Naturally death-resistant precursor cells revealed as the origin of retinoblastoma

The molecular mechanisms and the cell-of-origin leading to retinoblastoma are not well defined. In this issue of *Cancer Cell*, Bremner and colleagues describe the first inheritable model of retinoblastoma, revealing that loss of the pocket proteins pRb and p107 deregulates cell cycle exit in retinal precursors. The authors show that a subset of these precursors contain an inherent resistance to apoptosis, and that while most terminally differentiate, some are likely to acquire additional mutations, leading to tumor formation. Thus, this work defines the cell-of-origin of retinoblastoma and suggests that mutations giving increased proliferative capacity are required for retinoblastoma development.

Children carrying a germline mutation in one allele of *RB1* are highly predisposed to retinoblastoma, a tumor of the retina. Based on this hereditary predisposition, Knudson proposed the "two-hit" hypothesis, which was subsequently validated by the demonstration that the loss of the second allele of *RB1* is a rate-limiting step for retinoblastoma development (Knudson, 2001). However, the precise function of the retinoblastoma protein, pRB, in normal retinal homeostasis is still unknown. In addition, the cell of origin of retinoblastoma has not been established. That is, until now. In this issue, Bremner and colleagues describe the generation of the first inheritable mouse model for retinoblastoma (Chen et al., 2004). Interestingly, they show that loss of pRb and the pRb related protein, p107, specifically impairs cell cycle exit of retinal precursors, but does not prevent terminal differentiation. Based on a careful analysis of retinal development in the mouse model, they propose that tumors arise from cells that have escaped terminal differentiation rather than cell death, and point to an amacrine precursor as the "cell-of-origin" for retinoblastoma.

Unlike humans, *Rb* heterozygous mice do not develop retinoblastoma, but are instead highly predisposed to pituitary and thyroid tumors (for review, see Classon and Harlow, 2002). Due to the embryonic lethality of *Rb* null mice, it has been challenging to establish the function of pRb in the retina at later stages of development. The first clues came from an analysis of chimeric $Rb^{+/-}$mice, which demonstrated a poor contribution of $Rb^{-/-}$ cells to the adult retina. The lack of Rb null cells in the adult retina was ascribed to apoptosis (Maandag et al., 1994), suggesting that $Rb^{-/-}$ cells must escape apoptosis in the development of retinoblastoma. Consistent with this, expression of E7, which inactivates pRB family members, induces cell death in the retina, whereas its expression in $p53^{-/-}$ photoreceptors induces tumor formation (Classon and Harlow, 2002). Moreover, the simultaneous inactivation of both the pRb and p53 pathways by expression of SV40 T antigen in the murine retina also results in tumor formation (Classon and Harlow, 2002). However, the absence of p53 mutations in human retinoblastomas raises the concern of whether these mouse models mimic the development of human retinoblastomas.

The demonstration that $Rb^{+/-}$; $p107^{-/-}$ compound mice develop retinal dysplasia was a key step toward the generation of an inheritable mouse model for human retinoblastoma (Lee et al., 1996). This finding was further supported by the observation that chimeric mice derived from $Rb^{-/-}$; $p107^{-/-}$ embryonic stem cells suffer from retinoblastoma originating from the inner nuclear layer (INL) of the retina with amacrine cell characteristics (Robanus-Maandag et al., 1998). However, the fact that some but not all chimeric retinas form retinoblastomas and that the majority of $Rb^{-/-}$; $p107^{-/-}$ retinoblasts undergo apoptosis suggests that in addition to loss of pRb and p107, other mutations could be required to counteract cell death and allow tumor development.

The inheritable mouse model for retinoblastoma described by Bremner and colleagues is based on the retinal-specific ablation of pRb in a $p107^{-/-}$ genetic background. Interestingly, the careful analysis of the mice has led the

