The pRB subsystem

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Symbols used:

§	Data from a non-human model, generality uncertain
®	Reference thus tagged is a review article
[*number*]	Reference to the correspondingly labelled part of the table or figure last cited in the text

The pRB subsystem

Study of a rare hereditary paediatric cancer has led to the identification of pRB, a tumour-suppressor implicated in human cancer of many types. It plays a crucial role in embryogenesis, differentiation, cellular senescence, and proliferation. The manifold functions of pRB are mediated solely via interactions with over 100 proteins, both individually and in higher-order complexes. Its functions are modulated chiefly post-translationally, with regulated alterations in phosphorylation state being the best understood mechanism. Not surprisingly, many of the elements necessary for regulation of pRB function have themselves been implicated in tumour suppression or tumorigenesis, in particular, the cyclins, the CDKs, and the CKIs.

This article provides a general review of pRB structure, interaction, and regulation as a basis for a discussion of the mechanism by which pRB exerts control over cell-cycle progression. The relevance that this may have to tumorigenesis in general, and to melanoma in particular, is then addressed.

1 Retinoblastoma

Retinoblastoma is a paediatric intraocular tumour accounting for 5% of childhood blindness. It occurs as an inherited disease with autosomal dominant transmission[28] and 90% penetrance[76], in which tumours are usually bilateral and multifocal. Sporadic cases are also known, but these differ from the typical hereditary disease in that they are usually unilateral and unifocal, although a hereditary low-penetrance unifocal phenotype has been described[55]. Several modes of treatment exist, including surgery and radiotherapy, and these are usually curative[70] and preserve vision. However, significant mortality still occurs after successful treatment of hereditary cases due to the increased incidence of subsequent primary tumours of various types.

Since rodents infected with adenovirus often developed retinoblastoma-like symptoms[267] [268] it was thought that human retinoblastoma may have a similar cause, but no trace of the adenovirus genome could be found in cells from patients[362] [386]. A different interpretation began to emerge with the publication of a seminal paper by Knudson[202] that reported the results of a statistical analysis of retinoblastoma incidence. The clear inference to be drawn from the data was that retinoblastoma could develop after the occurrence of just two independent genetic events. In the case of the hereditary disease, one of these was presumed to be an inherited trait, while the second, and both in the case of the sporadic disease, were considered to be somatic changes. This is the 'two-hit' hypothesis. Although the two targets were not specifically identified in this work, given the diploid nature of the human genome, a reasonable working hypothesis was that a defect in only one gene was involved, with two events being required to disrupt both alleles. This was supported by loss-of-heterozygosity studies[41].

2 The retinoblastoma susceptibility gene, *RB1*

Mapping

Cytogenetic analysis of retinoblastoma tumours led to the discovery of a frequently deleted chromosomal region at 13q14, and linkage analysis within kindreds displaying hereditary disease led to the identification of closely linked microsatellite markers which co-segregated with the disease phenotype. These efforts ultimately resulted in the identification of a candidate retinoblastoma susceptibility gene, *RB1*[106] [227] and its authentication[110].

Gene structure and transcriptional regulation

RB1 comprises 27 exons spanning over 200 kbp of genomic DNA[28 371], and is transcribed into an mRNA of 4.6kb length[227]. No splice-variants appear to exist in normal tissue, but aberrant splicing resulting in truncation or skipped exons does occur in tumours[236].

The initial *RB1* promoter characterisation[371] was extended by Gill et al.[119], who, by using a series of 5'-deletion constructs, discovered that a region spanning nucleotides –215 to –179, relative to the initiating methionine codon, contains the major functional determinants of transcriptional regulation. They identified putative SP1, CREB/ATF, and E2F binding sites, together with a potential hormone-response element. Surprisingly, the protein that they found to associate with the SP1 site was not SP1, but another protein they dubbed 'RBF-1'. Further work established that it is the GA-binding protein component of the E4TF1 Ets-family transcription factor complex that binds to this site[326]. Mutation at this[55], or the CREB/ATF site[322] is associated with a mild, low-penetrance hereditary retinoblastoma phenotype.

A CpG island extends from the promoter into exon one[379] and there is evidence that this can be methylated, preventing binding of E4TF1 and ATF/CREB and causing a 92% reduction in transcription rate[285]. Transcriptional silencing due to promoter methylation coupled with deletion or mutation of the alternate allele has been causally linked to over 9% of unilateral sporadic retinoblastomas[128 284 285], and has been reported in oligodendroglial[72] tumours and glioblastoma[273].

There is a consensus that pRB contributes to transcriptional regulation of its own gene, but there is less accord over the nature of this. Some opine that E2F transcription factors, regulated by pRB, function as repressors[132 286 334], but others have established that the E2F binding site is dispensable for auto-repression[119]. Positive auto-regulation via the ATF/CREB site has also been reported[295].

3 The retinoblastoma-associated protein, pRB

Significance

Perhaps the best gauge of the importance of a protein is the consequence of its absence, as amply demonstrated in mouse knockout studies. A degree of perspective is afforded by comparing the effect of non-expression of two crucial tumour-suppressors: p53 and pRB. Mice engineered to be *Trp53*-null are born apparently normal, anatomically and physiologically. Only after about six months does their phenotype of increased tumour incidence emerge[§71]. *Trp53*, and by extension the human *TP53*, are tumour-suppressor genes, *par excellence*, but that is all they are. In contrast, mice engineered to be *Rb1*-null die before day 16 *in utero*, with major neural tube deformities, flaws in haematopoiesis, and liver and lens defects[§224]. Clearly, *Rb1*, and by extension *RB1*, have extremely important biological roles beyond tumour suppression. Perhaps the best generalisation of pRB function is to consider it as a key determiner of cellular fate. It profoundly influences proliferation, differentiation, senescence, and apoptosis[®135 ®184]. The retinoblastoma-associated protein is no less than the kismet of cells.

Translation

The *RB1* mRNA transcript contains an open reading frame encoding 928 amino acids, and SDS-PAGE immunoblotting detects at least five mobility variants with indicative molecular weights in the range 105–110 kD. These are believed to result from the adoption of multiple conformations determined by post-translational covalent modification, addressed further below. There is some evidence for translation from a second AUG start site resulting in an amino-terminally truncated variant seen by immunoblotting as a protein of 98–104 kD indicative molecular weight. The functional significance of this is unknown. It

has also been suggested that sequence variations in the 5' untranslated region may affect mRNA structure and thence translation efficiency[108].

Conservation and homology

Species including plants[®§57 ®§82], insects, fish, amphibians, birds, and other mammals have proteins clearly related to human pRB by sequence similarity {Table 1}. Interestingly, no close homologues exist among unicellular organisms such as yeast. This is entirely in keeping with the principal biological functions of pRB being the constraint of proliferation and the implementation of differentiation, neither of which is of great relevance to such an organism.

Species	Common name	Homology length (amino acids)	Sequence comparison	
			Identity (%)	Similarity (%)
Pan troglodytes	Chimpanzee	882	98	98
Mus musculus	Mouse	928	89	93
Rattus norvegicus	Norway rats	900	89	94
Gallus gallus	Chicken	937	71	81
Notophthalmus viridescens	Eastern red-spotted newt	914	59	75
Xenopus laevis	African clawed frog	936	57	74
Canis familiaris	Dog	518	95	97
Oncorhynchus mykiss	Rainbow trout	944	54	70
Oryzias latipes	Japanese medaka fish	942	50	67
Populus (hybrid)	Aspen	790	24	40
Chenopodium rubrum	Red goosefoot	805	24	40
Arabidopsis thaliana	Mouse-ear cress	895	23	40
Euphorbia esula	Leafy spurge	528	25	44
Zea mays	Maize	765	24	40
Drosophila melanogaster	Fruit fly	709	23	40
Pisum sativum	Garden pea	792	24	40
Caenorhabditis elegans	A nematode worm	870	21	36

Data from NCBI/BLAST. Comparison is with *Homo sapiens* pRB. Similarity implies identity or a conservative amino acid substitution.

Table 1: pRB protein sequence conservation

Within the human proteome, two proteins are sufficiently similar to pRB in terms of sequence conservation and function to support the notion of a 'pocket-protein' family {Table 2}. Their degree of similarity to pRB is of the same order as that of the nearest plant pRB homologues. Whether this implies that pRB is strongly conserved and p107 and p130 are closely related, or precisely the opposite, is entirely subjective. It is telling, however, that while pRB has been established as a bona fide tumour-suppressor, there is insufficient evidence to support such a role for either p107 or p130[®49].

Protein	Gene	Homology length (amino acids)	Sequence comparison	
			Identity (%)	Similarity (%)
p107	*RBL1*	559	27	44
p130	*RBL2*	703	24	41

Data from NCBI/BLAST. Comparison is with pRB. Similarity implies identity or a conservative amino acid substitution.

Table 2: Human proteins similar to pRB

Tissue-specificity of pRB expression

A comprehensive study of pRB expression in 53 human tissues was performed by Cordon-Cardo and Richon[54]. Expression was seen in all but interstitial matrix, which is essentially acellular. There was variability of expression between and within organs, however. In stratified epithelia, cells in the proliferating basal layer expressed low levels of pRB, while those in suprabasal layers expressed it strongly. In simple epithelia, expression was generally high, but where compartments differing in proliferation rate were distinguishable, an inverse correlation between expression and proliferation rate

was seen. Within the testis, this pattern was again repeated, with non-proliferating Sertoli cells having intense expression, while spermatogonial cells, spermatocytes, and spermatids had low or undetectable levels. Within tissues of the central nervous system, expression was low with the conspicuous exception of Purkinje cells, where it was intense. Intense staining was also seen in cells of the peripheral nervous system. Among haematopoietic cells, proliferating B-cells expressed high levels of pRB, while that seen in mature B-cells and in T-cells was much lower. It was the authors' overall conclusion that pRB regulated the proliferation of maturing cells.

Sub-cellular disposition of pRB

The pRB protein is predominantly nuclear during interphase, being associated with low-density euchromatin. In metaphase and anaphase, it disperses to the cytoplasm eventually to reassociate with euchromatin during telophase[364]. Hypophosphorylated pRB is tethered to the nucleus, but this linkage is weakened upon phosphorylation[88 373]. Nevertheless, a confocal microscopic study of HL60 cells has shown that the ratio of nuclear to cytoplasmic pRB is stable both throughout the cell-cycle and during differentiation, independent of its phosphorylation status[420]. However, these cells do not contain functional p16, an inhibitor of pRB phosphorylation, as they have only a single non-functional mutant *CDKN2A* allele[311]. Consequently, pRB phosphorylation status may be abnormally high in these cells, and greater partitioning of pRB to the cytoplasm through reduced tethering may result.

pRB phosphorylation and the role of CDK4 and p16 are discussed more fully below.

