Chapter 1

General Introduction

The p53 protein was discovered over thirty years ago as a target of the Large T-antigen of the oncogenic DNA virus SV40 [1;2]. Initial observations that p53 possesses oncogenic activity suggested that p53 functions as an oncogene, but it soon became evident that this was due to mutations in the initially isolated cDNAs and that normal p53 rather acts as a tumor suppressor [3]. The p53 protein provides essential protection against malignancies; mice deficient for p53 are prone to develop cancer [4;5] and the p53 gene is mutated in approximately 50% of all human tumors [6;7]. Germ line p53 mutations cause a rare type of cancer predisposition known as Li-Fraumeni Syndrome [8]. Because of its prominent role in cancer, p53 has been the subject of extensive research to understand the different aspects of its regulation and function in different cellular settings. A complex picture has emerged, of which the core message is that p53 functions to cease the growth of damaged cells by regulating the required responses to cellular insults, in order to prevent tumorigenesis. The anti-proliferative functions of p53 are primarily based on its ability to regulate transcription of genes involved in cell cycle arrest, apoptosis, senescence, DNA repair and prevention of angiogenesis [9]. Importantly, 95% of p53 mutations are located in the DNA binding domain, emphasizing the key role of p53 as a transcription factor [10]. However, also transcription-independent activities have been reported, including direct regulation of apoptosis at the mitochondria [11] and regulation of miRNA processing [12]. Interestingly, from an evolutionary perspective it has been argued that p53 was not selected for its tumor suppressor function [13], and p53 is probably involved in many other biological processes as well, such as stem cell homeostasis [14] and fertility [15].

The human p53 protein can be divided into several functional domains (Figure 1). It contains two N-terminal transactivation domains, that allow p53 to interact with transcriptional cofactors [16;17]. The N-terminus also contains the main Hdm2 and Hdmx binding site (see below). The central region of p53 holds a well-conserved DNA binding domain [18]. An oligomerization domain just C-terminal of the DNA binding domain mediates p53 tetramerization, which is crucial for target gene transactivation [19]. A nuclear localization signal [20] and a nuclear export domain [21] are located within the oligomerization domain. The carboxy-terminus consists of a basic domain, which is subject to distinct post-translational modifications and is considered to be a major regulator of p53 transcriptional activity [22]. The p53 tetramer binds to a p53 responsive element (RE) in promoter or intronic sequences of the target gene. A p53 RE consists of two copies of a 10 base-pair motif, separated by a 0-13 base-pair spacer [23]. The exact p53 RE composition determines the binding efficiency, thereby providing a possible mechanism for differential gene regulation by p53 [24].

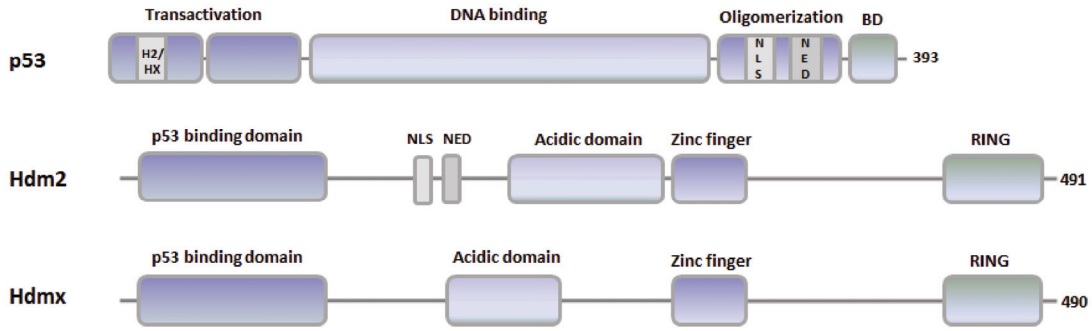

Figure 1 Domain architecture of p53, Hdm2 and Hdmx proteins. H2/HX, Hdm2/Hdmx binding site; NLS, nuclear localization signal; NED, nuclear export domain; BD, basic domain; RING, really interesting new gene.

P53 regulation by Hdm2 and Hdmx

Cells need to keep their p53 activity in check in order to proliferate. Two critical negative regulators of p53 are Mdm2 and Mdmx, in humans mostly referred to as Hdm2 and Hdmx. Mdm2 (murine double minute clone 2) was originally cloned from purified acentric chromosomes, also known as double minutes, which often contain amplified genes that contribute to cellular proliferation and tumorigenesis [25]. Mdm2 was found to directly bind p53, thereby abrogating its transcription regulatory properties [26;27]. The rescue of early embryonic lethality of Mdm2 deficient mice by simultaneous p53 knockout strongly established the importance of Mdm2 in p53 inhibition [28;29]. Later on, another protein showing sequence similarity with Mdm2 was identified to interact with p53 and named Mdmx [30]. Similar to Mdm2, loss of Mdmx also caused p53-dependent embryonic lethality, although occurring somewhat later (E10.5 vs. E4.5) in embryogenesis [31-33]. This indicates that Mdm2 and Mdmx are essential, non-redundant regulators of p53 during embryonic development. In adult tissues, the currently available data indicate that Mdm2 loss is almost invariably lethal, whereas Mdmx loss can be tolerated in some cases [34-39]. Thus, the contribution of Mdm2 and Mdmx to p53 regulation *in vivo* may be cell type-specific.

