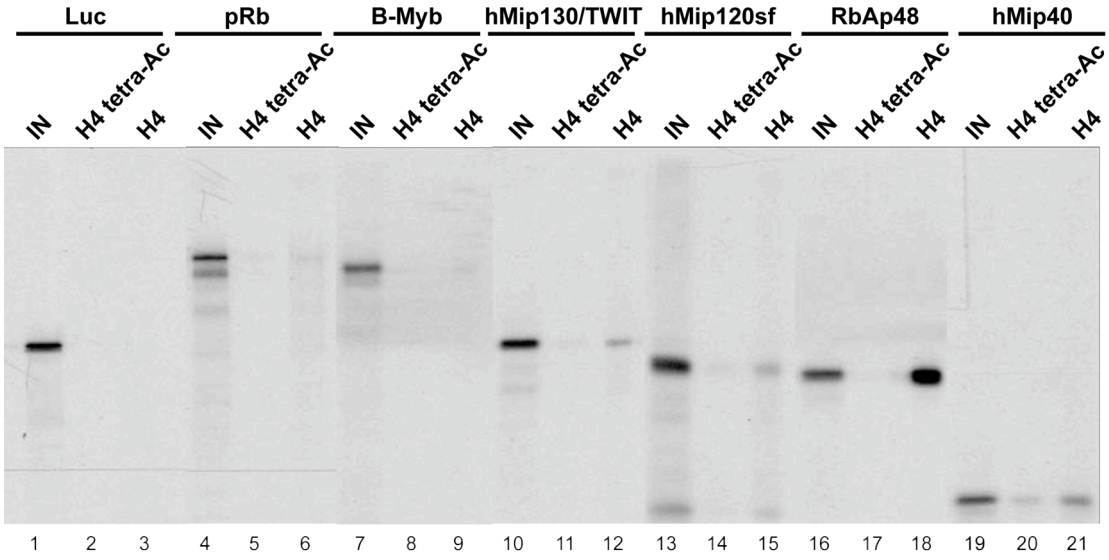


Figure 4.28: Several B-Myb complex subunits specifically interact with unmodified H4 tails
Autoradiography of the bound material from an H4-peptide pulldown with *in vitro* translated ^{35}S-labelled proteins. *In vitro* translated proteins and histone tail-peptides are denoted on top. Luc, Luciferase (control); hMip120sf, hMip120 short form; IN, input; H4 tetra-Ac, tetra-acetylated H4 tail; H4, unmodified H4 tail.

5 Discussion

In this PhD thesis advantage was taken of the streamlined pRb-E2F network in the fruitfly *Drosophila melanogaster*. High molecular weight dE2F2/RBF complexes, which only differ in their RBF subunit (RBF1 or RBF2), were purified from *Drosophila* embryo nuclear extract. These complexes contain several novel pocket protein-associated polypeptides and localize to transcriptionally silent chromatin. Furthermore, the complexes specifically repress a certain class of dE2F target genes, which show sex-specific expression patterns and are implicated in gametogenesis. Strikingly, dE2F2/RBF complexes seem to be highly conserved between different organisms: Genes encoding the *C. elegans* homologs of the complexes act within the same genetic pathway and cooperate in different developmental processes. Moreover, a complex with striking similarity to the *Drosophila* complexes also exists in human cells.

5.1 RBF-containing complexes in *Drosophila*

High molecular weight RBF-containing complexes exist in *Drosophila* embryos as well as in *Drosophila* KC cells. dE2F2/RBF complexes represent the predominant species in late embryos, although a dE2F1/RBF1 complex is also detectable (see Fig. 4.2 + 4.3). The subunit composition and function of the latter was not studied in more detail, but since dE2F2/RBF complexes do not seem to be involved in cell cycle regulation, it appears likely that dE2F1/RBF1 complexes act in cell cycle control. This is in agreement with the analysis of *de2f1* mutant flies, which show numerous deficiencies in cell cycle regulation and with results showing that dE2F1 is required for transcription of many cell cycle regulated dE2F target genes (Dimova et al. 2003).

Analysis of early embryos revealed that high molecular weight RBF2-containing complexes assemble earlier in development than RBF1 complexes (see Fig. 4.1). Given the redundant functions of dE2F2/RBF complexes, this discrepancy is unexpected. It cannot formally be excluded that this complex differs in its subunit composition from the dE2F2/RBF2 complex in 0 – 12 hr embryos. However, this seems unlikely since no additional RBF2 complex was detected in 0 – 12 hr embryos (see Fig. 4.2).

RBF1- and RBF2-containing complexes in KC cells differ in size (see Fig. 4.1), suggesting that in KC cells the complexes have a subunit composition distinct from embryos. Unlike cells of the *Drosophila* embryo, KC cells are highly proliferative. This property might explain a possible difference in the subunit composition of the complexes compared to embryos.