Turnover of pRB

In the normal course of events, pRB levels do not appear to be controlled by regulated proteolysis, although this does play a role in viral infection[123] and in apoptosis[95 370]. It has been suggested by one group[107] that a cathepsin-like protease, dubbed SPase, may be involved in the cell-cycle dependent regulation of pRB, but this has not been confirmed. There is doubt also over the validity of their methodology in that, having synchronised cells first by isoleucine starvation, and then by aphidicolin treatment, the induction of this protease in response to this treatment cannot be excluded. More recently, a gene over-expressed in some hepatocellular carcinomas was found to encode a protein, gankyrin, that binds pRB and facilitates its 26S-proteasome-mediated destruction[149]. Data are as yet too sparse to conclude what the normal role of this protein may be, but the recent finding that it binds CDK4 in competition with p16, but does not inhibit it, suggests that this role may be significant[230].

Function of pRB

Scope of review

With such a broad range of functions, the molecular biology of pRB, and its attendant literature, are necessarily extensive and complex. A comprehensive review would fill several volumes, and given the burgeoning of knowledge in this area, would likely be obsolete before it reached publication. While many aspects of pRB are presented below, the emphasis is very much on the role it plays in tumour-suppression, and in particular, in the regulation of proliferation.

Basis of pRB function

The retinoblastoma-associated protein appears to contain no inherent enzymatic activity and the great weight of evidence is in favour of protein-protein interaction being its dominant operative mode[®263]. If so, its influence depends on its ability to modify the inter-molecular interactions of the bound protein. This may be achieved by one of four major mechanisms, given here in order of decreasing apparent relevance to pRB: masking of interaction domains; constraint of physical location; molecular matchmaking; and alteration of physical conformation.

Several domains within pRB have been implicated in mediating protein interactions, and conserved motifs in proteins that bind pRB have also been identified.

pRB-binding motifs

The LXCXE motif

The basis for the retinoblastoma-like effects of adenovirus infection in rodents became clearer with the discovery that a viral protein, E1A, bound pRB in a step necessary for productive infection[403]. Similar proteins were soon found to be produced by other small DNA viruses[83]. When the sequences of these were determined, many were found to contain a pentapeptide motif, LXCXE, including the adenovirus E1A protein (LV**L**D**C**P**E**NP), the human papillomavirus E7 protein (VD**L**V**C**H**E**QL), and the large-T proteins of SV40 (EN**L**F**C**S**E**EM) and polyomavirus (PD**L**F**C**Y**E**EP). More recently, the sequence **L**P**C**A**E** has been implicated in the pRB binding of the NSP90 non-structural protein from the teratogenic human rubella virus, *Rubivirus*[103]. The novelty here is that *Rubivirus* is not a DNA virus, but a positive-strand RNA virus. This attests to both the crucial role of pRB in mediating cellular affairs, and to the efficacy of the LXCXE motif in modulating this. Once identified, the LXCXE motif was found in many cellular proteins known to interact with pRB {Table 3}, most notably the D-cyclins[74 89].

LXCXE relatives

Two variations on the LXCXE motif have been suggested to operate similarly. The first, IXCXE has been identified in the transcriptional repressor HBP1, however it was shown that it was the LXCXE motif also present that mediated its association with p130[377]. A stronger case for pRB binding by IXCXE exists with HEC[427], although it was not found to be essential for function. The second variant is LXSXE, suggested by Durfee et al.[81] as a possible basis for the binding of PPP1CA-2. They noted, however, that the domains of pRB associated with the binding of large-T and PPP1CA-2, while similar, were not identical, leaving open the possibility of a different mode of interaction. Further supportive evidence for a role for LXSXE comes from the directed-mutagenesis study in *Rubivirus* cited above[103]. In seeking to determine the importance of the **L**P**C**A**E** motif, Forng and Atreya altered the cysteine to arginine, and so showed that this was critical for proliferation. After approximately one generation time, however, the proliferation rate increased in correlation with a spontaneous mutation to **L**P**S**A**E** in the motif of interest.

The LXSXE motif is present in the transcription factors JUN, MYC, BRCA1, E2F4, and E2F1 {Table 3}, considered by many to be the most important pRB-interacting protein of all. Its presence in BRCA1 in addition to an LXCXE motif may account for the continuing ability of BRCA1 to bind pRB when this motif is disrupted[91]. In addition to these, it is present in ARID3B (ER**L**E**S**G**E**PA), ELF1[393] (VQ**L**L**S**S**E**EL), ENC1 (VQ**L**L**S**S**E**EL), GABPB1 (TG**L**V**S**S**E**NS), lamin A/C (AL**L**N**S**K**E**AA, RK**L**E**S**T**E**SR), RBBP6[323] (AL**L**E**S**D**E**HT), and TRIP11 (KK**L**S**S**A**E**ND, KS**L**L**S**Q**E**KE, QL**L**S**S**N**E**NF), all of which are known to bind pRB. Furthermore, it is present in p107 (KH**L**N**S**I**E**EQ) and in pRB itself (SM**L**K**S**E**E**ER), perhaps accounting for reports of oligomerisation in vitro[144], and the reported ability of the C-terminus of pRB to block repression by the A/B pocket *in trans*[136]. The possibility that LXSXE may have a major role in pRB interactions does not appear to have been fully appreciated as there is very little reported in the literature.

The DLXX(X)E motif

While inspection of the viral protein sequences revealed the importance of LXCXE, a further potential binding motif may have been overlooked. The LXCXE motif within the adenovirus E1A protein CR2 region also conforms to the pattern DLXXXE, as it does in polyomavirus large-T and HPV E7. In SV40 large-T, this overlap is absent, but a separate instance of DLXXXE exists (QLM**DL**LGL**E**RSA). A similar

motif, DLXXE, conserved among adenovirus strains, appears in the adjacent CE1 region. This composite motif, DLXX(X)E, is present in five of the proteins listed in Table 3, including two with no other recognised binding motif, notably MDM2. It is present also in MYOD (DSP**DL**RFF**E**DLD), and TRIP11 (LKQ**DL**ND**E**KKR), both of which bind pRB.

pRB protein structure

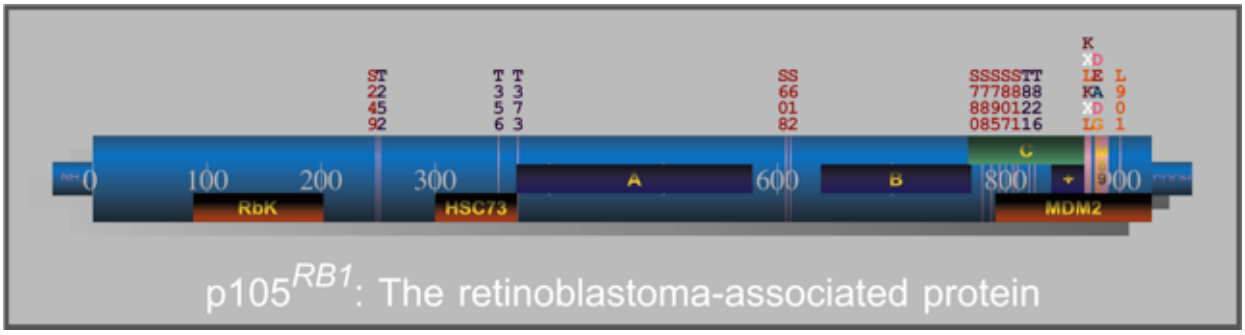


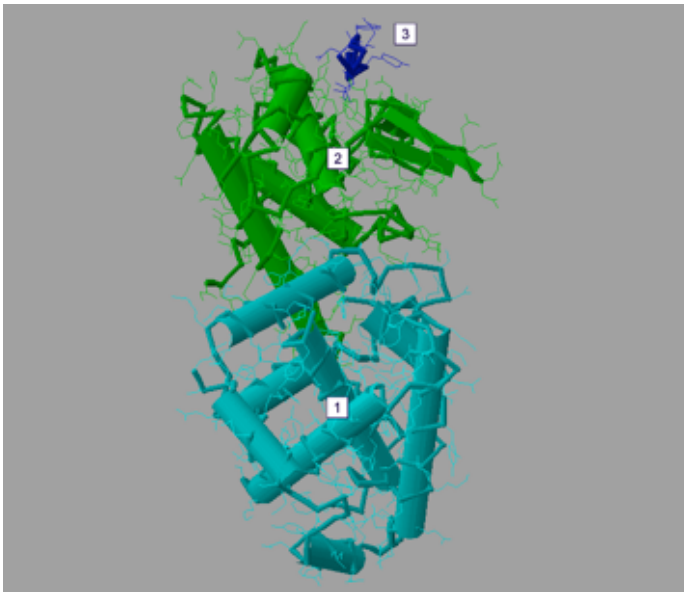
Figure 1: Salient pRB features

N-terminal domains

Sterner et al. have reported two related kinases, both referred to as RbK, that bind pRB within the 89–202 amino acid region, and phosphorylate pRB, and possibly the transactivation domain of MYC, in G_2/M. The pRB domain implicated appears essential for pRB-mediated growth suppression and is altered in some retinoblastoma patients[357 358]. RbK does not appear to have been further characterised. In addition, the heat-shock protein HSP73 associates with the pRB 301–372 amino acid region[170].

The 'A' domain and the 'B' pocket

The investigation of viral protein binding led to the identification of two jointly required pRB domains {Figure 2}: the 'A domain', spanning amino acids 372–578 [1], and the 'B pocket', spanning amino acids 639–770 [2]. These regions have also been shown to be necessary for nuclear tethering of pRB[374], but not for growth suppression[65]. Structural studies[225] suggest that the B pocket domain forms a lobe containing an apical cleft which is the principal binding site [3]. The conformation of B, and therefore of the binding cleft, seems to depend on the intact presence of the A domain. The functional combination of these domains is referred to as the 'small A/B pocket'[310], and it is from this feature that pRB, p107, and p130 derive their designation of 'pocket proteins'.



Key: pRB A domain = light blue; pRB B pocket = green; LXCXE-containing nonapeptide from HPV E7 = dark blue. Data from Lee et al. [225]. Rendered by Cn3D.

Figure 2: The pRB small A/B pocket

The large A/B pocket and the C-pocket

The pRB small A/B pocket is also necessary for binding of members of the E2F transcription factor family[310], and, while this may be sufficient for binding in vitro[185], it seems likely that an additional pRB C-terminal domain within the region spanning amino acids 841–870 [147] is required in vivo[162]. Together with the small A/B pocket, this is referred to as the 'large A/B pocket'. This additional requirement may

in part be a consequence of the absence of the LXCXE motif from E2F. This further suggests that distinct domains within the A/B region may mediate interaction between LXCXE-bearers and E2F, and therefore, this binding need not be competitive. Indeed, simultaneous binding may be essential for function.