The human Hdm2 and Hdmx proteins (depicted in Figure 1) show great structural similarities [40]. Best conserved is their N-terminal hydrophobic pocket, which binds to an N-terminal alpha-helix of p53. This binding shields p53's transcription activation domain, thereby inhibiting its activity [41]. In addition, Hdm2 has E3 ubiquitin ligase activity that can target p53 for proteasomal degradation via poly-ubiquitination, for which a central acidic domain as well as a C-terminal RING finger are necessary. Hdmx has no detectable

E3 ligase activity for p53 (or any other substrate), despite the presence of both the acidic domain and the RING finger [42-44]. Hdmx and Hdm2 dimerize via their RING finger domains [45]. The Hdm2-Hdmx hetero-oligomer promotes Hdm2 stability and is a more effective E3 ligase for p53 than the Hdm2 homo-dimer [46;47]. Because the ratio between Hdm2 and Hdmx levels strongly determines p53 stability, it is subject of tight regulation. Besides degrading p53, Hdm2 can also ubiquitinate itself [48;49] and Hdmx [50-52], thereby building positive feedback loops into this system. Moreover, ubiquitinations of Hdm2, Hdmx and p53 can all be counteracted by multiple deubiquitinating enzymes, such as HAUSP [53;54] and USP42 [55]. In addition to the long list of p53 target genes that execute the required biological responses to cellular stress, p53 also transactivates the Hdm2 gene. Since Hdm2 inhibits the p53 response, this provides an important negative feedback loop [56], resulting in out of phase oscillation of p53 and Hdm2 protein levels [57]. More recently, a p53 RE in the first intron of the Hdmx gene has been described. This initiates transcription from an alternative promoter (P2), resulting in an alternative first exon (1β) in the Hdmx mRNA and the synthesis of a slightly longer protein (Hdmx-L), which also contributes to the attenuation phase of the p53 response [58-60].

The p53 pathway as a protective mechanism against cancer

Multiple stress signals can induce the p53 network (Figure 2). These stresses are in many ways related to carcinogenesis and include for example double-strand DNA breaks, DNA replication stress, telomere erosion, oncogene activation, oxidative stress, nitric oxide, hypoxia, ribonucleotide depletion and mitotic apparatus dysfunction [61]. One of the most extensively studied p53 activating triggers is DNA damage. Diverse types of DNA damage lead to the activation of the ataxia-telangiectasia mutated (ATM) and the ataxia-telangiectasia and Rad3-related (ATR) kinases, which subsequently phosphorylate a multitude of proteins, including the checkpoint kinases Chk1 and Chk2 [62]. ATM, ATR, Chk1 and Chk2 have all been reported to directly mediate N-terminal phosphorylations on p53, including the transactivation domain and the Hdm2/Hdmx binding alpha helix [63-65]. These phosphorylations weaken the interaction with Hdm2 and Hdmx, allowing some p53 to escape from functional inhibition. Many other p53 modifications may occur sequentially following phosphorylation [66]. For example, p53 Ser15 phosphorylation stimulates the recruitment of factors involved in transcription, including the acetyl-transferases p300 and CBP [67;68]. This leads to the acetylation of a number of C-terminal lysines, several of which are also ubiquitination sites, thereby contributing to the stabilization of p53 [69]. In addition to p53 modifications, DNA damage signaling also induces the phosphorylation of Hdm2 and Hdmx, resulting in their ubiquitination and degradation [49;70-73].