The presence of monomeric RBF1 in *Drosophila* embryos is in agreement with its role in cell cycle progression and the dissociation of phosphorylated RBF1 from dE2F factors. The detection of uncomplexed RBF1 implies the presence of free dE2F/dDP heterodimers. However, no separate dE2F/dDP peak was observed in the embryo extract (see Fig. 4.2). It is formally possible that dissociation of RBF1 from dE2F1/dDP and/or association with coactivators does not affect interaction with the ion exchange resin. However, it seems more likely that *Drosophila* embryos might contain an excess of RBF1 protein to "neutralize" uncontrolled dE2F activity, whereas the transient, cell cycle-dependent changes in RBF-dE2F interaction are not detected.

5.2 dE2F2/RBF complexes act in transcriptional regulation

Several of the subunits of dE2F2/RBF complexes are implicated in transcriptional regulation. RBF1, RBF2 and dE2F2 are well characterized transcriptional repressors (Du et al. 1996a; Sawado et al. 1998; Stevaux et al. 2002; Dimova et al. 2003). In addition, dMyb has been implicated in progression from the G2 to the M phase of the cell cycle due to transcriptional activation of the cyclin B gene (Katzen et al. 1998; Okada et al. 2002). Furthermore, CAF1p55 is a component of several chromatin modifying complexes (Henikoff 2003), implicated in both, activation and repression of transcription. Mip130/TWIT, Mip120 and Mip40, however, have not been shown to function in transcriptional regulation before, but rather are involved in the selective amplification of the chorion gene cluster in *Drosophila* ovarian follicle cells (see below).

5.2.1 dE2F2/RBF complexes repress transcription of a special class of dE2F target genes

dE2F2, dDP and the redundant function of RBF1 and RBF2 are required for the stable repression of E group genes, a class of dE2F target genes, many of which show sex-specific expression patterns and have been implicated in gametogenesis (Dimova et al. 2003). In agreement with their presence in dE2F2/RBF1 and dE2F2/RBF2 complexes, knockdown of Mip130/TWIT, Mip120 and CAF1p55 results in the de-repression of E group genes (see Fig. 4.12) (Taylor-Harding et al. 2004). Interestingly, downregulation of dMyb protein level does not result in an upregulation of E group gene expression, even though it is an integral complex subunit (see Fig. 4.13). Moreover, on E group gene promoters E2F binding sites but no Myb binding sites are found. Therefore, it remains possible that dMyb in the context of

dE2F2/RBF complexes is crucial for the regulation of genes containing Myb binding sites, whose expression was not tested in this work. Alternatively, the integrity of the complexes might be essential for their function and most dE2F2/RBF complex subunits might be crucial for this, whereas dMyb might be dispensable. Moreover, dMyb might not be needed for the function of dE2F2/RBF complexes in transcriptional repression but might be a crucial component for their role in chorion gene amplification, which will be discussed below.

Strikingly, several dE2F2/RBF complex subunits are not required for the regulation of the cell cycle-regulated A group genes (see Fig. 4.12) (Dimova et al. 2003; Taylor-Harding et al. 2004). Nevertheless, complex subunits are physically present at A group gene promoters. The effect of Mip130/TWIT, Mip120, dMyb and CAF1p55 knockdown on the expression of A group genes was tested only for a subset of these genes and cannot exclude a possible regulation of other A group genes. All A group genes tested for regulation by dE2F2/RBF complexes are implicated in the G1/S progression of the cell cycle. However, in addition to genes important for G1/S progression, A group genes also comprise genes encoding factors with functions in other aspects of cell cycle regulation, such as DNA repair, mitosis and with still unknown roles (Dimova et al. 2003). A regulation of these distinct sets of genes by dE2F2/RBF complexes cannot formerly be excluded.

The involvement of dE2F2/RBF complexes in transcriptional repression is supported by the association of complex subunits with transcriptionally silent chromatin on polytene chromosomes (see Fig. 4.7 and 4.8). The numerous chromosomal regions bound by complex subunits, which exceeds the number of known E group genes, could reflect association with other dE2F target genes (e.g. A group genes, see above), dMyb target genes, sites of DNA replication or yet other DNA sequences.

5.2.2 Transcription repression mechanism of dE2F2/RBF complexes

dE2F2/RBF complexes block E group gene transcription in a cell cycle-independent manner in proliferating *Drosophila* cells. This implies a relatively stable repression mechanism that has to be unresponsive to cyclin/cdk-mediated phosphorylation of RBF proteins during the cell cycle. Moreover, such a stable repression mechanism is very likely to involve the formation of a repressive chromatin conformation at the target gene promoters.

Indeed, RBF1 is not dissociated from the dE2F2/RBF1 complex upon phosphorylation by cyclin E/cdk2 complexes (Lewis et al. 2004). The mechanism responsible for this escape is not known, but it seems likely that the additional complex subunits play a crucial role.