This additional domain intersects with the binding domain of the ABL tyrosine kinase, located at amino acids 768–869 and termed the 'C-pocket'[402]. Despite the overlap, it appears that simultaneous binding by pRB of ABL via the C-pocket and either E2F via the large, or cyclin-D2 via the small A/B pocket is possible[401]. Within the C-pocket, at amino acid 792, begins a domain implicated in the binding of MDM2[411]. This same region, albeit imprecisely defined, has also been shown to be necessary and sufficient for the binding of PPP1CA[81].

At the extreme C-terminal end of the C-pocket, a motif 870KXLKXL875 exists that is believed to constitute the principal pRB–cyclin interaction domain for those that do not carry the LXCXE motif, that is, non-D-cyclins. It may also provide an alternative interaction mode for those that do[4]. One consequence of this is that it is required for effective targeting of pRB by CDK2, but not CDK4. Unlike the relatively stable and abiding interaction between the small A/B pocket and cyclin-D1, that between a cyclin and the KXLKXL motif appears to be transitory, serving more to direct and orient the associated kinase with respect to its substrate than to promote an on-going association.

The C-terminal region: amino acids 876–928

Driscoll et al.[78] have identified a region spanning amino acids 880–900, dubbed 'M89', that appears to be a critical determinant of C-terminal pRB conformation, and can significantly affect the accessibility of pRB targets to modifying enzymes, in particular, CDKs. Their work extended to the identification of other key determinants of pRB conformation, noted in Table 5, and provided the first insight into the structural basis for the multiple electrophoretic species of pRB seen.

Cyclin-D1 may have a third mode of interaction with pRB. Pan et al.[292] report that pRB L901 mediates a productive cyclin-D1 interaction that appears to be distinct from that involving the nearby KXLKXL motif. Whether interaction here influences the role of the immediately adjacent M89 region is unknown.

Within M89 is a sequence 883DEADG887, that is a site for caspase-dependent cleavage of pRB during apoptosis[370]. It seems likely that such cleavage would prevent both the association of MDM2, and that of cyclin-D1 mediated via L901.

pRB-binding proteins

Scope of pRB–protein interactions

At least 129 proteins are believed to interact directly with pRB®[263], and a selection of these that have been, or potentially may be, associated with tumorigenesis, is listed in Table 3.

Competition for pRB binding

There appear to have been no definitive and comprehensive studies either of the mutual competition among potential pRB binding proteins for access, or of any precedence among any such competitors. In some cases, specific data are available, and in others, reasonable inferences can be drawn based on the apparent necessity of a single, well-defined pRB domain for binding of more than one protein, as with the B pocket. Slightly less robust implication of non-competition exists in the form of apparent spatial separation and non-intersection of binding requirements. The situation is extremely complicated, as there are undoubtedly multiple interactions among protein binding, covalent modification, and conformation. Such data as pertains to representative proteins interacting via the better-defined pRB domains is given in Table 4.

Protein	Motif(s)	pRB domain(s)	Significance
AATF[92]	DDLGSSEEE LKDLDEEIFD	–	Binding prevents pRB repression of E2F[92] AATF also mediates apoptosis[290]
ABL[402]	VVLDSTEAL	C[402]	Binding inhibits ABL kinase[402]
AHR[307]	DMLYCAESH	Probably AB[307]	Dioxin carcinogenesis[307]
ATF2[295]	–	C-terminus[295]	JUN induction[388] pRB autoinduction[295]
BRCA1[22 91]	QKLPCSENP KKLESSEEN	1) A?B 2) Another [91]	BRCA1 regulates genome surveillance[397]
Cyclin-A[4]	–	870KXLKXL[4]	Proliferation regulation[®64]
Cyclin-E[4]	–	870KXLKXL[4]	Proliferation regulation[®193]
Cyclin-D1[74]	HQLLCCEVE	1) A?B[74 89] 2) C-terminus[§292]	Mitogen response; proliferation regulation[®342]
Cyclin-D2[§89 402]	MELLCHEVT	A?B[§89]	Mitogen response; proliferation regulation[®342]
Cyclin-D3[74]	MELLCCEGT	A?B[74 §89]	Mitogen response; proliferation regulation[®342]
E2F1	QSLLSLEQE	AB+[147 310]	Proliferation regulation[180]; apoptosis[®304]
E2F4[231]	EELMSSEVF	AB+?	Cell-cycle arrest[114]
HDAC1[244]	KRIACEEEF?[1 91]	1) AB? 2) indirect?[213]	Chromatin modelling[244] Modulation of p53 activity[242]
HSP75[45]	EVLFCFEQF	AB[45]	pRB chaperone in M-phase and after heat shock[45]
ID2[218]	–	AB[166]	Implicated in proliferation, differentiation, and apoptosis[102 219]
JUN[278]	LKLASPELE	1) A?B[278] 2) C-terminus[278]	Implicated in proliferation, oncogenic transformation, and apoptosis[®337]
MCM7[358]	–	N-terminal to amino acid 380[358]	DNA replication licensing
MDM2[411]	QKDLVQELQ	C-terminus[411]	Regulation of p53 activity
MYC[320]	QKLISEEDL SLLSSTESS	B[320]	Cellular growth, proliferation, and apoptosis[300]
p21[275]	–	1) AB[275] 2) C-terminus?[275]	Proliferation regulation; senescence
POLD1[208]	GKLPCLEIS	AB[208]	Binding stimulates enzyme activity[208] Required for S-phase DNA synthesis[®150] Required for DNA mismatch[237] and UVR repair[425]
PPP1CA[81]	PDLQSMEQI	C-terminus[81 368]	Regulation of pRB by dephosphorylation[®369]
PRDM2[34]	VNDLGEEEEE PEDLLEEPK TEDLPKEPL GIDLPVENP	A?B[33]	Tumour-suppressing, proapoptotic methyltransferase[®35]
prohibitin[396]	–	B[396]	Inhibitor of E2F transactivation[396]
RAF1[395]	QILSSIELL	A?B[395]	Major receptor tyrosine kinase signal transduction element[®192]
RBBP1[94]	ETLVCHEVD	Probably AB[94]	Repression of E2F-dependent transcription[214]
RBBP4[309]	LKLHSFESH	1) A?B?[309] 2) Indirect[281]	Chromatin remodelling[281 407]
RBBP7[309]	–	Probably A?B[161]	Modulation of BRCA1 function[46]
RBBP8[112]	AELECEEDV	1) Probably AB[112] 2) Another?[65]	Modulation of BRCA1 function
RBBP9[405]	TELHCDEKT	Probably AB[405]	Role in cellular transformation[405]
RFC1[265]	ASLVCQELG KALGSKEIP GVLESIERD	Probably AB[301]	Component of replication factor C; necessary for processive DNA synthesis.
TAF1[335]	KVLSSTEVL SDLDSDE	1) C[335] 2) AB+[346]	RNA polymerase II regulation[®398]
UBTF[40]	YSLYCAELM	Probably AB[40]	RNA polymerase I (ribosomal RNA) regulation[390]

Key: – = no recognised motif, or no binding domain data; B = B-pocket; C = C-pocket; AB = small A/B pocket; AB+ = large A/B pocket; ? = domain implicated, but not proven to be necessary. Binding motifs and domains are described in the text.

Table 3: Selected pRB-interacting proteins

pRB	RbK	HSP73	Cyclin-D1[a]	E2F	ABL	MDM2	PP1α	Cyclin-A
HSP73	–							
Cyclin-D1[a]	(+)	(+)						
E2F1	(+)	(+)	+					
ABL	(+)	(+)	+	+				
MDM2	(+)	(+)	(+)	X	(X)			
PP1α	(+)	(+)	+	(X)	(X)	(X)		
Cyclin-A	(+)	(+)	(+)	+	–	–	–	
Cyclin-D1[b]	(+)	(+)	(+)	(+)	–	–	–	–

[a] = binding via LXCXE motif and small A/B pocket. [b] = binding via pRB C-terminal domain

Key: X = compete for binding; + = can bind simultaneously; – = no data; (+), (X) = inferred

Table 4: Competition matrix for pRB binding

Phosphorylation of pRB

The earliest studies of the retinoblastoma-associated protein revealed that it was a nuclear phosphoprotein[228], and that differences in phosphorylation status accounted for the multiplicity of electrophoretic species[111] seen. This observation facilitated the discovery that the phosphorylation state of pRB altered in synchrony with progression through the cell division cycle, with it being minimally phosphorylated upon synthesis and rapidly and sequentially phosphorylated at the G_1–S transition[255]. The basis for this sequencing lies partly in subtle differences in substrate specificity of the relevant kinases[424] and partly in their successive activation. It is also believed that conformational changes wrought by earlier phosphorylations are necessary to allow subsequent access to other sites. The significance of this sequential phosphorylation lies in the apparent independence of control of protein binding among the different interaction domains within pRB[200]. The proportion of phosphorylated pRB decreases at the beginning at anaphase[238], indicating the existence of regulated phosphatase activity.

pRB kinases

It was soon found that pRB was a substrate for the CDC2 kinase in vitro[234], and of this[229], or related kinases in vivo[198]. The latter possibility was confirmed with the discovery that pRB was a substrate of CDK2[6], CDK4[188], and the closely related CDK6[254]. Of the sixteen potential SER/THR–PRO CDK targets in pRB, thirteen have been found to be phosphorylated in vivo {Figure 1} and considerable data concerning the timing, kinase-specificity and consequence of these phosphorylations have been gathered {Table 5}.

Upon mitogen stimulation, pRB is phosphorylated by RAF1 before it is by cyclin-D–CDK4[395]. This may provide an efficient link between RTK activation and the abrogation of pRB growth-suppression operative independently of that supplied by cyclin-D regulated kinases. This also places pRB downstream of RAS, and so may contribute to the oncogenic potential of the latter[299].

The RbK kinases of Sterner et al., also phosphorylate the pRB N-terminus during G_2/M, and are apparently distinct from CDC2, CDK2, CDK4, MAPK1, and MAPK3[357].

pRB phosphatases

Given the established importance of pRB phosphorylation, and the emerging biological importance of balanced antagonistic kinase/phosphatase pairs, there is a surprising dearth of data concerning the identity and regulation of pRB phosphatases. Using a system based upon the yeast two-hybrid screen of Fields and Song[98], Durfee et al.[81] identified and cloned a protein that directly interacted with pRB, and was found to be the catalytic subunit of a type I protein phosphatase complex (PP1), PP1CA2. Through pRB immunoprecipitation of extracts of human cells at intervals after release from density-arrest, they found that the association of PP1CA2 with pRB was cell-cyclical, occurring in G_1, diminishing throughout S and G_2, and returning in M-phase. By gel-mobility shift, PP1CA2 was inferred to bind the

hypophosphorylated form of pRB, although binding to phosphorylated pRB was not ruled out. Ludlow et al.[238 239] have pursued the timing of dephosphorylation and found that it progresses sequentially.