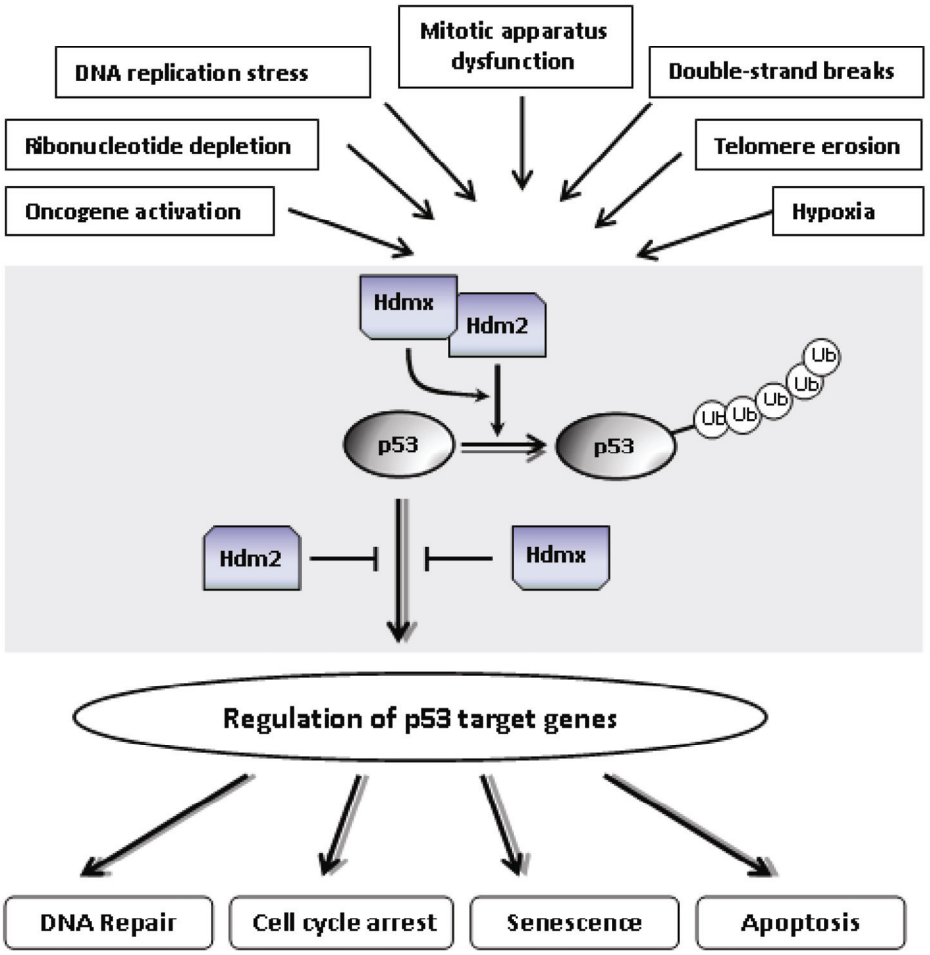

Figure 2 Simplified scheme of the p53 tumor suppressor pathway. Multiple stress signals act upon the inhibitory activities of Hdm2 and Hdmx toward p53. This results in the release and activation of p53, regulation of p53 target genes and the induction of the required cellular response.

Another major p53 activating mechanism involves the deregulation of various mitogenic signaling pathways. The induction of p53 by aberrant activation of oncogenes such as Ras [74], c-myc [75], E2F1 [76], and adenovirus E1A [77] mostly relies on the tumor suppressor protein $p14^{ARF}$. $p14^{ARF}$ (alternative reading frame) is the product of an alternative transcript of the CDKN2A locus which also encodes another tumor suppressor, $p16^{INK4A}$ [78]. $p14^{ARF}$ interacts with Hdm2, thereby directly inhibiting the E3 ligase activity of Hdm2 and also sequestering Hdm2 in the nucleolus [79-81]. This stimulates p53-mediated cellular responses, thereby serving as protection against the tumorigenic consequences of oncogene activation. Loss of this signaling pathway may predispose cells to malignant transformation. Although the resulting tumor cells are no longer growth inhibited by oncogene-induced p53, p53 reactivation might still be achieved through alternative pathways, which could be beneficial for targeting a subset of cancers.

The p53 pathway as target for tumor therapy

As mentioned, about half of all human cancers contain p53 mutations. Mutant p53 can exert tumor-promoting effects, owing to dominant-negative inactivation of wild-type p53 function, as well as to certain oncogenic gain-of-function activities [82]. There is considerable variation of p53 mutation frequency among different cancer types. For instance, p53 mutation occurs in up to 70% of ovarian [83], colorectal [84] and head and neck [85] cancers, whereas it is rare in leukemia's [86], retinoblastoma [87] and melanoma's [88]. The tumors that retain wild-type p53 are assumed to have other defects in p53 regulation or downstream signaling that impede a proper p53 response [89]. For example, some cancers harbor Hdm2 amplification [90], and others show upregulation of Hdmx expression, mostly correlating with wild-type p53 status [91-93].

The pivotal position of p53 in tumor suppression makes it a potentially attractive target for cancer treatment. Several low-molecular-mass compounds aiming at specific reactivation of p53 function have been developed, in order to aid or substitute conventional cancer therapies. For example PRIMA1 [94] has been designed to interact with mutant p53, thereby altering its conformation and restoring p53 activity. This thesis mostly focuses on tumors that express wild-type, but functionally impaired p53. Reactivation of p53 in these tumors may be achieved by inhibiting the p53-Hdm2 interaction. This strategy is exemplified by the small molecule Nutlin-3, which binds Hdm2 in its p53-binding pocket, thereby releasing, stabilizing and activating p53 [95]. Nutlin-3 is a non-genotoxic p53 activator and normal (non-tumor) cells are relatively insensitive to Nutlin-3 induced apoptosis [96]. The biological outcome of p53 activation by Nutlin-3 is highly dependent on the cellular context. Factors contributing to apoptosis induction may include the status of Hdmx [97-99] and E2F1 [100]. Nutlin-3 appeared promising in retinoblastoma treatment, especially when combined with the topoisomerase I inhibitor Topotecan [101]. The use of Nutlin-3 in combination with chemotherapeutics may enhance their effectiveness and allow such drugs to be administered at lower doses [102-105]. Other examples of Hdm2 antagonists include Benzodiazepinedione [106], MI-63 and MI-219 [107]. Hdmx antagonists have also been described, including SJ172550 [108] and SAH-p53-8 [109], and recently a compound was identified that activated p53 through inhibition of Hdmx transcription [110]. In addition to these Hdm2 and Hdmx targeting drugs, RITA (reactivation of p53 and induction of tumor cell apoptosis) was identified to suppress *in vivo* growth of transformed cells in a p53-dependent manner [111], supposedly by direct binding to p53. In addition to p53 stabilization, other effects are likely to contribute to the