In order to establish a repressive chromatin conformation, enzymes capable of covalently modifying histones or remodeling nucleosomes are essential. In this respect it is rather surprising that no subunits with known enzymatic activity directed towards histones were identified as complex components. On the other hand, the characterization of dE2F2/RBF complexes by another group revealed the histone deacetylase dRPD3 and the subunits of the NURF chromatin remodeling complex as Mip120 and Mip130/TWIT interaction partners (Lewis et al. 2004). However, these proteins are present in substoichiometric amounts and knockdown of the proteins in S2 cells does not result in a de-repression of E group genes (Taylor-Harding et al. 2004). Furthermore, treatment of S2 cells with the histone deacteylase inhibitor Trichostatin A does not affect the repression of E group genes (Taylor-Harding et al. 2004). Therefore, it is unlikely that loss of dRPD3 can be compensated by other histone deacetylases. Furthermore, several other chromatin modifying enzymes, implicated in active repression by pRb, are not essential for E group gene repression (Taylor-Harding et al. 2004). In *C. elegans*, LIN-37 (Mip40) is part of a protein complex containing LIN-35 (RBF), DPL-1 (dDP) and LIN-53 (CAF1p55), but it is not associated with HDA-1 (dRPD3) (M. Harrison and R. Horvitz, personal communication).

Despite being not important for the repression of E group genes in *Drosophila* S2 cells, the presented data cannot exclude a possible role for chromatin modifying proteins in the establishment of the repressive chromatin conformation at E group gene promoters early in development. Therefore, a model is conceivable in which chromatin modifying factors might get recruited to E group genes by dE2F, RBF or unknown factors early in development in order to establish the repression of the genes. In late embryos and differentiated *Drosophila* S2 cells, however, the maintenance of this repression is taken over by dE2F2/RBF complexes and the repression mechanism might involve stable chromatin/histone binding (Fig. 5.1).

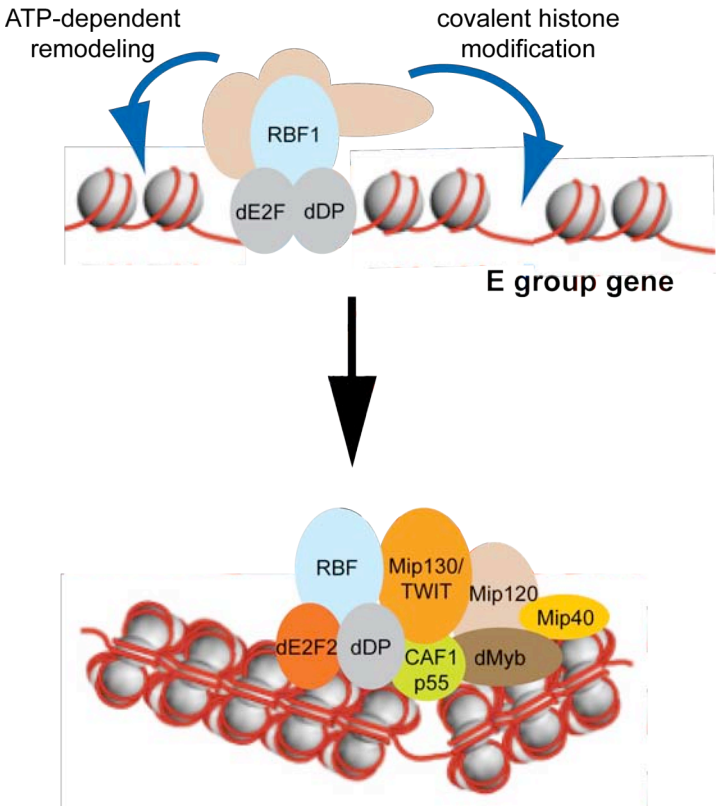


Figure 5.1: Model for the repression of E group genes during development
See text for details.

The idea of dE2F2/RBF complexes acting by stable chromatin/histone binding is supported by the specific association of the complexes with deacetylated H4 tail-peptides *in vitro* (see Fig. 4.10). Moreover, studies of individual human homologs of complex subunits revealed a strong interaction of RbAp48 (CAF1p55) with deacetylated H4 tails, whereas hMip40, hMip120, hMip130/TWIT and B-Myb showed modest or no interaction (see Fig. 4.28). Interestingly, RbAp46 and RbAp48 have previously been shown to interact with a region within the globular domain of histone H4 (Verreault et al. 1998). The data presented here provide evidence that RbAp48 can also specifically associate with histone H4 tails, when they are deacetylated. In contrast to the other human proteins tested for H4 tail interaction, RbAp48 is a known component of several chromatin modifying complexes. Therefore, it cannot formerly be excluded that RbAp48 is assembled into chromatin binding complexes with proteins from the reticulocyte lysate, which has been used for the *in vitro* translation reactions. The lack of interaction between pRb and the H4 tail, however, argues against this possibility because pRb is also known to interact with many chromatin modifying and binding proteins.