The mode of physical interaction between pRB and PP1 has not been determined unequivocally. Several authors[81 369] have suggested that the LXSXE sequences present imply association via the small A/B pocket, and therefore in competition with, and susceptible to the same regulation as, carriers of the LXCXE motif. Such an interaction is difficult to reconcile with the ability of PP1CA2 to bind a pRB construct that lacks the entire B domain, but the inability to bind one lacking only the region C-terminal to this[81]. More recent work has provided strong evidence that it is in fact the C-terminal region of pRB that associates with PP1, and in so doing, non-competitively inhibits its phosphatase function[368]. This does not necessarily preclude the involvement of LXSXE, or the overlapping DLXXXE, in this interaction {Table 3}, or that there may also be some affinity between PP1 and the small A/B pocket. It has been established that, as with other pRB-interacting proteins, the binding of PP1 is regulated by the phosphorylation state of pRB, specifically, that phosphorylation of S249, T373, S811, T821, or T826 prevents association at the C-terminus, while that of S608, S612, S780, or S807 does not[369] {Table 5}.

On initial consideration, it appears paradoxical that an enzyme should be inhibited by its principal substrate: how could it ever function? Further reflection in the context of cyclical control of pRB phosphorylation, yields an attractive explanation for this. With PP1 bound to pRB and inhibited, any newly activated pRB kinase can phosphorylate pRB unopposed. In so doing, it may cause the release of proteins bound to pRB, with potentially far-reaching effect. In some cases, the particular pRB molecule that is phosphorylated may have been sequestering PP1, and this too would be released and disinhibited. If the kinase phosphorylated sites that also prevented re-association of PP1 with pRB, then it would be free to oppose the kinase and dephosphorylate pRB. This in turn may render pRB once again able to bind and inhibit PP1, completing the cycle. The net result of these interactions is to provide a limited period during which a variety of pRB regulated enzymes may be activated. This is consistent with the observed cell-cyclical nature of the pRB–PP1 association. In addition to being attractive from a mechanistic viewpoint, such a scenario also explains the otherwise problematic observation that despite inhibition of PP1 by pRB, the former is able to dephosphorylate the latter in vitro. The sequence of events would be that phosphorylated pRB, unable to bind and inhibit PP1, is dephosphorylated by it, whereupon it immediately proceeds to bind and inhibit it.

pRB acetylation

Chan[43] et al. have established that pRB is also the subject of cell-cycle synchronised acetylation, and that this materially affects pRB function by hindering phosphorylation by CDKs and enhancing its affinity for MDM2. The ramifications of this novel aspect of pRB regulation remain to be explored.

S/T	Phosphorylation	Dephosphorylation	Relevance of phosphorylation
T5	In vivo phosphorylation not reported		
S230	In vivo phosphorylation not reported		
S249	Inaccessible when LXCXE bound[424] Phosphorylated by cyclin-D1–CDK4, but may require prior T826 phosphorylation[424]	Begins at M; complete by M+60 min[§319] Dephosphorylated in response to TGFβ1[159]	May prevent PP1α binding[369]
T252	Inaccessible when LXCXE bound[424] Phosphorylated by cyclin-D1–CDK4, but may require prior T826 phosphorylation[424]	Begins at M; complete by M+60 min[§319] Dephosphorylated in response to TGFβ1[159]	No data available
T356	Phosphorylated by cyclin-D1–CDK4[424] Not phosphorylated by cyclin-A–CDK2[424]	Begins at M; complete by M+60 min[§319]	Likely to affect pRB conformation[78]
T373	Phosphorylated by cyclin-D1–CDK4[424] Begins at M+30 min; complete by G_1[§319]	Begins at M; complete by M+30 min[§319] Dephosphorylated in response to TGFβ1[159]	May prevent PP1α binding[369]
S567	No in vivo phosphorylation reported, but suggested based on in vitro data[136]. Not solvent accessible[225]. Mutation prevents pRB-mediated growth arrest and affects protein binding and phosphorylation[374].		
S608	Phosphorylated by cyclin-D1–CDK4 and cyclin-A–CDK2, but not cyclin-E–CDK2[424] Increases during M-phase, peaks at M+30 min[§319]	Begins after M+30 min[369] Complete after M+4 h[§319], that is, in G_1	Probably prevents E2F binding[201]
S612	Phosphorylated by cyclin-A/E–CDK2 but not cyclin-D1–CDK4[424]	No data available	Probably prevents E2F binding[201]
S780	Phosphorylated by cyclin-D1–CDK4 but not cyclin-E–CDK2[197] Increases during M-phase[369] Peaks at M+30 min[§319]	Begins after M+30 min ; complete after M+6 h[§319], that is, in G_1 Dephosphorylated in response to TGFβ1[159]	Probably prevents E2F binding[197] [201]
S788	Phosphorylated by cyclin-D1–CDK4[424]	Begins at M; complete by M+ 60 min[§319]	Probably prevents E2F binding[201]
S795	Phosphorylated by cyclin-D1–CDK4 and cyclin-A/E–CDK2[424] Inaccessible when LXCXE bound[424] Begins at M+30 min; complete by G_1[§319]	Begins at M; complete by M+30 min[§319]	Probably prevents E2F binding[201]
S807	Inaccessible when LXCXE bound[424] Phosphorylation increases during early M-phase[369]	Begins at M; complete by M+40 min[§319] Dephosphorylated in response to TGFβ1[159]	Likely to affect pRB conformation[78] Facilitates further pRB phosphorylation[78] Probably prevents E2F binding[201] Causes dissociation of pRB–ABL complex[200]
S811	Phosphorylated by cyclin-D1–CDK4[424]	Dephosphorylated in response to TGFβ1[159]	Likely to affect pRB conformation[78] Facilitates further pRB phosphorylation[78] Probably prevents E2F binding[201] Causes dissociation of pRB–ABL complex[200] May prevent PP1α binding[369]
T821	Phosphorylated by cyclin-A/E–CDK2 but not cyclin-D1–CDK4[424] Increases from soon after M-phase onset, by M+ 40 min[§319]	Never fully dephosphorylated[§319] May not be a target of PP1 isoforms[§319] Rapid, partial dephosphorylation begins at M[§319] Second partial dephosphorylation begins at M+40 min[§319]	Likely to affect pRB conformation[78] Probably[424] prevents LXCXE binding, but some doubt exists[§319] May prevent PP1α binding[369] May dissociate preformed pRB–LXCXE[424]
T826	Inaccessible when LXCXE bound[424] Phosphorylated by cyclin-D1–CDK4 but not cyclin-A/E–CDK2[424]	Begins at M-phase onset; complete by M+10 min[§319] Preferentially targeted by PP1δ[369]	Prevents LXCXE binding[424] May prevent PP1α binding[369] Does not dissociate existing pRB–LXCXE[424] Prerequisite for S249 and T252 phosphorylation[424]

M = time of release of green monkey kidney fibroblast cells from nocodazole inhibition[§319].

Table 5: pRB phosphorylation summary

4 Phosphorylation-dependent regulation of proliferation by pRB

A minimal proof

That pRB could influence the progression through the cell division cycle was unambiguously demonstrated by Goodrich et al., who injected purified pRB into proliferating cells and discovered that it prevented passage into S phase from G_1[124]. This effect could be overcome by the simultaneous expression of cyclin-A or cyclin-E[151], suggesting that it was phosphorylation of pRB by a CDK that was critical, a possibility supported by the increased phosphorylation of pRB seen in this experiment. Co-expression of E2F1 was also able to overcome the G_1 arrest, and do so without influencing pRB phosphorylation[180], establishing that E2F1 acted either downstream, or independently of pRB. The former appeared the more likely as E2F1 was known to bind pRB and thereby be functionally inhibited[101]. Further support came from the finding that E2F1 bound unphosphorylated pRB, but not that phosphorylated by cyclin-A–CDK2, cyclin-E–CDK2 or cyclin-D1–CDK4[363]. The final link necessary to connect pRB with entry into S-phase, and therefore control of cellular proliferation, is provided by the preponderance of genes among the transcriptional targets of E2F1 whose encoded proteins are critical to this progression. Among these proteins are DNA pol-α, TS, PCNA, cyclin-E, cyclin-A, and CDC2[59]. Therefore, it can reasonably be concluded that the phosphorylation-dependent release of E2F1 from pRB inhibition regulates progression from G_1 to S phase. As a corollary, whatever influences the phosphorylation status of pRB is likely to influence progression through the cell-cycle[58].

A model scenario

Caveat lector

The enormous complexity of pRB interactions defies exposition in any readily assimilable manner. Nevertheless, a 'thought experiment' involving a model system, wherein cells arrested in G_1 by virtue of an absence of mitogens are stimulated to proliferate, can provide a basis from which a possible sequence of events can be deduced from experimental observations. Of necessity, simplifying assumptions have been made. For each of the proteins cited, multiple close relatives with overlapping but distinct characteristics exist, and their expression and interactions may vary with organism, cell-type, and physiological context. As a result, the scenario presented may be neither generally applicable, nor even applicable in any particular case.

G_1 arrest

When cells arrest in G_1 for want of mitogenic stimulation, pRB is essentially unphosphorylated and therefore competent to bind proteins via any of its interaction domains. E2F1/2/3–DP1/2/3 transcription factors, able to associate via the pRB large A/B pocket are favoured candidates, and in this way pRB is localised to the promoter of E2F-regulated genes. The interaction between these molecules involves the transactivation domain of E2F, and this is thought to contribute to gene repression.

This binding does not prevent pRB interacting with additional proteins through other domains. There is general agreement[31 244] that pRB is able to recruit active HDAC1 to E2F, but opinion is divided over how this occurs. Much of the controversy centres on the putative binding of the HDAC1 `IXCXE` sequence to the pRB small A/B pocket. Magnaghi-Jaulin et al.[244] found that deletion of this sequence strongly decreased binding, as did the presence of a synthetic `IXCXE` peptide, while an `LXCXE` peptide was an even better competitor. Consistent with this, Dahiya et al.[56] found that mutation of the pRB `LXCXE` binding cleft prevented HDAC1 association. Conversely, two groups have arrived at precisely the opposite conclusion[65 191]. The second area of controversy is over whether the interaction between pRB and HDAC1 is direct or mediated by an additional protein. The results of Magnaghi-Jaulin et al.[244]

support the notion of a direct interaction between the two, involving the A/B pocket, but not the C-terminal region of pRB. Others have proposed a matchmaking role for RBBP1[213] or RBBP4[191]. These apparently contradictory results are perhaps most easily reconciled by assuming that all of these interaction modes occur, and that differences in experimental conditions are responsible for the discordant results.