activation of a pro-apoptotic program by RITA in tumor cells [112], although the exact mechanisms are still unclear and require further investigations.

Scope of thesis

Much of the work described in this thesis is centered on two types of eye cancer that typically express a wild-type p53 protein: retinoblastoma and uveal melanoma. Retinoblastoma is a rare childhood cancer (approximately 1-2 cases per million people per year [113]) initiating in the retina, a specialized light-sensitive stack of neuronal layers at the back of the eye. Mutations in the RB1 gene, encoding the Rb tumor suppressor protein, are responsible for most cases of retinoblastoma [114]. Loss of Rb in the developing retina activates the tumor surveillance pathway mediated by $p14^{ARF}$, Hdm2 and p53. Interestingly, a particularly high proportion of retinoblastomas apparently select for increased Hdmx levels as a mechanism to suppress the p53 response in Rb-deficient cells [101]. Although most primary melanoma's originate from melanocytes in the skin, in some cases (5.3%) the eye is affected [115]. This type of tumor arises in the uveal tract, comprising iris, ciliary body and choroid. The annual incidence of uveal melanoma in the western world is 6-8 cases per million people [116]. Metastases occur in up to 50% of patients, mostly to the liver, but also to other distant sites such as the lung, bone and skin [117-119]. Prognosis is poor when the tumor has metastasized; median survival is about 10 - 18 months [120;121]. Interestingly, p53 mutations are infrequent in uveal melanoma [122-125].

This thesis presents novel studies regarding the role of Hdmx in p53 inactivation during tumorigenesis, as well as the use of specific drugs for p53 reactivation as cancer treatment. Chapter 2 shows that constitutive Hdmx over-expression contributes to the neoplastic transformation of human fibroblasts and embryonic retinoblasts, thereby functionally resembling loss of p53. Chapter 3 establishes the importance of Hdmx as an oncogene in a subset of uveal melanomas. Importantly, the results described in this chapter extend the function of Hdmx beyond p53 inhibition. Chapter 4 evaluates the use of the specific p53 activating drugs Nutlin-3 and RITA in synergy studies as potential therapy for uveal melanoma. Chapter 5 is a more detailed analysis of the cellular responses to RITA. In particular, Chk2 is shown to be an essential mediator of the RITA-induced effects. Chapter 6 is a general discussion of the results presented in this thesis, and their implications for clinical exploitation and future research.

Reference List

1. Lane,D.P. and Crawford,L.V. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature*, **278**, 261-263.

2. Linzer,D.I. and Levine,A.J. (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*, **17**, 43-52.

3. Nigro,J.M., Baker,S.J., Preisinger,A.C., Jessup,J.M., Hostetter,R., Cleary,K., Bigner,S.H., Davidson,N., Baylin,S., and Devilee,P. (1989) Mutations in the p53 gene occur in diverse human tumour types. *Nature*, **342**, 705-708.

4. Donehower,L.A., Harvey,M., Slagle,B.L., McArthur,M.J., Montgomery,C.A., Jr., Butel,J.S., and Bradley,A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215-221.

5. Jacks,T., Remington,L., Williams,B.O., Schmitt,E.M., Halachmi,S., Bronson,R.T., and Weinberg,R.A. (1994) Tumor spectrum analysis in p53-mutant mice. *Curr.Biol.*, **4**, 1-7.

6. Hainaut,P. and Hollstein,M. (2000) p53 and human cancer: the first ten thousand mutations. *Adv.Cancer Res.*, **77**, 81-137.

7. Hollstein,M., Sidransky,D., Vogelstein,B., and Harris,C.C. (1991) p53 mutations in human cancers. *Science*, **253**, 49-53.

8. Malkin,D., Li,F.P., Strong,L.C., Fraumeni,J.F., Jr., Nelson,C.E., Kim,D.H., Kassel,J., Gryka,M.A., Bischoff,F.Z., Tainsky,M.A., and . (1990) Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*, **250**, 1233-1238.

9. Vogelstein,B., Lane,D., and Levine,A.J. (2000) Surfing the p53 network. *Nature*, **408**, 307-310.

10. Olivier,M., Eeles,R., Hollstein,M., Khan,M.A., Harris,C.C., and Hainaut,P. (2002) The IARC TP53 database: new online mutation analysis and recommendations to users. *Hum.Mutat.*, **19**, 607-614.