The interaction between RbAp48 (CAF1p55) and the H4 tail very likely involves charge effects. Histone tails have a strong positive charge, whereas RbAp48 (CAF1p55) has a net

negative overall charge (pI = 4.74). In addition, RbAp48 (CAF1p55) contains WD40 repeats, which have been shown to fold into a beta-propeller domain. Recently, another WD40 repeat protein, WDR5, has been shown to specifically bind to methylated histone tails (Dou et al. 2005; Wysocka et al. 2005), suggesting that also RbAp48 (CAF1p55) might be able to specifically bind to histone tails via its WD40 repeats. In addition to WD40 repeats, Tudor and MBT domains have also been implicated in the binding of specific histone lysine methylation marks (Huyen et al. 2004; Kim et al. 2006; Klymenko et al. 2006). Strikingly, Mip130/TWIT contains a region with similarity to a Tudor domain. Moreover, Lewis et al. identified L(3)MBT as a substoichiometric component of dE2F2/RBF complexes and showed that it is required for the stable repression of some E group genes (Lewis et al. 2004), and L(3)MBT contains 3 MBT domains. However, dE2F2/RBF complexes show no specific association with histone tails dimethylated at H3K4, H3K9, H3K27 and H4K20 (see Fig. 4.9). Regardless of this lack of interaction, two lines of evidence argue for a more thorough investigation of this hypothesis: First, many more histone lysine residues are known to be methylated and binding is often very specific for the modification of one certain lysine residue. Heterochromatin Protein 1 (HP1), for example, binds preferentially to methylated H3K9, whereas little binding to the same modification on H3K27 is observed, even though the amino acids surrounding the lysine are identical. Second, the specific recognition of lysine methylation marks by some WD40, Tudor and MBT domain proteins depends on the methylation state of the residue (mono-, di- or trimethylation).

5.3 dE2F2/RBF complexes are highly conserved among different species

An interesting feature of dE2F2/RBF complexes is the high degree of conservation of their subunits between different species. Besides *Drosophila*, *C. elegans* and mammals represent the model systems that provide the largest body of knowledge about proteins homologous to dE2F2/RBF complex subunits.

5.3.1 Worms, vulval development and dE2F2/RBF complex homologs

Homologs of all complex subunits exist in the nematode worm *Caenorhabditis elegans*. Intriguingly, most of them act in the same genetic pathway to determine cell fate during vulval differentiation. With the exception of dMyb, homologs of all dE2F2/RBF complex subunits in

C. elegans are members of the class B synMuv genes (Table 5.I). Whereas some of these genes have already been characterized before, *JC8.6*/Mip120 and *lin-37*/Mip40 were shown to function as a class B synMuv gene, and to encode a worm homolog of the dE2F2/RBF complex component Mip40, respectively (see 4.1.10 and (Owen et al. 2003)). LIN-52 is the homolog of dLin52, which has been shown to be an integral component of dE2F2/RBF complexes and to be required for the stable repression of E group genes (Lewis et al. 2004). The fact that dLin52 and the substoichiometric proteins L(3)MBT and dRPD3 were identified in the purification by Lewis et al. but not in the work presented in this PhD thesis might be due to the different purification strategies that have been used. While Lewis et al. used an immunoaffinity purification, in this work a more stringent classical chromatographic purification was applied. This difference might account for the loss of the integral complex subunit dLin52, but might also explain why the putative contaminant dRPD3 was not picked up in the work presented here.

Table 5.I: *C. elegans* homologs of dE2F2/RBF complex subunits act in the same genetic pathway

dE2F2/RBF complex subunits	*C. elegans* homolog	synMuv B gene
Mip130/TWIT	LIN-9	+
Mip120	LIN-54/JC8.6	+
RBF1/RBF2	LIN-35	+
dMyb*	GEI-11	-
CAF1p55	LIN-53	+
dDP	DPL-1	+
dE2F2	EFL-1	+
Mip40	LIN-37	+
dLin52	LIN-52	+
dRPD3*§	HDA-1	+
L(3)MBT§	LIN-61	+

*dMyb and dRPD3 are not required for the repression of E group genes in *Drosophila* S2 cells (Korenjak et al. 2004; Lewis et al. 2004; Taylor-Harding et al. 2004)

§dRPD3 and L(3)MBT are substoichiometric components of dE2F2/RBF complexes

See text for details.

It has been proposed that synMuv gene products might act together in transcriptional repressor complexes regulating the expression of vulva-specific genes (Ceol and Horvitz 2001). Recent work, however, suggests that the adoption of the vulval fate by additional precursor cells upon synMuv gene knockout is non-cell-autonomous and probably due to a failure in the transcriptional repression of the signaling molecule (LIN-3/EGF), that triggers the vulval differentiation program, in the surrounding tissue (Myers and Greenwald 2005; Cui et al. 2006). Future experiments should clarify if synMuv gene products regulate the expression of additional vulval differentiation genes or whether regulation of *lin-3*/EGF transcription is sufficient for the role of synMuv genes in vulval differentiation.