However HDAC1 binds pRB, it does so coincidently with pRB dephosphorylation[308], being bound in early G_1. At that time, it deacetylates amino-terminal lysine amino acids of nucleosomal core histones, reinstating the positive charge there. This is thought to enhance the affinity of the core for DNA, and thereby deny access to the promoter by the transcriptional apparatus and thus repress the gene. It is released at the transition to S-phase[308], coincident with the observed acetylation of histone H4[97] and nucleosomal relaxation.

With many genes whose transcription is necessary for S-phase progression having E2F binding sites in their promoters, unphosphorylated pRB, will cause cell-cycle arrest at this point.

Release from inhibition

Cyclin-D1 elevation

There is a low level of constitutive expression of *CCND1* mediated through CREs in its promoter[272], but in the absence of mitogenic stimulus, cyclin-D1 is rapidly degraded via the ubiquitin-directed proteasomal subsystem[67 117], its half-life being of the order of ten minutes. This situation changes abruptly upon mitogen stimulation, when cyclin-D1 levels rise dramatically[240]. Two mechanisms are though to be involved in this elevation.

Firstly, the rate of transcription of *CCND1* is increased. While studies in a variety of cell-types have uncovered elements of the signal transduction path leading to this activation, no overall pattern of general applicability has yet emerged, and apparent contradictions exist. The transcription factor MYC directly induces cyclin-D2[30], and probably also cyclin-D1[302], and consistent with this, the level of cyclin-D1 expression closely parallels the activation of MYC. The transcription factor LEF1 has also been shown to contribute to *CCND1* expression[344]. Strongly implicated are proteins with homology to RAS. RAS itself may initiate multiple independent molecular cascades leading to increased *CCND1* transcription. When activated by ectopic expression[§99], or by PDGF[291] stimulation, it can increase *CCND1* transcription via MEK1, MAPK1, and ultimately SP1 sites[272] in the promoter. Additionally, it may operate via MAPK3 and JUN, ultimately via an AP-1 promoter site[8]. The role of the different MAPK enzymes is not entirely clear as p38MAPK has been reported both to enhance *CCND1* transcription via ATF2 promoter sites in response to HGF stimulation[314], but also to cause a reduction in this rate[221]. Two RAS homologues, Rac1[§183] and Ral[§143] have been shown to influence *CCND1* transcription, apparently via the NF-κB subsystem.

The second mechanism of cyclin-D1 elevation is the enhancement of protein stability, and here, members of the PI3K family are involved. In addition to possible activation by RAS, PI3K is also downstream of G-protein-coupled membrane receptors[331], providing a further link between extracellular conditions and cyclin-D1 regulation. However activated, PI3K, probably via AKT1[120] or another protein kinase B, can inhibit the GSK3β enzyme that is responsible for phosphorylation of cyclin-D1 `T286`[67] which would otherwise mark it for nuclear export[12] and accelerated degradation[68]. Without this proteolysis, the half-life of cyclin-D1 rises to over one hour.

The mechanisms of enhanced cyclin-D1 expression are very complex, with multiple inter-links among the RAS, MYC, MAPK, and PI3K subsystems, multiple binding sites in the promoter, and multiple independent degradative pathways[120 ®332].

CDK4 activation
With cyclin-D1 levels elevated, and its cellular disposition increasingly nuclear, the opportunity for interaction with CDK4 increases. With three provisos, this will enable the CDK4 kinase function. Firstly, the association of cyclin-D1 with CDK4 is dependent on a serum-inducible assembly factor[251], possibly p21[297]. Secondly, CDK4 activity depends on its phosphorylation state, which in turn depends on the relative activities of CAK and CDC25A, which is itself subject to upstream regulation. Finally, complex assembly and kinase activation are both subject to inhibition by CKIs, particularly $p16^{CDKN2A}$ and its relatives, and this may be further influenced by gankyrin {*See 'Turnover of pRB', above*}. Clearly, CDK4 is at a major regulatory node.

Initial pRB phosphorylation
Cyclin-D1, in this case, with its attendant activated CDK4 partner, can bind pRB either via the latter's small A/B pocket and its own LXCXE motif, or via an additional C-terminal pRB domain {Table 3}. Within the constraints of the model scenario being explored, only the second docking mode is available since the small A/B pocket is hypothesised to be occupied by HDAC1 or its linking protein. This has important implications for the functional scope of CDK4 since when docking is via the pRB C-terminus, S807 and S811 cannot be phosphorylated[292]. Furthermore, a number of pRB CDK4 target sites are inaccessible when a protein is occupying the B pocket[424]. Phosphorylation at one of these, T826, appears to be a prerequisite for subsequent phosphorylation at S249 and T252, possibly influencing the regulation of N-terminal interacting proteins. These phosphorylations cannot therefore proceed at this time. Of the thirteen in vivo phosphorylation targets within pRB, given the substrate specificities, pRB conformation and steric constraints, the immediate CDK4 targets available in the model scenario are T356, T373, S608, S780, and S788.

Persistence of small A/B pocket interactions
These initial phosphorylations do not appear to suffice to cause the general dissociation of proteins interacting with pRB via the small A/B pocket as phosphorylation of T821 may be essential for this, and it is not a substrate for CDK4[424]. While T826 is a potential CDK4 target, phosphorylation here may not cause dissociation of existing complexes, even if it can prevent their formation[424]. This may be moot in this instance since T826 appears to be inaccessible when any protein is occupying the B pocket, as is assumed here. Hence, proteins interacting with pRB via their LXCXE motif and the small A/B pocket are immune to eviction by cyclin-D1–CDK4.

The situation is less clear with respect to HDAC1, as the mode of its attachment is uncertain. It has been suggested by Harbour et al.[136] that phosphorylation of pRB by CDK4 is sufficient to cause dissociation of pRB-HDAC1 complexes, but some doubt exists over this. Certainly, in co-transfection experiments they were able to establish that the ability of HDAC1 to bind via the pRB small A/B pocket is disrupted in the presence of cyclin-D2. Simultaneously, they found that a co-expressed pRB C-terminal fragment became phosphorylated, and that irrespective of its phosphorylation state, it was able to bind the pRB small A/B pocket, even when HDAC1 could not. However, their conclusion that the C-terminal domain is involved in inhibiting binding of HDAC1 is questionable. They appear to have given no consideration to the ability of co-expressed cyclin-D2 to interact directly with the small A/B pocket via its LXCXE motif. Within the context of a co-transfection, expressed cyclin-D2 could simply have out-competed HDAC1 or

its linking protein for binding. Nor did they address the possibility that cyclin-D2-dependent phosphorylation of the small A/B pocket itself may have inhibited HDAC1 binding. Unfortunately, based on this report, the suggestion that cyclin-D–CDK4 can displace HDAC1 from pRB has entered the literature and been adopted[369].

Transcriptional activation

Notwithstanding this uncertainty, a mechanism exists whereby HDAC1 can be removed from the proximity of the promoter in consequence of CDK4 phosphorylation. It depends not on the severance of the link between pRB and HDAC1, but on that between pRB–HDAC1 and E2F. Phosphorylation at `S608`, `S780`, or `S788` is sufficient to prevent binding of E2F to pRB[201], and while there appear to have been no definitive studies, it is assumed to suffice to dissociate existing complexes. If so, an early consequence of CDK4 activation will be the detachment of pRB, with its attendant histone deacetylase complex, from the promoter-bound E2F transcription factor. With the local deacetylase concentration reduced, acetylation of the core histones becomes possible, and with it, a loosening of the nucleosomal structure and the granting of access for the transcription apparatus to the E2F-regulated gene. This process has been reported recently in some detail by Morrison et al. with respect to the gene for cyclin-E1[§264].

Interestingly, TAF1, a component of the RNA polymerase II complex with serine kinase[69], histone acetyltransferase[258], and ubiquitin ligase capacity[§303], also binds pRB via the large A/B pocket, resulting in the inhibition of its kinase, but not its acetyltransferase function[346]. While it has not been established experimentally, the apparent coincidence of pRB domains mediating E2F and TAF1 interaction suggests that TAF1 may also be evicted from pRB complexes by activated CDK4. This would be consistent with the reported ability of cyclin-D1 to bind TAF1 independently of pRB and prevent the inhibition of its kinase function by the latter[347]. This interaction may also affect transcription from promoters containing SP1 binding sites[5]. This modulation of TAF1 function may well influence RNA polymerase II transcriptional rate or specificity at exactly the time when such a control is required: the onset of S-phase[®398].

Following the de-repression of E2F-regulated genes, many of which encode proteins essential for the synthesis and repair of DNA[305], there follows a period of active transcription and protein synthesis in preparation for S-phase. It is at some point during this period that entry into S-phase becomes inevitable.

Passage through the restriction point into S-phase

The term 'restriction point' was coined by Arthur B. Pardee[294] to describe:

> ...a single switching point in G_1 ... that regulates the reentry [sic] of a cell into a new round of the cell cycle.
>
> *Proceedings of the National Academy of Sciences of the USA*, **71**:1286–90, 1974

Factors that cells may encounter in vivo, such as 'high cell density, nutrient or serum insufficiency, or high cAMP [levels]' would cause an arrest at this point, while 'non-physiological agents such as hydroxyurea or colchicine' would not. The reference to cAMP as a cause of arrest in its own right reveals that its role in signal transduction was then unrecognised. The principle that Pardee wished to establish was that stimuli of diverse origins converged at a unique, crucial, biochemical decision point. If passed, a cell would be committed to continuing through the cell-cycle.

For a time, it was thought that passage beyond this point signified a commitment to execute a complete cellular division, and that it was the only physiological determinant of this progression. This has proven not to be the case, nor was it ever suggested by Pardee, who proposed only that it controlled re-entry to the cycle. The restriction point must be considered only as a point of commitment to enter S-phase, but this is still a very significant function that is now recognised to contribute not only to the integration of extracellular growth signals, but also to purely internal signals, particularly those related to differentiation and senescence. From this definition, it is reasonable to conclude that it is the de-repression of E2F that is the crucial step that constitutes this transition. CDK4-sponsored release of E2F from pRB may be an initial step, but it does not suffice. As described, phosphorylation of pRB by CDK4 may lead to the release and disinhibition of PP1, an antagonistic phosphatase, and the phosphorylation state of pRB becomes dependent on which of the two predominates. If mitogen stimulation continues, cyclin-D continues to be elevated, and CDK4 remains active. If mitogen stimulation abates, or a CDK4 inhibitor is induced, the phosphatase will prevail and E2F will be again sequestered. To this point, the process remains reversible, and the restriction point has not been passed.