11. Moll,U.M., Wolff,S., Speidel,D., and Deppert,W. (2005) Transcription-independent pro-apoptotic functions of p53. *Curr.Opin.Cell Biol.*, **17**, 631-636.

12. Suzuki,H.I., Yamagata,K., Sugimoto,K., Iwamoto,T., Kato,S., and Miyazono,K. (2009) Modulation of microRNA processing by p53. *Nature*, **460**, 529-533.

13. Aranda-Anzaldo,A. and Dent,M.A. (2007) Reassessing the role of p53 in cancer and ageing from an evolutionary perspective. *Mech.Ageing Dev.*, **128**, 293-302.

14. Pearson,B.J. and Sanchez,A.A. (2010) A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. *Development*, **137**, 213-221.

15. Kang,H.J., Feng,Z., Sun,Y., Atwal,G., Murphy,M.E., Rebbeck,T.R., Rosenwaks,Z., Levine,A.J., and Hu,W. (2009) Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans. *Proc.Natl.Acad.Sci.U.S.A*, **106**, 9761-9766.

16. Lill,N.L., Grossman,S.R., Ginsberg,D., DeCaprio,J., and Livingston,D.M. (1997) Binding and modulation of p53 by p300/CBP coactivators. *Nature*, **387**, 823-827.

17. Van Orden,K., Giebler,H.A., Lemasson,I., Gonzales,M., and Nyborg,J.K. (1999) Binding of p53 to the KIX domain of CREB binding protein. A potential link to human T-cell leukemia virus, type I-associated leukemogenesis. *J.Biol.Chem.*, **274**, 26321-26328.

18. Cho,Y., Gorina,S., Jeffrey,P.D., and Pavletich,N.P. (1994) Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science*, **265**, 346-355.

19. Chene,P. (2001) The role of tetramerization in p53 function. *Oncogene*, **20**, 2611-2617.

20. Liang,S.H. and Clarke,M.F. (1999) A bipartite nuclear localization signal is required for p53 nuclear import regulated by a carboxyl-terminal domain. *J.Biol.Chem.*, **274**, 32699-32703.

21. Stommel,J.M., Marchenko,N.D., Jimenez,G.S., Moll,U.M., Hope,T.J., and Wahl,G.M. (1999) A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J.*, **18**, 1660-1672.

22. Scoumanne,A., Harms,K.L., and Chen,X. (2005) Structural basis for gene activation by p53 family members. *Cancer Biol.Ther.*, **4**, 1178-1185.

23. el-Deiry,W.S., Kern,S.E., Pietenpol,J.A., Kinzler,K.W., and Vogelstein,B. (1992) Definition of a consensus binding site for p53. *Nat.Genet.*, **1**, 45-49.

24. Qian,H., Wang,T., Naumovski,L., Lopez,C.D., and Brachmann,R.K. (2002) Groups of p53 target genes involved in specific p53 downstream effects cluster into different classes of DNA binding sites. *Oncogene*, **21**, 7901-7911.

25. Cahilly-Snyder,L., Yang-Feng,T., Francke,U., and George,D.L. (1987) Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somat.Cell Mol.Genet.*, **13**, 235-244.

26. Barak,Y. and Oren,M. (1992) Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53-mediated growth arrest. *EMBO J.*, **11**, 2115-2121.

27. Momand,J., Zambetti,G.P., Olson,D.C., George,D., and Levine,A.J. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, **69**, 1237-1245.

28. Jones,S.N., Roe,A.E., Donehower,L.A., and Bradley,A. (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature*, **378**, 206-208.

29. Montes de Oca,L.R., Wagner,D.S., and Lozano,G. (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature*, **378**, 203-206.

30. Shvarts,A., Steegenga,W.T., Riteco,N., van Laar,T., Dekker,P., Bazuine,M., van Ham,R.C., van der Houven van Oordt,W., Hateboer,G., van der Eb,A.J., and Jochemsen,A.G. (1996) MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J.*, **15**, 5349-5357.

31. Finch,R.A., Donoviel,D.B., Potter,D., Shi,M., Fan,A., Freed,D.D., Wang,C.Y., Zambrowicz,B.P., Ramirez-Solis,R., Sands,A.T., and Zhang,N. (2002) mdmx is a negative regulator of p53 activity in vivo. *Cancer Res.*, **62**, 3221-3225.

32. Migliorini,D., Lazzerini,D.E., Danovi,D., Jochemsen,A.G., Capillo,M., Gobbi,A., Helin,K., Pelicci,P.G., and Marine,J.C. (2002) Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol.Cell Biol.*, **22**, 5527-5538.

33. Parant,J., Chavez-Reyes,A., Little,N.A., Yan,W., Reinke,V., Jochemsen,A.G., and Lozano,G. (2001) Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. *Nat.Genet.*, **29**, 92-95.