Regardless of the place of action and the exact nature of the target gene(s), there is general agreement on the idea that synMuv gene products might form multi-subunit transcription repressor complexes. This hypothesis is supported by several observations: The homologs of many synMuv gene products act as transcriptional regulators in a variety of organisms. They comprise sequence specific DNA binding proteins (e.g. EFL-1/E2F) as well as proteins with chromatin modifying (e.g. CHD3/Mi-2, HDA-1/HDAC) and binding (e.g. LIN-53/RbAp46,RbAp48) properties. Several of these proteins can interact and form multi-subunit complexes in other organisms (e.g. E2F-pocket protein complexes, NuRD complex). *C. elegans* LIN-35/Rb can interact with EFL-1/E2F, DPL-1/DP and LIN-53/RbAp46,RbAp48 and the latter one associates with HDA-1/HDAC *in vitro* (Lu and Horvitz 1998; Ceol and Horvitz 2001). Moreover, LIN-37/Mip40 is part of a protein complex containing LIN-35/Rb, DPL-1/DP and LIN-53/RbAp46,RbAp48 (M. Harrison and R. Horvitz, personal communication).

Together with the data implicating the *Drosophila* homologs of a variety of class B synMuv genes in a transcription repressor complex, this strongly supports the hypothesis that complexes similar to dE2F2/RBF act in *C. elegans*.

5.3.2 A distinct function for a homologous mammalian E2F4/p107 complex?

The human genome encodes homologs of all dE2F2/RBF complex subunits and, interestingly, several of these proteins have been shown to associate with each other. Besides the well characterized interaction between E2F and pocket proteins, RbAp46 and RbAp48 have originally been identified as pRb interacting proteins (Qian et al. 1993; Qian and Lee 1995). Moreover, an association between p107 and B-Myb and a pRb – hMip130/TWIT interaction have been established more recently (Joaquin et al. 2002; Gagrica et al. 2004).

Indeed, several lines of evidence argue for the existence of a complex homologous to dE2F2/RBF in mammalian cells. The human homologs of complex components are assembled into high molecular weight complexes (see Fig. 4.21). Moreover, B-Myb, hMip130/TWIT, hMip120, RbAp48 and hMip40, representing the homologs of dMyb complex subunits (Beall et al. 2002), copurifiy over six chromatography columns and elute in fractions representing a molecular weight >669 kDa (see Fig. 4.22). The strongest evidence for the existence of a complex comprising also pocket and E2F proteins comes from the analysis of hMip40 interacting proteins (see Fig. 4.23 and 4.24). Strikingly, p107 coprecipitates with hMip40 in stoichiometric amounts compared to B-Myb, hMip120 and hMip130/TWIT in nuclear extract derived from MOLT-4 cells (see Fig. 4.23). Moreover, also E2F4 associates with hMip40. Interestingly, E2F4 is a mammalian repressive E2F factor, thereby representing the closest homolog of dE2F2. However, no other E2F factors were tested for their association with hMip40 in this work. Therefore, it cannot be excluded that additional human E2F proteins can interact with hMip40.

What argues against the existence of a complex comprising all homologs of dE2F2/RBF subunits, is the slight shift in the peak fractions of dMyb complex homologs (B-Myb, hMip120, hMip130/TWIT, RbAp48 and hMip40) and pocket proteins observed during the purification procedure (see Fig. 4.22 + 4.25). A possible explanation for this discrepancy could be the existence of two distinct hMip40-containing complexes, one representing the human homolog of the dMyb complex and the second one comprising hMip40, p107 and E2F4. However, the fact that the association of hMip40 with p107 and E2F4 gets disrupted when MOLT-4 nuclear extraxct is separated on a Q Sepharose HP ion exchange column rather argues for the presence of a single, large complex like in *Drosophila*, which gets disrupted during the purification (see Fig. 4.25 + 4.26).

No interaction between p107 and hMip40 was observed in HEK 293 cells, consistent with the idea that this association might be cell type-specific (see Fig. 4.19 and 4.20). A possible explanation might be the viral transformation of HEK 293 cells. Viral oncoproteins like Ad5-E1A or SV40-T have been shown to disrupt the interaction between pocket proteins and some of their binding partners. Since this is thought to be an important transformation strategy of oncoviruses, dissociation of pocket proteins from the human complex might be a critical step in oncogenic transformation.

B-Myb does not only interact with p107, but also associates, more weakly, with p130 (Joaquin et al. 2002). Furthermore, in MOLT-4 cells hMip40 interacts with p130, although much less efficiently than with p107 (data not shown). Therefore, it remains possible that, like in the fly, two redundantly acting complexes also exist in human cells.