Under these conditions, there will be some transcription of E2F targets, although this may be intermittent. Among these is *CCNE1*, the gene for one isoform of cyclin-E[115]. *CCNE2*, the second cyclin-E gene may also be under E2F regulation, but this has not yet been established conclusively[§116]. In time, with continuing CDK4-dependent partial activation of E2F, production of cyclin-E will outpace its degradation, and activated CDK2 will enter the equation. Two properties of cyclin-E–CDK2 are of note at this point. Firstly, it is not subject to inhibition by a major class of CDK4 inhibitors, the p16-related CKIs. Thus, if activation of CDK4 had been being constrained by the presence of such inhibitors, but still had managed to rise to a level sufficient to allow the accumulation of cyclin-E, the inhibitors immediately lose any ability to constrain further progression. The second salient feature is that activation of CDK2 by ectopic expression of cyclin-E is sufficient to promote S-phase entry, and, most importantly, do so even in the presence of a non-phosphorylatable form of pRB[241]. The inference therefore is that the only critical target of E2F may be cyclin-E. The production of other proteins from E2F-regulated genes may be rate-limiting for DNA synthesis, but it seems that even constitutive levels of expression are sufficient to allow its commencement.

While immune to inhibition by p16-related CKIs, CDK2 is subject to regulation by p21-related CKIs, in particular, p27. Cyclin-D1–CDK4 also binds and is inhibited by p27[380], and an interesting dynamism exists in the inter-relationships among p16, p27, cyclin-E–CDK2, and cyclin-D1–CDK4. When p16-related inhibitors are absent, whatever p27 is present in the cell will bind cyclin-D1–CDK4 as it is produced, delaying the onset of pRB phosphorylation. However, once it starts, and cyclin-E–CDK2 begins to accumulate, it will do so in the absence of competition from p27. Furthermore, p27 is itself a CDK2 substrate, and when phosphorylated, becomes the subject of ubiquitin-directed proteolysis[389], further enhancing CDK2 activity. Conversely, if p16-related inhibitors are present, such p27 as exists is free to inhibit the low levels of activated CDK2 that may be produced under these circumstances, and thus forestall the self-reinforcing accumulation of CDK2. The apparent induction of p16 upon pRB phosphorylation would contribute to this[232].

While the critical CDK2 target has not been identified, a strong candidate is CDC6, a component of the DNA replication licensing subsystem. CDC6 is an excellent in vitro substrate for cyclin-E–CDK2, with the same pattern of phosphorylation as is seen in vivo, and this phosphorylation is required for the

initiation of DNA synthesis[178]. In serum-deprived cells, ectopically expressed CDC6, in conjunction with cyclin-E–CDK2, but not cyclin-A–CDK2, results in the commencement of DNA replication[§53].

Further pRB phosphorylation

Within the model scenario under consideration, the activation of CDK2 assures entry into S-phase, and synchronisation with the centrosomal division cycle. If these functions were considered insufficiently noteworthy, it has yet another role: further phosphorylation of pRB, probably mediated via the pRB C-terminal KXLKXL sequence[4]. Immediate CDK2 targets include S612 and T821. The significance of the first is unknown, but the second is thought to bring about a conformation change[78] that reduces the affinity of the small A/B pocket for LXCXE-bearing proteins, and probably causes dissociation of such complexes[424]. With their departure, other sites previously masked from the cyclin-D1–CDK4 complex docked at the C-terminus become available including S795, also a target of CDK2, and T826. Phosphorylation at the latter then renders S249 and T252 available to cyclin-D1–CDK4[424]. In the final step, cyclin-D1–CDK4 complexes can now dock via the vacant small A/B pocket, even if only transiently, and effect the phosphorylation of S807 and S811, inaccessible from the C-terminus. In consequence of these alterations, ABL is released and disinhibited whereupon it is thought to take part in the monitoring of genomic integrity in conjunction with ATM and p53[194]. There is a functional parallel here with the simultaneous induction of ARF by E2F1 resulting in increased levels of p53.

Phosphorylation at all of the sites where it is seen in vivo has now been completed. Interestingly, it occurred in five stages, the same as the number of major pRB electrophoretic species discernible in Western blots of asynchronous populations[78]. The functional consequences of these final phosphorylations have yet to be fully explored, and given the very large number of proteins that interact with pRB, this will be no small feat.

Maintenance of pRB phosphorylation

The reign of cyclin-E–CDK2 is relatively short-lived. By activating CDK2, cyclin-E has been the author of its own demise since its phosphorylation at T380 by CDK2 results in its degradation via ubiquitin-directed proteolysis[408]. The preferred model has it that this phosphorylation causes the dissociation of cyclin-E from CDK2, rendering it subject to the ubiquitin-ligase function of CUL3[350]. Nevertheless, phosphorylation of pRB can be maintained as rising cyclin-A, another E2F1 target, continues to activate CDK2.

Dephosphorylation of pRB

This too comes to an end in metaphase, when cyclin-A also becomes a target of proteasomal degradation, here at the instigation of the cyclosome. Only then does the driving force behind pRB phosphorylation abate sufficiently to allow the opposing phosphatase any opportunity to reverse the process. Like its phosphorylation, the dephosphorylation of pRB is synchronised with the cell-cycle and appears to be incremental[§319].

Variations on the theme

Continuous cycling

The extent of dephosphorylation depends in large measure on the cellular context at the time. In particular, if mitogens are still present and p16-related inhibitors absent, cyclin-D1–CDK4 will still be active, although RAS stimulation of *CCND1* may only be operative in G_2[§153]. Not only will this prevent complete dephosphorylation of pRB by antagonising PP1 activity, it may modify PP1 directly through phosphorylation[197]. In any case, cyclin-D1–CDK4 can only oppose PP1 with respect to sites that are substrates for both. Thus, the initial dephosphorylation may be limited to S612 and T821. However,

transient dephosphorylation of T826 may occur, and during the period when both T821 and T826 are dephosphorylated, pRB again has the capacity to interact via the small A/B pocket. Subsequent re-phosphorylation of T826 by cyclin-D1–CDK4 may be insufficient to dissociate such a newly formed complex. One consequence of this that S249 and T252 may also be subject to dephosphorylation as access by cyclin-D1–CDK4 here depends on prior T826 phosphorylation and is hindered by B-pocket occupancy, unless, presumably, the occupant is cyclin-D1 itself. This raises a further distinction between the situation that pertains in cells released from mitogen deprivation and those cycling continuously. In the latter case, this mode of docking is available to cyclin-D1, whereas in the former, it is denied access by the presence of HDAC1 or its linking protein. Now, the tables are turned, and cyclin-D1 is in the position to prevent the recruitment of the deacetylase complex. In addition, S807 and S811 will be subject to phosphorylation, albeit transiently, and can partially impede re-sequestration of ABL. Finally, the continuing phosphorylation of S795 probably suffices to prevent re-association between pRB and E2F.

In all probability then, in the continuing presence of mitogenic stimulation, all of the recognised means by which pRB constrains proliferation are disabled. This does not imply that such cells can cycle freely. Requirements of chromatin decondensation, E2F production, CDK2 activation, and DNA replication licensing must still be met. A change in cyclin-D1 status before the next passage through the restriction point would alter the situation markedly.

Inhibitory cytokines

Inhibitory cytokines have the capacity to prevent cellular proliferation even in the presence of mitogens. One of the better studied and understood of these is TGFβ, a potent inhibitor of epithelial cell division. It has been found to operate through several signal transduction channels including SMAD®[177], MAPK[158], and PI3K[17] subsystems, and several mechanisms of engendering cell-cycle arrest in G_1 have been identified. It depresses MYC transcription[414] and possibly via this, reduces cyclin-D1 expression[203] and induces p15^{CDKN2B}[333], an inhibitor of CDK4; it induces p21^{CDKN1A}[293], an inhibitor of CDK2; it decreases the activity of both CDC25A[164 165] and CAK[271], contributing to the inactivation of existing CDKs; and it may interfere with the translation of CDK4 mRNA[257]. These results suggest very strongly that modulation of the pRB subsystem is an important component of the growth inhibitory effect of TGFβ.

Cellular senescence

Observations by Leonard Hayflick[155 ®339] revealed that cultured human fibroblasts could sustain only a limited number of population doublings prior to undergoing a phenotypic change and ceasing to proliferate. In contrast, cultures derived from tumours appeared to be immortal. This established as the norm the concept of cellular, or replicative, senescence, an inherent proliferative limitation, and its defeat as a feature of neoplastic transformation. Its existence implies a cellular memory that survives mitosis, but the molecular basis of this memory is still a subject of experiment and debate.

An extremely attractive candidate mechanism involves the maintenance of the distinctive base sequences found at the termini of chromosomal DNA, known as telomeres®[359]. The normal process of DNA replication cannot access these final bases since new bases are appended at the trailing edge of the polymerase as it proceeds along the template strand. When it reaches the terminus and dissociates, the single-stranded sequence to which it had been binding must remain unreplicated. This is a progressive process, and in most tissues, telomeres are seen to shorten with each round of DNA synthesis[11]. In some tissues however, the enzyme telomerase is expressed that has the capacity to concatenate telomere

sequences onto these termini using an inherent RNA template; it is, therefore, a reverse-transcriptase, the first found in eukaryotes. Such tissues include the germ-line and those with an extremely high cellular turnover rate, such as haematopoietic cells and cells of the intestinal lining. Aberrant expression of telomerase is also a feature of cancer cells[154]. An alternative explanation of this cellular memory may involve the simple mechanism of gradual accumulation of a regulatory protein due to a slight bias in favour of expression over degradation[118]. It is also entirely possible, and suggested by many[181 270], that several independent mechanisms of replicative senescence exist, and that the relative importance of these may differ among cell-types.

While the details of replicative senescence remain elusive, a number of critical elements have been characterised. These include the telomerase reverse-transcriptase, TERT[66], ATM[253], p53[27], CDC25A[324], CDK4[313], p16[79], p21[327], p27[10], and pRB[199]. There is thus very strong circumstantial evidence that modulation of pRB subsystem activity, probably through altered phosphorylation, is involved in the regulation of senescence.

Viral infection

Viruses are able to carry out their vital and defining functions utilising a genome of tens of genes, in stark contrast to all other classes of organism, where thousands to hundreds of thousands are more usual. They are able to do so by usurping cellular regulation and perverting the host cell metabolism to their own ends. It is therefore of great interest that in many DNA and retroviruses, a large proportion of the reduced viral genome is dedicated to the nullification of the pRB subsystem. Typically, this is achieved by carrying a gene that encodes a protein that binds to the pRB small A/B pocket via an `LXCXE` motif. This is often portrayed as a means of defeating the pRB-dependent constraint on cellular proliferation, but there is no reason why this should be required for viral infection to proceed, nor is it sufficient to achieve this. To do so would require that the binding of a viral protein to pRB interfered with the constraint of E2F activity. This is not the case, however, as it is insufficient to disrupt pRB-E2F complexes[422] and furthermore, the ability of pRB to bind such proteins and to impose a cell-cycle arrest are functionally separable[44 65].