34. Francoz,S., Froment,P., Bogaerts,S., De,C.S., Maetens,M., Doumont,G., Bellefroid,E., and Marine,J.C. (2006) Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. *Proc.Natl.Acad.Sci.U.S.A*, **103**, 3232-3237.

35. Grier,J.D., Xiong,S., Elizondo-Fraire,A.C., Parant,J.M., and Lozano,G. (2006) Tissue-specific differences of p53 inhibition by Mdm2 and Mdm4. *Mol.Cell Biol.*, **26**, 192-198.

36. Maetens,M., Doumont,G., Clercq,S.D., Francoz,S., Froment,P., Bellefroid,E., Klingmuller,U., Lozano,G., and Marine,J.C. (2007) Distinct roles of Mdm2 and Mdm4 in red cell production. *Blood*, **109**, 2630-2633.

37. Ringshausen,I., O'Shea,C.C., Finch,A.J., Swigart,L.B., and Evan,G.I. (2006) Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo. *Cancer Cell*, **10**, 501-514.

38. Valentin-Vega,Y.A., Okano,H., and Lozano,G. (2008) The intestinal epithelium compensates for p53-mediated cell death and guarantees organismal survival. *Cell Death.Differ.*, **15**, 1772-1781.

39. Valentin-Vega,Y.A., Box,N., Terzian,T., and Lozano,G. (2009) Mdm4 loss in the intestinal epithelium leads to compartmentalized cell death but no tissue abnormalities. *Differentiation*, **77**, 442-449.

40. Marine,J.C. and Jochemsen,A.G. (2004) Mdmx and Mdm2: brothers in arms? *Cell Cycle*, **3**, 900-904.

41. Bottger,V., Bottger,A., Garcia-Echeverria,C., Ramos,Y.F., van der Eb,A.J., Jochemsen,A.G., and Lane,D.P. (1999) Comparative study of the p53-mdm2 and p53-MDMX interfaces. *Oncogene*, **18**, 189-199.

42. Haupt,Y., Barak,Y., and Oren,M. (1996) Cell type-specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J.*, **15**, 1596-1606.

43. Kawai,H., Wiederschain,D., and Yuan,Z.M. (2003) Critical contribution of the MDM2 acidic domain to p53 ubiquitination. *Mol.Cell Biol.*, **23**, 4939-4947.

44. Meulmeester,E., Frenk,R., Stad,R., de Graaf,P., Marine,J.C., Vousden,K.H., and Jochemsen,A.G. (2003) Critical role for a central part of Mdm2 in the ubiquitylation of p53. *Mol.Cell Biol.*, **23**, 4929-4938.

45. Sharp,D.A., Kratowicz,S.A., Sank,M.J., and George,D.L. (1999) Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J.Biol.Chem.*, **274**, 38189-38196.

46. Gu,J., Kawai,H., Nie,L., Kitao,H., Wiederschain,D., Jochemsen,A.G., Parant,J., Lozano,G., and Yuan,Z.M. (2002) Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. *J.Biol.Chem.*, **277**, 19251-19254.

47. Linares,L.K., Hengstermann,A., Ciechanover,A., Muller,S., and Scheffner,M. (2003) HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. *Proc.Natl.Acad.Sci.U.S.A*, **100**, 12009-12014.

48. Fang,S., Jensen,J.P., Ludwig,R.L., Vousden,K.H., and Weissman,A.M. (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J.Biol.Chem.*, **275**, 8945-8951.

49. Stommel,J.M. and Wahl,G.M. (2004) Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation. *EMBO J.*, **23**, 1547-1556.

50. de Graaf,P., Little,N.A., Ramos,Y.F., Meulmeester,E., Letteboer,S.J., and Jochemsen,A.G. (2003) Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2. *J.Biol.Chem.*, **278**, 38315-38324.

51. Kawai,H., Wiederschain,D., Kitao,H., Stuart,J., Tsai,K.K., and Yuan,Z.M. (2003) DNA damage-induced MDMX degradation is mediated by MDM2. *J.Biol.Chem.*, **278**, 45946-45953.

52. Pan,Y. and Chen,J. (2003) MDM2 promotes ubiquitination and degradation of MDMX. *Mol.Cell Biol.*, **23**, 5113-5121.

53. Li,M., Brooks,C.L., Kon,N., and Gu,W. (2004) A dynamic role of HAUSP in the p53-Mdm2 pathway. *Mol.Cell*, **13**, 879-886.

54. Meulmeester,E., Maurice,M.M., Boutell,C., Teunisse,A.F., Ovaa,H., Abraham,T.E., Dirks,R.W., and Jochemsen,A.G. (2005) Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol.Cell*, **18**, 565-576.

55. Hock,A.K., Vigneron,A.M., Carter,S., Ludwig,R.L., and Vousden,K.H. (2011) Regulation of p53 stability and function by the deubiquitinating enzyme USP42. *EMBO J.*.

56. Momand,J., Zambetti,G.P., Olson,D.C., George,D., and Levine,A.J. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, **69**, 1237-1245.