Regarding the function of the human complex, the specific binding of the partially purified B-Myb subcomplex and the individual proteins, most notably RbAp48, to deacetylated histone H4 tail-peptides are consistent with a role in transcriptional repression, as shown for the *Drosophila* complexes (see Fig. 4.27 and 4.28).

hMip130/TWIT mutant mice do not show any obvious phenotype. However, the simultaneous mutation of hMip130/TWIT and the cell cycle regulatory factor cdk4, whose single inactivation results in decreased cell proliferation and downregulation of genes important for the G1/S transition of the cell cycle, can restore the protein levels of several of the G1/S regulators to some extent (Sandoval et al. 2006). This would be in agreement with a role for hMip130/TWIT in transcriptional repression. Furthermore, overexpression of hMip130/TWIT in mouse cells significantly decreases the number of S-phase cells (Sandoval et al. 2006). In contrast to the function of Mip130/TWIT in *Drosophila* cells, these findings implicate hMip130/TWIT in the control of G1/S phase progression. The data presented by Sandoval et al. closely resemble a study in *C. elegans*, where opposing functions for LIN-9/TWIT and CYD-1 in G1 regulation have been shown in double mutant animals (Boxem and van den Heuvel 2002). LIN-35/Rb, EFL-1/E2F and DPL-1/DP behave like LIN-9/TWIT, whereas LIN-37/Mip40 and LIN-53/RbAp46,RbAp48 do not act in G1 regulation. This argues for a separate function of pRb, E2F, DP and Mip130/TWIT proteins in the worm that might not involve other complex subunits. The same might be the case in human cells, although no additional hMip130/TWIT complexes were detected during the purification procedure. These interactions might, however, be transient which would explain the lack of such complexes that are amenable to biochemical purification. In *Drosophila*, a role for Mip130/TWIT in G1 regulation in the context of cyclin/cdk knockout has not been studied.

Regarding the analysis of the mutant mice it should be noted that single knockout of hMip130/TWIT did not result in any obvious phenotype, except of an increase in body size (Sandoval et al. 2006). This mild phenotype might be explained by fact that the “knockout” does probably not result in a complete null allele. It rather only removes the N-terminal 84 amino acids, giving rise to a truncated protein. Importantly, the critical functional domains of hMip130/TWIT might reside in the Box 1 and 2 regions, which appear to be the most highly conserved stretches within the protein (Gagrica et al. 2004), and they are still present in the truncated protein.

In contrast to its proposed role in cell cycle regulation, another study does not implicate hMip130/TWIT in G1/S phase progression. Neither overexpression nor RNAi-mediated knockdown of hMip130/TWIT reveals significant changes in the cell cycle profile of human cells (Gagrica et al. 2004). Although hMip130/TWIT and pRb interact, they do not cooperate

in transcriptional repression. Moreover, hMip130/TWIT does not control expression from the cell cycle-regulated cyclin E gene promoter, which is a target of the E2F transcription factor family (Gagrica et al. 2004). It rather cooperates with pRb in the induction of the “flat cell” phenotype, which shows some of the hallmarks of senescent and differentiated cells, and in the activation of a differentiation-specific gene (Gagrica et al. 2004).

The discussed findings suggest rather diverse functions for hMip130/TWIT in human cells. Together with the role of dE2F2/RBF complexes in the repression of sex- and differentiation-specific dE2F target genes and the fact that a homologous complex also exists in human cells, these results put another twist to the possible role of the complex in mammalian cells. In order to unravel the actual function of the human complex, future work will have to address the question of endogenous target genes of the complex.

5.4 A conserved role for E2F and pocket proteins in germ line – soma distinction

Many E group genes are known to act during gametogenesis and show sex-specific expression patterns (Dimova et al. 2003), suggesting that their expression is essential for proper development of germ cells and, hence, fertility. Accordingly, their expression is stably repressed in somatic *Drosophila* S2 cells. This is in agreement with the idea that their expression has to be specifically restricted to the germ line. It is tempting to speculate that dE2F2/RBF complexes are crucial for the repression of germ line-specific genes in somatic tissues. This assumes that mechanisms exist that override repression in the germ line. Interestingly, *Drosophila* spermatocytes express the always early (Aly) gene product, a paralog of Mip130/TWIT (White-Cooper et al. 1998). Aly is a chromatin-associated protein that is essential for progression through meiosis and terminal differentiation (White-Cooper et al. 2000) and might, therefore, be involved in the activation of germ line-specific genes. However, a direct role for Aly in the activation of these genes remains to be shown. The comparison of genes that are repressed by dE2F2 in S2 cells and ovaries revealed only very limited overlap (Stevaux et al. 2005). This suggests that the majority of genes that are stably inactivated by dE2F2/RBF complexes in S2 cells are not negatively regulated by the same complexes in the germ line.