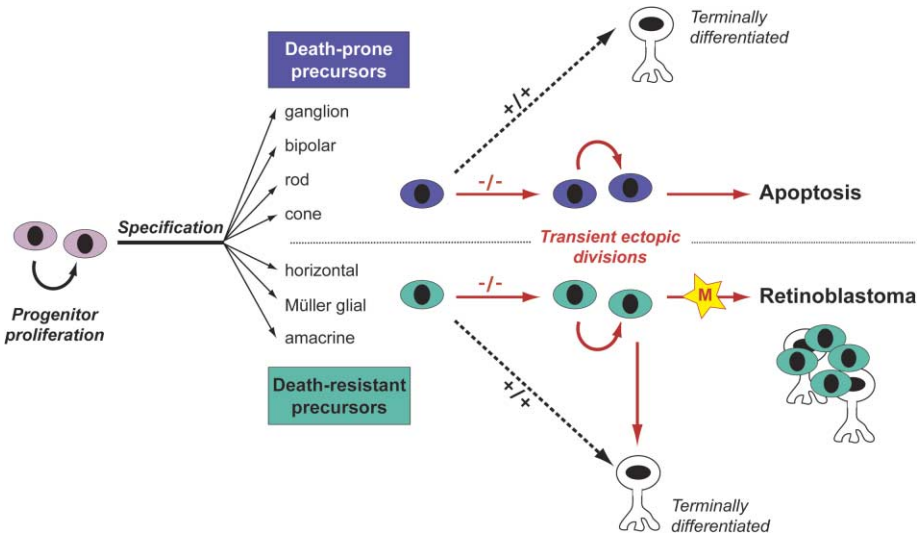

Figure 1. Model for retinoblastoma development

Retinal development involves 7 different cell types and is characterized by expansion of progenitor cells, specification of precursors, and terminal differentiation. Mice lacking pRb and p107 (–/–) carry out all of these retinal developmental processes in a manner similar to wild-type mice (+/+, dashed lines). However, pRb/p107 deficiency results in ectopic division of all retinal precursors (–/–, in red). One group of precursors subsequently undergoes apoptosis (death-prone), while another group is resistant (naturally death-resistant). Retinoblastoma arises from these naturally death-resistant precursors after they have acquired additional mutations (M). These as yet to be identified mutations presumably facilitate continued proliferation, ultimately leading to retinoblastoma.

authors to challenge the view that tumors arise from acquired resistance to apoptosis. Critically, the work provides a very comprehensive analysis of the cell-specific effects of pRb loss during retinal development. Specifically, they demonstrate that loss of pRb or combined loss of pRb/p107 does not affect retinal progenitor expansion, precursor specification, or terminal differentiation. In contrast, loss of pRb and p107 results in ectopic division of all precursors at a time when proliferation normally ceases (Figure 1). This supports a model in which retinoblastoma arises from the ectopically dividing precursors rather than progenitors. Surprisingly, however, the large majority of double knockout precursors divide ectopically only for a finite period of time and ultimately undergo terminal differentiation. This result demonstrates that pRb and p107 loss is not sufficient to provide infinite proliferating capacity to precursors, and strongly suggests that additional mutations are required for the subsequent development of retinoblastomas. Consistent with the cell death reported for the chimeric mice, massive apoptosis in the *Rb*$^{-/-}$; *p107*$^{-/-}$ retina was also observed. Interestingly, the loss of pRb and p107 results in apoptosis in four precursor subtypes (ganglion, bipolar, rod, and cone), whereas the remaining INL precursors (horizontal, amacrine, and Müller cells) undergo ectopic cell proliferation without displaying signs of apoptosis. The accelerated proliferation induced by loss of pRb and p107 combined with the natural death resistance of the INL precursors suggests that these cells are the prime candidates for the cell-of-origin in retinoblastomas. Indeed, emerging and mature tumors express markers characteristic for amacrine cells and, to a lesser extent, Müller cells. These results are consistent with other reports (Robanus-Maandag et al., 1998; Chen et al., 2004; MacPherson et al., 2004), and the authors therefore propose a model in which intrinsically death-resistant INL precursors with limited proliferative capacity require additional mutation(s) to overcome differentiation-associated growth arrest. Importantly, this implies that the most critical barrier to retinal transformation is terminal differentiation rather than apoptosis, as previously suspected.

Whether INL precursors are also the origin of human retinoblastoma remains unclear. In fact, both outer (ONL) and inner nuclear layer (INL) precursor markers have been detected in human retinal tumors. However, Gallie et al. demonstrated that early stage human retinoblastomas in fact exhibit a nuclear morphology typical of INL (Gallie et al., 1999), suggesting that the ONL characteristics observed in late stage tumors may be due to transdifferentiation events.

The work presented in this issue of *Cancer Cell* and by the Jacks group in *Genes & Development* firmly establishes that while pRb loss is not sufficient to induce retinoblastomas in mice, the compound *Rb*$^{-/-}$; *p107*$^{-/-}$ or *Rb*$^{-/-}$; *p130*$^{-/-}$ mutant mice develop retinoblastoma with high incidence (Robanus-Maandag et al., 1998; Chen et al., 2004; MacPherson et al., 2004). This points to overlapping functions between the pocket proteins during retinal development and suggests that p107 and p130 can compensate for loss of pRB in the control of retinal homeostasis. Since the pRB family members display overlapping functions, it is tempting to speculate that ablation of all three pRB family proteins in murine retina would further enhance the tumor formation phenotype.