57. Pigolotti,S., Krishna,S., and Jensen,M.H. (2007) Oscillation patterns in negative feedback loops. *Proc.Natl.Acad.Sci.U.S.A*, **104**, 6533-6537.

58. Li,B., Cheng,Q., Li,Z., and Chen,J. (2010) p53 inactivation by MDM2 and MDMX negative feedback loops in testicular germ cell tumors. *Cell Cycle*, **9**, 1411-1420.

59. Phillips,A., Teunisse,A., Lam,S., Lodder,K., Darley,M., Emaduddin,M., Wolf,A., Richter,J., De Lange,J., Verlaan-de,V.M., Lenos,K., Bohnke,A., Bartel,F., Blaydes,J.P., and Jochemsen,A.G. (2010) HDMX-L is expressed from a functional p53-responsive promoter in the first intron of the HDMX gene and participates in an autoregulatory feedback loop to control p53 activity. *J.Biol.Chem.*, **285**, 29111-29127.

60. Wei,C.L., Wu,Q., Vega,V.B., Chiu,K.P., Ng,P., Zhang,T., Shahab,A., Yong,H.C., Fu,Y., Weng,Z., Liu,J., Zhao,X.D., Chew,J.L., Lee,Y.L., Kuznetsov,V.A., Sung,W.K., Miller,L.D., Lim,B., Liu,E.T.,

Yu,Q., Ng,H.H., and Ruan,Y. (2006) A global map of p53 transcription-factor binding sites in the human genome. *Cell*, **124**, 207-219.

61. Levine,A.J. and Oren,M. (2009) The first 30 years of p53: growing ever more complex. *Nat.Rev.Cancer*, **9**, 749-758.

62. Smith,J., Tho,L.M., Xu,N., and Gillespie,D.A. (2010) The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv.Cancer Res.*, **108**, 73-112.

63. Banin,S., Moyal,L., Shieh,S., Taya,Y., Anderson,C.W., Chessa,L., Smorodinsky,N.I., Prives,C., Reiss,Y., Shiloh,Y., and Ziv,Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, **281**, 1674-1677.

64. Lakin,N.D., Hann,B.C., and Jackson,S.P. (1999) The ataxia-telangiectasia related protein ATR mediates DNA-dependent phosphorylation of p53. *Oncogene*, **18**, 3989-3995.

65. Shieh,S.Y., Ahn,J., Tamai,K., Taya,Y., and Prives,C. (2000) The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev.*, **14**, 289-300.

66. Meek,D.W. (2009) Tumour suppression by p53: a role for the DNA damage response? *Nat.Rev.Cancer*, **9**, 714-723.

67. Dornan,D. and Hupp,T.R. (2001) Inhibition of p53-dependent transcription by BOX-I phospho-peptide mimetics that bind to p300. *EMBO Rep.*, **2**, 139-144.

68. Lambert,P.F., Kashanchi,F., Radonovich,M.F., Shiekhattar,R., and Brady,J.N. (1998) Phosphorylation of p53 serine 15 increases interaction with CBP. *J.Biol.Chem.*, **273**, 33048-33053.

69. Ito,A., Kawaguchi,Y., Lai,C.H., Kovacs,J.J., Higashimoto,Y., Appella,E., and Yao,T.P. (2002) MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. *EMBO J.*, **21**, 6236-6245.

70. Khosravi,R., Maya,R., Gottlieb,T., Oren,M., Shiloh,Y., and Shkedy,D. (1999) Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. *Proc.Natl.Acad.Sci.U.S.A*, **96**, 14973-14977.

71. Maya,R., Balass,M., Kim,S.T., Shkedy,D., Leal,J.F., Shifman,O., Moas,M., Buschmann,T., Ronai,Z., Shiloh,Y., Kastan,M.B., Katzir,E., and Oren,M. (2001) ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev.*, **15**, 1067-1077.

72. Chen,L., Gilkes,D.M., Pan,Y., Lane,W.S., and Chen,J. (2005) ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. *EMBO J.*, **24**, 3411-3422.

73. LeBron,C., Chen,L., Gilkes,D.M., and Chen,J. (2006) Regulation of MDMX nuclear import and degradation by Chk2 and 14-3-3. *EMBO J.*, **25**, 1196-1206.

74. Palmero,I., Pantoja,C., and Serrano,M. (1998) p19ARF links the tumour suppressor p53 to Ras. *Nature*, **395**, 125-126.

75. Zindy,F., Eischen,C.M., Randle,D.H., Kamijo,T., Cleveland,J.L., Sherr,C.J., and Roussel,M.F. (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev.*, **12**, 2424-2433.

76. Bates,S., Phillips,A.C., Clark,P.A., Stott,F., Peters,G., Ludwig,R.L., and Vousden,K.H. (1998) p14ARF links the tumour suppressors RB and p53. *Nature*, **395**, 124-125.

77. De Stanchina E., McCurrach,M.E., Zindy,F., Shieh,S.Y., Ferbeyre,G., Samuelson,A.V., Prives,C., Roussel,M.F., Sherr,C.J., and Lowe,S.W. (1998) E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes Dev.*, **12**, 2434-2442.