What then is the function served, from the viewpoint of the virus, or defeated, from the viewpoint of the cell, by such binding? By binding in the small A/B pocket, a viral protein will prevent the recruitment of the histone deacetylase complex to gene promoters and so diminish the ability of pRB to repress transcription of genes used in the synthesis of DNA, something beneficial to the virus. When bound there, it will also deny this docking mode to cyclin-D1, and thereby prevent phosphorylation of `S807` and `S811`, as these are not accessible from the C-terminal docking domain[424]. In consequence, ABL will not dissociate from pRB[200] and it will remain inhibited[402]. One substrate of the ABL kinase is MDM2, and its phosphorylation prevents it binding to, and directing the degradation of p53[121]. Therefore, on-going inhibition of ABL by pRB may contribute to the suppression of the p53-dependent apoptotic response that could otherwise be triggered during the infection of mitogen-stimulated cells. ABL can also promote apoptosis via p73[®394], and this effect is also nullified by the continuing association of ABL with pRB. While the suppression of apoptosis may be required in order to give the infecting virus the opportunity to replicate, this interpretation is difficult to reconcile with the general observation that expression of a viral pRB-binding protein such as E7[361], E1A[375], or large-T[52], promotes rather than inhibits apoptosis, especially where p53 is not disabled by an additional viral protein[375].

Complications

As noted above, the model scenario presented incorporates many simplifying assumptions, particularly regarding the multiplicity of related proteins of each type involved. At last count, there are three pRB-related pocket proteins[®49 ®360], six E2F transcription factors[®382] that may dimerise with one of three DP co-factors[®418], three D-cyclins[®342], two E-cyclins[298 ®325], two A-cyclins[®26 ®64], perhaps four CDKs implicated in G_1–S transition regulation[®142], four CKIs related to p16[®318 ®378], and three related to p21[®277]. This discussion could not be complete without some indication of the distinctions among these.

Generally, depending on their lineage, cells express cyclin-D2 and either cyclin-D1 or cyclin-D3. All contain the `LXCXE` motif {Table 3} and all are thought to bind pRB. All can bind CDK2, CDK4, and CDK6, and all can activate them, except in the case of cyclin-D1–CDK2[148]. This may well account for the biphasic response seen with ectopic expression of cyclin-D1, wherein a small increment of expression accelerates S-phase entry, but a larger increment causes a G_1 arrest. In the first instance, increased CDK4 activity would cause the acceleration, but when the available CDK4 is saturated, additional cyclin-D1 would act as a competitive inhibitor of CDK2[109], preventing its activation by cyclin-E.

E2F1, -2, and -3 associate with pRB, rather than p107 or p130; have an N-terminal domain that binds cyclin-A, but not cyclin-E, that is essential for phosphorylation of the DP co-factor; and are exclusively nuclear. E2F4 and -5 associate with p107 and, particularly so in the case of E2F5, p130. An association between E2F4 and pRB has also been reported commencing at the G_1–S transition[259] and in the growth-suppressive response to TGFβ[231]. E2F4 is the predominant form found in quiescent cells, when it is essentially nuclear, this localisation depending on DP2, and p107 or p130, but not pRB. As cells approach S-phase, it becomes increasingly cytoplasmic[235], and when engineered to remain nuclear, is functionally indistinguishable from E2F1[269]. E2F6 has no transactivation domain or pocket-protein-binding domain and may be a natural inhibitor of the other E2Fs[36].

The CKI p15^{CDKN2B} has a more polarised tissue-dependent expression than p16, being present at high levels in lung, but scarce or absent in kidney. Also unlike p16, its expression is not regulated by pRB, nor is its mRNA level different in proliferating versus quiescent cells, but it does increase some thirty-fold in response to TGFβ treatment of epithelial cells[133]. Like *CDKN2A*, it has been reported to be subject to transcriptional silencing through promoter methylation[146]. The p18^{CDKN2C} inhibitor has greatest expression in skeletal muscle, and may[378] be a better inhibitor of CDK6 than of CDK4[130 282]. The p19^{CDKN2D} inhibitor has expression linked to the cell-cycle that peaks at the G_1–S transition and then declines until mitosis.

Protein levels of the pRB-relatives, p107 and p130 vary cell-cyclically, and at least in the case of p130, this is due to alteration of protein translation or stability as the mRNA level stays essentially constant. Interestingly, their patterns of expression are mutually inverted. Levels of p107 are low in quiescent cells as a consequence of repression via E2F4, and rise during G_1, while those of p130 are high in quiescent cells and low during proliferation[351]. Both are subject to cell-cyclical phosphorylation, and while both are substrates for CDK4, neither is a substrate for CDK2[23]. Indeed, they are either inhibitors of CDK2[38 410], or influence its substrate specificity[139]. Consistent with this, phosphorylation of both begins in mid-G_1 coincidently with CDK4 activation[412]. In the case of p130, this proceeds rapidly and completes before that of pRB[252].

5 The pRB subsystem and cancer

The pRB-related pocket proteins

While homozygous mutant *Rb1* mice die *in utero* with severe developmental flaws, the corresponding heterozygotes are viable, but spontaneously develop pituitary tumours[§157]. In the analogous human situation, it is of course predisposition to retinoblastoma that is seen. It is widely reported that despite having been cured of their initial tumour, survivors of hereditary retinoblastoma are at increased risk of developing second and subsequent primary tumours[2 3 63 75 80 85 260 404], notably osteosarcoma[134], and often die during childhood or adolescence as a result. While there may be an iatrogenic component to this, as with increased bladder leiomyosarcoma after cyclophosphamide treatment[190 266], the major effect is thought to be due to the functional loss of pRB upon mutation of the intact allele in other tissues. The nature of subsequent primary tumours is probably a joint reflection of the vulnerability to mutation, and the importance of the tumour-suppressor function of pRB in different tissues. Among tumours other than, or as sequelae of retinoblastoma, alterations of *RB1* or expression of pRB are also widely reported, instances being in breast carcinoma[125], chodrosarcoma[14], glioma[140], small-cell lung cancer[421], non-small-cell lung cancer[126], oesophageal squamous cell carcinoma[187], pituitary adenoma[349], hepatocellular carcinoma[163], osteosarcoma[24], thymic carcinoma[152], and head and neck squamous cell carcinoma[215]. In addition, aberrant over-expression of pRB has been reported in bladder carcinoma[25] and hepatocellular carcinoma[163].

In contrast, the other members of the pRB-related pocket protein family appear to be less important in tumour suppression. *Rbl1*-null mice are viable, and are reported to be either phenotypically normal[§226], or growth-impaired and exhibiting myeloid hyperplasia[§222]. A similar disparity exists for *Rbl2*-null mice, with both apparent normality[§51] and embryonic lethality[§223] being reported. It has been suggested that the particular genetic backgrounds of the differing mouse strains used in these experiments may account for this phenomenon. Nevertheless, even in the more permissive C57BL/6 strain the double, *Rbl1*/*Rbl2* homozygous knockout results in early neonatal death[§51 ®§127], indicating that they may have overlapping abilities to perform a function critical for survival. Alterations affecting p130 have been reported in a few human tumour types, including vulvar squamous cell carcinoma[423], nasopharyngeal carcinoma[50], Burkitt's lymphoma[48], and small-cell lung cancer[141]. Alterations affecting p107 appear to be very rare[367].

The D-cyclins

The oncogenic potential of cyclin-D1 is well established[®73], indeed it was the search for an 11q13 oncogene associated with BCL and parathyroid adenoma that led to its identification[317]. In the case of BCL, it was found that chromosomal translocation resulted in aberrant expression of cyclin-D1, not normally produced by B or T lymphocytes. Moderate over-expression has been reported in many carcinomas including hepatocellular (58%)[182], lung non-small-cell (37%)[415], head and neck squamous cell (48%)[20], and those of the breast (35%)[430], and bladder (31%)[288].

Over-expression of cyclin-D2 has been reported in a number of myeloid malignancies[61], sometimes as a consequence of BCR–ABL activity[60]. It is seen in male germ-cell tumours[156]; and in gastric cancer, it correlates with progression, while over-expression of cyclin-D1 does not[366]. Conversely, loss of expression due to promoter methylation has been reported in breast carcinoma[87].

Chromosomal translocations resulting in the aberrant expression of cyclin-D3 have been found in a subset of multiple myeloma cell-lines and tumours[338] and *CCND3* has been found to be amplified in a

glioblastoma[209]. Over-expression has been reported in pancreatic adenocarcinoma[174], non-Hodgkin's lymphoma[262], and breast carcinoma[21].

The cyclin-dependent kinases

There is no strong case to support a direct role for CDK2 in tumorigenesis, although some over-expression and increased activity have been reported[196 246 256]. While the same is true for CDK6[216], the corresponding case for CDK4 is very substantial. A germ-line *CDK4* `R24C` mutation that prevents binding and inhibition by p16 has been found in melanoma[406], and mice engineered to be homozygous for this allele spontaneously develop multiple tumours[352], particularly invasive melanoma[353]. A mutation in the corresponding position in CDK6 has been sought, but not found[340]. *CDK4* is amplified in cervical carcinoma[47], osteosarcoma[400], breast carcinoma[13], glioblastoma[316], and Ewing's sarcoma[212]; and CDK4 is over-expressed in oral and pharyngeal carcinoma[206], glioblastoma[216], cervical carcinoma[47], breast carcinoma[13], hepatoblastoma[195], and ovarian carcinoma[248].

The cyclin-dependent kinase inhibitors

p16 and relatives

Three proteins structurally similar to p16^{CDKN2A} and with overlapping function are known: p15^{CDKN2B}, p18^{CDKN2C}, and p19^{CDKN2D}. Assessing the contributions toward tumour suppression of the closely-linked 9p21 genes *CDKN2A* and *CDKN2B* and their encoded proteins ARF, p16, p15, and its p10[384] and p15.5[113] splice variants is no simple task. Co-deletion of the genes is commonly reported, as is simultaneous transcriptional silencing due to methylation, but combined inactivation by different mechanisms is also known. In consequence, it is difficult to determine if only one, either, or both are the functional targets, and what, if any, tissue specificity there may be among these alternatives.

CDKN2A is undoubtedly a tumour-suppressor gene of stature rivalling *TP53*. It seems likely that the two proteins it encodes, p16[®104] and ARF, contribute independently toward this[®341]. This is perhaps best demonstrated by the phenotypes of mice engineered to be functionally deficient in each of these proteins without compromise of the function of the other. When ARF was selectively ablated, mice displayed a cancer-prone phenotype, with spontaneous tumour development in 19 of 24 animals, the most common type being sarcoma[§186]. Similar results were seen for p16, with spontaneous tumour development in 10 of 39 homozygotes, with the predominant type being sarcoma, while lymphoma and melanoma were also seen[§336]. Interestingly, a melanoma kindred has been reported wherein two members are homozygous for a non-functional *CDKN2A* allele: one has melanoma, the other is unaffected[129]. Clearly, while loss of p16 function may predispose toward the development of melanoma, it does not guarantee it. Other genetic or environmental factors must be involved.