An important question is whether the conclusions derived from the mouse models are relevant for human retinoblastomas. Significantly, whereas germline mutation of one *RB1* allele in humans leads to the development of retinoblastoma with 100% penetrance, the tumor incidence drops to 60% in the mouse model described by Chen and colleagues (Chen et al., 2004). Since previous data have shown that the phenotypes arising in p107 or p130 mutant mice are strongly dependent on their genetic background (Classon and Harlow, 2002), it is possible that genetic modifiers partially compensate for combined loss of p107 and pRB in this mouse model, thus suggesting that the penetrance might be higher in other mice strains. Moreover, the safeguard mechanisms operating in human and mouse may be different, because mutations in the *p107* gene have not been found in human retinoblastoma. However, it is possible that pRB-related proteins are also involved in retinal tumor development in humans, since p130 expression has been reported to be strongly downregulated in a subset of retinoblastomas (Bellan et al., 2002). Furthermore, additional genetic alterations in addition to *RB1* mutations have been described in human retinoblastomas. Specifically, the vast majority of retinoblastomas examined exhibit either a discrete genomic amplification of chromosome 6p (isochromosome of 6p) or extra copies of chromosome 1q (Chen et al., 2001). Identification of the crucial genes involved in these cytogenetic alterations could provide important information to help further understand the genesis of human retinoblastoma. A candidate oncogene, the human kinesin gene RBKIN/KIF13A, has been found located within the amplified region of chromosome 6p, and consistently, it is highly expressed in retinoblastomas and required for growth of retinoblastoma cell lines.

As in human retinoblastoma, the mouse model described by Bremner and colleagues shows that genetic alterations, in addition to pRb inactivation, are required for the development of retinoblastomas. It will be extremely interesting to identify these genes and to test their relevance in human cancer. Therefore, it is likely this inheritable mouse model of retinoblastoma will be an invaluable tool in our efforts to understand not only the genesis of retinoblastoma, but cancer in general. Already, it has provided the provocative possibility that the cell-of-origin of retinoblastoma in humans might have to escape the barrier of terminal differentiation, as opposed to apoptosis as previously thought.

Emmanuelle Trinh,[1]
Eros Lazzerini Denchi,[2] and
Kristian Helin[1,2,3,*]

[1]European Institute of Oncology, Department of Experimental Oncology, Via Ripamonti 435, 20141 Milan, Italy
[2]FIRC Institute of Oncology, via Adamello 16, 20139 Milan, Italy
[3]Biotech Research & Innovation Centre, Symbion Science Park, Fruebjergvej 3, 2100 Copenhagen, Denmark
*E-mail: khelin@ieo.it

Selected reading

Bellan, C., De Falco, G., Tosi, G.M., Lazzi, S., Ferrari, F., Morbini, G., Bartolomei, S., Toti, P., Mangiavacchi, P., Cevenini, G., et al. (2002). Invest. Ophthalmol. Vis. Sci. *43*, 3602–3608.

Chen, D., Gallie, B.L., and Squire, J.A. (2001). Cancer Genet. Cytogenet. *129*, 57–63.

Chen, D., Livne-bar, I., Vanderluit, J.L., Slack, R.S., Agochiya, M., and Bremner, R. (2004). Cancer Cell *5*, this issue.

Classon, M., and Harlow, E. (2002). Nat. Rev. Cancer *2*, 910–917.

Gallie, B.L., Campbell, C., Devlin, H., Duckett, A., and Squire, J.A. (1999). Cancer Res. *59*, 1731s–1735s.

Knudson, A.G. (2001). Nat. Rev. Cancer *1*, 157–162.

Lee, M.H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., and Jacks, T. (1996). Genes Dev. *10*, 1621–1632.

Maandag, E.C., van der Valk, M., Vlaar, M., Feltkamp, C., O'Brien, J., van Roon, M., van der Lugt, N., Berns, A., and te Riele, H. (1994). EMBO J. *13*, 4260–4268.

MacPherson, D., Sage, J., Kim, T., Ho, D., McLaughling, M.E., and Jacks, T. (2004). Genes Dev., in press.

Robanus-Maandag, E., Dekker, M., van der Valk, M., Carrozza, M.L., Jeanny, J.C., Dannenberg, J.H., Berns, A., and te Riele, H. (1998). Genes Dev. *12*, 1599–1609.