Further support for a role of E2F and pocket proteins in germ line – soma distinction comes from studies in *C. elegans*. Inactivation of *lin-35*/Rb results in the detection of PGL-1, a component of the strictly germ line-specific P-granules, in somatic cells. In addition, structures

resembling P-granules can be observed in the soma (Wang et al. 2005). These findings suggest that *lin-35*/Rb functions to repress germ line-specific genes in somatic tissues. In contrast, LIN-35/Rb does not repress a group of genes implicated in oogenesis, which is positively regulated by EFL-1/E2F and DPL-1/DP, in the hermaphrodite gonad, suggesting that the LIN-35/Rb-dependent repression of germ line-specific genes might be overcome in the gonad (Chi and Reinke 2006). These findings are in agreement with the observation that inactivation of *efl-1*/E2F and *dpl-1*/DP results in sterility due to oocyte degeneration, whereas *lin-35*/Rb mutant animals are fertile with a morphologically normal germ line (Lu and Horvitz 1998; Ceol and Horvitz 2001; Chi and Reinke 2006).

Several dE2F2/RBF complex components are conserved in plants, including members of the RBR1 (RBF), MSI1 (CAF1p55), ALWAYS EARLY (Mip130/TWIT) and MYB (dMyb) families, and they appear to play an important role during reproductive development (Hennig et al. 2003; Kohler et al. 2003; Bhatt et al. 2004; Ebel et al. 2004; Hennig et al. 2004).

In mammalian cells, E2F6 has recently been shown to be crucial for the stable repression of testis-specific genes in somatic tissues. These targets include genes specifically required during meiosis (Pohlers et al. 2005; Storre et al. 2005). In contrast to the situation in *Drosophila* and *C. elegans*, repression by E2F6 is pocket protein-independent. Rather, E2F6 is known to be assembled into complexes with PcG proteins, some of which have chromatin modifying activity (Trimarchi et al. 2001; Ogawa et al. 2002; Attwooll et al. 2005). In agreement with this, loss of E2F6 results in a decrease in repressive histone modifications at target gene promoters (Storre et al. 2005). It is conceivable that E2F6 has evolved as a specialized member of the E2F transcription factor family in higher organisms to adopt the role of pocket proteins in the stable repression of germ line-specific genes in somatic tissues. This hypothesis is supported by the lack of E2F6-like factors in flies, worms and plants.

5.5 A role for dE2F2/RBF complexes in DNA replication?

Resolving the subunit composition of dE2F2/RBF complexes revealed an intriguing feature of the complex components. Several of the subunits (Mip130/TWIT, Mip120, dMyb, CAF1p55 and Mip40) have previously been shown to be assembled into a dMyb complex (Beall et al. 2002). This complex functions during the endoreplication of chorion genes in *Drosophila* ovarian follicle cells. In *Drosophila*, the developing oocyte is surrounded by a layer of follicle cells, which produce large amounts of eggshell protein, required for proper oocyte development. During oogenesis, the chorion (eggshell) genes in follicle cells are subject to specialized DNA replication events. These comprise the shutdown of genomic DNA

replication and subsequent specific amplification of four chorion gene loci (Calvi et al. 1998; Claycomb et al. 2004). Chorion gene amplification differs from normal DNA replication by re-replication events within a single cell cycle. The dMyb complex binds site-specifically to chorion gene amplification control elements (ACE). Moreover, dMyb interacts with origin recognition complex (Orc) proteins, and both, dMyb and Mip120 binding sites in an ACE, are required for proper amplification in a replication reporter assay (Beall et al. 2002). The analysis of *dmyb* and *mip130/twit* mutants revealed opposing effects on chorion gene amplification. *dmyb* mutant follicle cells shut down genomic DNA replication but they are unable to initiate amplification of the chorion gene loci (Beall et al. 2002). In contrast, *mip130/twit* mutants fail to shut down genomic DNA replication (Beall et al. 2004). This led to a model implicating the dMyb complex in the cessation of genomic DNA replication and the dMyb protein in overriding this negative regulation at chorion gene loci (Beall et al. 2004). As an integral subunit of the “repressor complex”, specific activation of dMyb seems essential for the initiation of chorion gene amplification. However, the underlying mechanism is still unclear.