In contrast, the *Cdkn2b* knockout mouse has a relatively mild phenotype, with an 8% tumour incidence after 18 months[§220]. Nevertheless, there is probably a sufficient weight of evidence to suggest that it is a tumour-suppressor in its own right, albeit relatively minor. In particular, homozygous deletion of *CDKN2B*, but not *CDKN2A* has been reported in bladder cancer[84], multiple myeloma[372], and non-Hodgkin's lymphoma[345]; and methylation of *CDKN2B*, without alteration of p16 expression is almost universal in adult acute myelogenous leukaemia, and very common in adult acute lymphocytic leukaemia, paediatric acute myelogenous leukaemia, and in glioma[145 146]. This same pattern is seen in radiation-induced murine T-cell lymphomas[§245]. Other data supports a joint role for these tumour-suppressors. Simultaneous functional loss of p15 and p16 may be important in the development of T-cell acute lymphoblastic leukaemia[172 287], glioma[348], and multiple myeloma[280]. In oesophageal squamous

carcinoma, promoter methylation of *CDKN2A* is seen either alone, or in combination with methylation of *CDKN2B*, but the latter rarely occurs alone[413].

The p18^{Cdkn2c} knockout mouse exhibited pituitary hyperplasia leading to the formation of primary tumours that were fatal due to their large size. They appeared to have little invasive or metastatic propensity however. Other tumour types were also seen, including lymphoma, and renal, adrenal and testicular tumours[§220]. There is evidence to support a tumour-suppressor function for p18 in humans, particularly in multiple myeloma[210], and perhaps acute lymphoblastic leukaemia[171], meningioma[29], and breast cancer, where a *CDKN2C* mutation leading to a p18 unable to bind CDK6 has been reported[217].

An extensive study of human haematopoietic malignancies found only very few instances of p19 alteration[77], nor is it implicated in other tumour types. The phenotype of the p19-deficient mouse supports the hypothesis that it is not a tumour-suppressor, but rather, regulates testicular development[§429].

p21 and relatives

The initial report of the p21^{Cdkn1a}-null mouse[§62] concluded that while aberrations of G_1-arrest were evident in cell cultures, there was no significant disposition toward spontaneous tumour formation by six months of age. However, when such mice were followed for an extended period it was found that spontaneous tumours did arise at a mean age of sixteen months, the predominant type being haematopoietic[§247]. Among human tumours, mutations of *CDKN1A* are known, but in general, are infrequent[376]. Among 81 gliomas[233], 28 pituitary adenomas[168], and 20 gastric carcinomas[296], no mutation was detected by PCR-SSCP or sequencing. Intragenic deletions or point mutations have been found in adrenocortical adenoma[167], 5 of 40 thyroid carcinomas[343], 3 of 28 brain tumours[385], and 7 of 102 tumours of assorted types[399]. Interestingly, a polymorphism that may affect the ability of p21 to interact with PCNA was identified in 42 of 50 cases of oesophageal squamous cell carcinomas in contrast to only 8 of 50 putatively normal individuals[16].

The most evident characteristic of the p27^{Cdkn1b} knockout mouse it that it is significantly larger than its wild-type litter-mates, an apparent consequence of increased general cellular proliferation resulting in enlarged organs[§96 §276]. Spontaneous development of pituitary tumours is seen, a feature also present in the phenotype of *Cdkn2c*-null[§105] and *Rb1*+/- animals, suggesting an important functional overlap in this tissue. In human solid tumours, reduced expression of p27 is frequently associated with rapid tumour progression and poor prognosis[160 249 328], while the converse may be true in some lymphomas[®261]. *CDKN1B* alterations are only rarely seen in tumours[189], however a mutation with simultaneous loss of heterozygosity at 12p13 has been found in 1 of 36 breast carcinomas[356].

Mice lacking *Cdkn1c* had cleft palates and skeletal deformities and usually died neonatally. In the ~10% of instances where they survived beyond weaning, their growth was markedly retarded and developmental flaws in reproductive organs become apparent in both males and females. While no increased cancer predisposition was detected during the five months of the study, increased incidence with later onset cannot be excluded[§365]. Mutation of *CDKN1C* has not been reported in human tumours, but loss of expression and loss of heterozygosity at 11p15.5 has been seen in thyroid[176], bladder[289], and hepatocellular[173] carcinomas, and in pancreatic adenocarcinoma[175]. *CDKN1C* is a strong candidate for the Beckwith-Wiedemann syndrome gene[37], a disease in which there is a mild predisposition toward cancer, particularly Wilms' tumour. While mutation has been found in some cases[138], conclusive proof is

proving difficult to obtain, not least because the implicated locus also contains *IGF2*, an equally viable candidate, and both are subject to parental imprinting[179].

The E2F transcription factors

The E2F transcription factors are involved in both the induction and repression of genes, and mediate both proliferation and apoptosis, hence, it is not possible to predict, a priori, whether their normal role is tumour-suppressive, excessive function oncogenic, neither, or even both for different E2F types or under different circumstances[®416]. The very real nature of this difficulty is demonstrated by the case of E2F1, one of the better studied E2Fs. *E2F1* is amplified in the HEL erythroleukaemia cell-line[321], E2F1 was over-expressed in 24 of 26 small-cell lung cancers[90], and its expression correlated with invasiveness in head and neck carcinoma[426], all suggesting a role in tumorigenesis. However, the *E2f1*-null mouse has an elevated rate of spontaneous tumour formation, particularly reproductive tract sarcomas[§419], suggesting a role in tumour suppression. How these effects come about is unknown, but it seems unlikely to involve interaction with pRB since no mutations in the pRB interaction domain of E2F1 were found in a survey of 406 human tumours[274], and concurrent ablation of *E2f1* reduces tumour incidence and increases the longevity of *Rb1* +/- heterozygous mice[§417].

E2F4 appears to influence tumour development significantly, seemingly due to the presence of an unstable $(\mathtt{CAG})_{13–18}$ trinucleotide repeat that encodes a polyserine tract. Alterations have been found here in various digestive[250 330 355 409 428] and haematological[205] tumours. It has been suggested that at least in some instances, this instability is due to a mutation within *MSH3*, whose encoded protein plays a prominent role in DNA mismatch repair[169].

There is little if any evidence to suggest a role for the other E2F transcription factors in tumorigenesis, with the possible exception of E2F5, which has been found to be amplified and over-expressed in some breast cancers[306].

6 The pRB subsystem and melanoma

The genetic analysis of hereditary tumour kindreds is a rich source of information pertinent to the molecular aetiology of cancer, and this is the case with melanoma[®32 ®39]. In some syndromes, melanoma occurs as the only, first, or predominant tumour type, notably when the disease phenotype is linked to 9p21[137 392], 12q14[354], or 1p36[18]. Here, the implicated genes are, respectively, *CDKN2A*[100 312], *CDK4*[431], and possibly *CDC2L1*[279] or even *PINK1*[387], but probably not *TP73*[207 329 383]. In others, melanoma is just one component of a more complex cancer predisposition as in xeroderma pigmentosum[204], with multiple linkage groups; hereditary retinoblastoma[9 22 260 381], implicating *RB1*; type I multiple endocrine neoplasia[283], implicating *MEN1*; multiple hamartoma syndrome, implicating *PTEN*[42]; and melanoma-astrocytoma syndrome, implicating *CDKN2A* exon 1β[312]. Among these, alterations in *RB1*, *CDKN2A*, and *CDK4* may be expected to affect the pRB subsystem directly[®131].

Most interestingly, extensive surveys have failed to provide any evidence for a role for CDK2[391] or CDK6[340] in the tumorigenesis of melanoma, and there appears to be no report of amplification or mutation of *CCNE1*. Deregulated phosphorylation of pRB, per se, may therefore be insufficient to predispose toward melanoma. This hints that the critical role for pRB is modulated by CDK4, but not CDK2, and that it may be inconsequential in tissues where the dominant cyclin-D-associated CDK is CDK6. Heretical though it may seem, this is consistent with the possibility that the ability of pRB to repress E2F activity may not be the critical aspect. The disparity between incidences of *CDK4* and *CDKN2A* mutations in hereditary melanoma[122] further suggests that there may be partial functional

overlap between CDK4 and another kinase less susceptible to p16 inhibition, or that some function other than inhibition of CDK4 may also be involved.

Hence, suspicion must fall upon ARF as a further, possibly subordinate, contributor to melanoma tumorigenesis. Fitzgerald et al. reported finding no *CDKN2A* mutations that would alter ARF, but not p16, in 33 consecutive melanoma patients who had one or more first or second-degree affected relations[100], nor were any sequence alterations found in *CDKN2A* exon 1β among ten 9p21-linked melanoma kindreds by Fargnoli et al.[93]. However, one melanoma-astrocytoma syndrome kindred has been reported in which there is a germ-line mutation in *CDKN2A* exon 1β. More data are required before a definitive assessment can be made of what role, if any, is played by ARF in the tumorigenesis of melanoma.

The hypothesis has been raised that it is the integrity of the pRB subsystem as a functional whole that protects against melanoma, and hence, failure of any critical component predisposes toward it. The strongest evidence to support this is the common finding that in melanomas, there is very often a functional defect in a single element of the subsystem, generally p16, pRB, or CDK4[19 243 392]. Nevertheless, multiple flaws have been found in individual cases, with amplification of *CCND1* or mutation of *CDK4* being seen in conjunction with *CDKN2A* deletion[315 392]. Clearly, pRB cannot be the only significant target of alterations affecting cyclin-D1 or CDK4, and some additional advantage is conferred by their presence. The basis for this advantage is unknown, but the most probable explanation is that further, as yet uncharacterised, substrates for cyclin-D1–CDK4 exist. The rationale for this is that the implicated CDK4 mutation involves its escape from inhibition by p16. For this to be significant in a cellular context where p16 or pRB are absent, the necessity of a substrate other than pRB, and an inhibitor other than p16 is implied. Potentially, where p16 is absent, some degree of constraint may still operate through induction of p15, unless CDK4 is impervious to this. Furthermore, if the amplification of cyclin-D1 were serving some purpose other than increasing CDK4 activity, then it could be expected to act as an inhibitor of CDK2 activation, hindering, rather than helping proliferation. As to the identity of such a substrate, nothing is known with certainty. There is one report of a cytoplasmic p88 CDK4 substrate[211], but this does not appear to have been confirmed. It is also possible that it corresponds to a product of caspase cleavage of pRB. The cited report relies on the lack of recognition of p88 by the pRB monoclonal antibody employed to exclude this, but it is quite possible that upon cleavage, the necessary epitope is lost or its conformation modified. The particular antibody is not defined sufficiently well in the report to establish if this may be the case. While the principal caspase degradation products of pRB are p44 and p68, there is evidence of the early production of larger, and the subsequent production of smaller products[95].

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