The linchpin? Pin1 meets p73

A recent paper shows that the peptidyl-prolyl isomerase Pin1 conformationally alters p73, promoting its acetylation by p300 in a c-Abl dependent manner. Given previous findings with p53, Pin1 may represent a common mediator linking pro-apoptotic cooperative activity of the p53 family members.

When considering the p53 gene family, one is struck by their conspicuous similarities as well as their intriguing differences. Structurally, the p53 family members' kinship is obvious, as they share a common domain topology and bear significant sequence homology within their transactivation, DNA binding, and tetramerization domains (reviewed in Melino et al., 2002). At this point, however, far more is understood about p53 and its function as a pivotal node in the DNA damage checkpoint. Upon genotoxic insult, p53 becomes modified, stabilized, and thereby activated to regulate transcription of its downstream target genes important in many cellular functions, including cell cycle arrest and apoptosis. While p53's central role in apoptosis and tumor suppression is proven, the extent to which the other members of the p53 family can support or recapitulate its functions is unclear. Nevertheless, p73 can activate at least a subset of p53 target genes independently of p53 and also can mediate p53-independent apoptosis in response to a number of chemotherapeutic agents (Irwin et al., 2003). Interestingly, however, p63 and p73 are required for p53-dependent apoptosis as well as activation of some proapoptotic p53 target genes in mouse embryo fibroblasts expressing E1a in response to cytotoxic agents (Flores et al., 2002). Understanding the mechanisms by which the p53 family members individually and together regulate transcription continues to be an important challenge for revealing their respective roles in tumor suppression.

A recent elegant paper from Montovani et al. links p73 with the Pin1 peptidyl-prolyl-isomerase in apoptosis, and as such may begin to provide a mechanism for coregulation of the p53 family (Montovani et al., 2004). Pin1 mediates *cis*/*trans* isomerization of proteins at serine-proline or threonine-proline (SP or TP) motifs. The ensuing conformational change produces a variety of functional outcomes in many proteins central to tumorigenesis, including cyclin D1, β catenin, NF-κB, and even p53 itself. Substrate recognition by Pin1 requires phosphorylation of SP or TP motifs by multiple families of proline-directed kinases, including cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs) (reviewed in Lu, 2003).

Montovani et al. demonstrate that Pin1 strongly enhances p73-dependent apoptosis in p53 null cell lines, an observation that correlates with the ability of Pin1 to augment p73's ability to induce proapoptotic target genes, including Bax, Pig3, and p53AIP1. The Pin1-p73 interaction is phosphorylation-dependent and seems to be mediated by three key phosphorylation sites within the poly-proline region of p73 that exist only in the α and β isoforms of this protein. Mutation of these 3 residues abrogates Pin1 binding as well as transcriptional regulatory activity of p73. Furthermore, the authors show by a partial proteolysis assay that Pin1 can directly induce conformational change(s) in p73 in vitro, indicating that the Pin1-p73 interaction is direct and that p73 is a bona fide substrate for Pin1 isomerization. Binding of Pin1 often enhances substrate half-life, and indeed, siRNA-mediated knockdown of Pin1 destabilizes p73 protein both in the presence and absence of DNA damage.

The signaling pathway converging on p73, activated by two chemotherapeutic agents cisplatinum (CDDP) and adriamycin (ADR), relies principally on the c-Abl tyrosine kinase (White and Prives, 1999). Direct phosphorylation of p73 by c-Abl on Y99, among other sites, can stabilize and activate p73 protein. Connecting this pathway with Pin1 is the finding that c-Abl activates proline-directed p38 MAPK to phosphorylate TP sites within p73 (Sanchez-Prieto et al., 2002). Thus, the c-Abl pathway likely creates Pin1 recognition motifs on p73 through MAPK. Indeed, Montovani et al. show that CDDP and ADR as well as overexpression of c-Abl or p38-MAPK stimulate Pin1-p73 association. This is dependent on c-Abl, as p73 mutated at Y99 is not active in these assays, and siRNA depletion of c-Abl inhibits the p73-Pin1 association. Conversely, c-Abl cannot stabilize or activate p73 in the context of Pin1 knockdown by siRNA. Taken together, these observations strongly implicate a strict functional interdependence between c-Abl and Pin1 in activating p73 after genotoxicity.

In addition to tyrosine phosphorylation, c-Abl is also required for p300-dependent acetylation of p73, and this modification enhances activation of proapoptotic p73 target promoters (Costanzo et al., 2002). Indeed, Montovani et al. found that DNA damage-dependent p73 acetylation is defective in Pin1 null cells, and coexpression of Pin1 enhances both the p300-p73 interaction as well as the