Excitingly, the gene amplification defect observed in *dmyb* mutant flies is closely resembled by a hypomorphic allele of *de2f1* (Royzman et al. 1999). Therefore, dE2F1 might also act in overriding the activity of the “chorion gene amplification repressor complex”. On the other hand, *rbf1* and *e2f2* flies continue genomic DNA replication instead of restricting replication to chorion gene loci, as seen for the inactivation of *mip130/twit* (Bosco et al. 2001; Cayirlioglu et al. 2001). These findings are especially interesting with respect to the subunit composition of dE2F2/RBF complexes. Several of the complex components seem to play a role in the amplification of chorion genes. Moreover, they associate with ACEs and interact with Orc proteins (Bosco et al. 2001; Beall et al. 2002). Therefore, it is conceivable that dE2F2/RBF complexes, in addition to their role in transcriptional regulation, have an unexpected role in DNA replication control at chorion gene loci. Replication of chorion genes in *Drosophila*, however, is a rather specialized event and mutation of *de2f2* and *mip130/twit* does not result in more general replication defects (Frolov et al. 2001; Beall et al. 2004). Nevertheless, pocket proteins have also been implicated in DNA replication in human cells. In early S phase, they specifically localize to sites of active DNA replication and pRb localizes to specific replication control sites after S phase DNA damage, where it suppresses abnormal endoreplication events (Kennedy et al. 2000; Avni et al. 2003).

Taken together, it is conceivable that dE2F2/RBF complexes execute rather distinct functions. They act in the stable repression of sex- and differentiation-specific genes, which might, for example, be crucial in germ line – soma distinction. On the other hand, they might also be

directly involved in the regulation of DNA replication. Therefore, dE2F2/RBF complexes might provide an important link for the coordination between cell cycle regulation, DNA replication and the onset of a specific differentiation program.

Pocket protein-mediated recruitment of several transcription repressor complexes has been shown to play an essential role in the regulation of E2F target genes important for cell cycle progression. This study, for the first time, implicates native pocket protein-containing complexes in the specific regulation of developmentally controlled E2F target genes. Based on the data presented in this work, further characterization of these complexes will be another step forward in our understanding of the pRb-E2F network as a “master regulator of cell cycle and differentiation”.

List of abbreviations and acronyms

aa	amin acid
ACE	amplification control element
Ad-E1A	Adenovirus E1A protein
Aly	always early
arp	actin related protein
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BRG1	brahma related gene 1
BRM	brahma
BSA	bovine serum albumine
CAF1	chromatin assembly factor 1
cdk	cyclin dependent kinase
cdki	cyclin dependent kinase inhibitor
CHD	chromatin organization modifier/helicase/DNA binding domains
ChIP	chromatin immunoprecipitation
CV	column volume
CYD	cyclin D
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dnk	deoxyribonucleoside monophosphate kinase
DP	differentiation regulated transcription factor protein
DPL	DP like
DTT	dithiotreithol
EDTA	ethylene diamine tetraacetic acid
EFL	E2F like
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid
E2F	E2 promoter binding factor
FCS	fetal calf serum
GEI	GEX interacting protein
G1 phase	gap phase 1
GST	glutathione-S-transferase
G2 phase	gap phase 2
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEK 293	human embryonic kidney epithelial cell line

HEPES	N(2-hydroxyethyl)piperazine-N´(2-ethanesulphonic acid)
HMT	histone methyltransferase
HPC2	human polycomb 2
HPL	HP1 like
HP1	heterochromatin protein 1
HPV16-E7	Human Papilloma Virus E7 protein
HRP	horseradish peroxidase
HSP	heat shock protein
Ig	immunoglobuline
IP	immunoprecipitation
IVT	*in vitro* translation
kDa	kilodalton
LIN	abnormal cell lineage
L(3)MBT	lethal (3) malignant brain tumor
MALDI-TOF	matrix-assisted laser desorption ionization – time of flight
MAP	mitogen activated protein
MBT	malignant brain tumor
MDa	megadalton
Mip	Myb interacting protein
M phase	mitosis
MSI1	mulitcopy suppressor of IRA 1
Myb	myeloblastosis
NE	nuclear extract
NuRD	nucleosome remodeling/deacetylation
NURF	nucleosome remodeling factor
Orc	origin recognition complex
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
Pc	polycomb
PcG	polycomb group
pcna	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PCV	packed cell volume
PEI	poly(ethylenimine)
PGL	P granule abnormality
PMSF	phenylmethane sulfonyl fluoride
Pol	polymerase
pRb	Retinoblastoma protein
PRMT	protein arginine methyltransferase
PVDF	polyvinylidine difluoride

RbAp	Retinoblastoma associated protein
RBF	Retinoblastoma like factor
RBR1	Retinoblastoma related 1
RNA	ribonucleic acid
rnr	ribonucleotide reductase
RPD3	reduced potassium dependency 3
rp49	ribosomal protein 49
SANT	SWI/SNF, ADA, N-CoR, TFIIIB
SDS	sodium dodecyl sulfate
S phase	DNA synthesis phase
SUV39H1	suppressor of variegation 3-9 H1
SV40-T	Simian Virus 40 T antigen
SWI/SNF	mating type switch/sucrose non-fermenting
synMuv	synthetic multivulva
TBP	TATA binding protein
TCA	trichloroacetic acid
TFIIH	transcription factor II H
TRAX	*Drosophila* transcription extract
TWIT	Twilight
VPC	vulval precursor cell
WCE	whole cell extract

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