78. Quelle,D.E., Zindy,F., Ashmun,R.A., and Sherr,C.J. (1995) Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell*, **83**, 993-1000.

79. Honda,R. and Yasuda,H. (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J.*, **18**, 22-27.

80. Tao,W. and Levine,A.J. (1999) P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. *Proc.Natl.Acad.Sci.U.S.A*, **96**, 6937-6941.

81. Weber,J.D., Taylor,L.J., Roussel,M.F., Sherr,C.J., and Bar-Sagi,D. (1999) Nucleolar Arf sequesters Mdm2 and activates p53. *Nat.Cell Biol.*, **1**, 20-26.

82. Brosh,R. and Rotter,V. (2009) When mutants gain new powers: news from the mutant p53 field. *Nat.Rev.Cancer*, **9**, 701-713.

83. Schuijer,M. and Berns,E.M. (2003) TP53 and ovarian cancer. *Hum.Mutat.*, **21**, 285-291.

84. Iacopetta,B. (2003) TP53 mutation in colorectal cancer. *Hum.Mutat.*, **21**, 271-276.

85. Blons,H. and Laurent-Puig,P. (2003) TP53 and head and neck neoplasms. *Hum.Mutat.*, **21**, 252-257.

86. Peller,S. and Rotter,V. (2003) TP53 in hematological cancer: low incidence of mutations with significant clinical relevance. *Hum.Mutat.*, **21**, 277-284.

87. Kato,M.V., Shimizu,T., Ishizaki,K., Kaneko,A., Yandell,D.W., Toguchida,J., and Sasaki,M.S. (1996) Loss of heterozygosity on chromosome 17 and mutation of the p53 gene in retinoblastoma. *Cancer Lett.*, **106**, 75-82.

88. Houben,R., Hesbacher,S., Schmid,C.P., Kauczok,C.S., Flohr,U., Haferkamp,S., Muller,C.S., Schrama,D., Wischhusen,J., and Becker,J.C. (2011) High-level expression of wild-type p53 in melanoma cells is frequently associated with inactivity in p53 reporter gene assays. *PLoS.One.*, **6**, e22096.

89. Vousden,K.H. and Lu,X. (2002) Live or let die: the cell's response to p53. *Nat.Rev.Cancer*, **2**, 594-604.

90. Momand,J., Jung,D., Wilczynski,S., and Niland,J. (1998) The MDM2 gene amplification database. *Nucleic Acids Res.*, **26**, 3453-3459.

91. Danovi,D., Meulmeester,E., Pasini,D., Migliorini,D., Capra,M., Frenk,R., de Graaf,P., Francoz,S., Gasparini,P., Gobbi,A., Helin,K., Pelicci,P.G., Jochemsen,A.G., and Marine,J.C. (2004) Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol.Cell Biol.*, **24**, 5835-5843.

92. Riemenschneider,M.J., Knobbe,C.B., and Reifenberger,G. (2003) Refined mapping of 1q32 amplicons in malignant gliomas confirms MDM4 as the main amplification target. *Int.J.Cancer*, **104**, 752-757.

93. Ramos,Y.F., Stad,R., Attema,J., Peltenburg,L.T., van der Eb,A.J., and Jochemsen,A.G. (2001) Aberrant expression of HDMX proteins in tumor cells correlates with wild-type p53. *Cancer Res.*, **61**, 1839-1842.

94. Bykov,V.J., Issaeva,N., Shilov,A., Hultcrantz,M., Pugacheva,E., Chumakov,P., Bergman,J., Wiman,K.G., and Selivanova,G. (2002) Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat.Med.*, **8**, 282-288.

95. Vassilev,L.T., Vu,B.T., Graves,B., Carvajal,D., Podlaski,F., Filipovic,Z., Kong,N., Kammlott,U., Lukacs,C., Klein,C., Fotouhi,N., and Liu,E.A. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*, **303**, 844-848.

96. Vassilev,L.T. (2007) MDM2 inhibitors for cancer therapy. *Trends Mol.Med.*, **13**, 23-31.

97. Hu,B., Gilkes,D.M., Farooqi,B., Sebti,S.M., and Chen,J. (2006) MDMX overexpression prevents p53 activation by the MDM2 inhibitor Nutlin. *J.Biol.Chem.*, **281**, 33030-33035.

98. Patton,J.T., Mayo,L.D., Singhi,A.D., Gudkov,A.V., Stark,G.R., and Jackson,M.W. (2006) Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3. *Cancer Res.*, **66**, 3169-3176.

99. Wade,M., Wong,E.T., Tang,M., Stommel,J.M., and Wahl,G.M. (2006) Hdmx modulates the outcome of p53 activation in human tumor cells. *J.Biol.Chem.*, **281**, 33036-33044.

100. Kitagawa,M., Aonuma,M., Lee,S.H., Fukutake,S., and McCormick,F. (2008) E2F-1 transcriptional activity is a critical determinant of Mdm2 antagonist-induced apoptosis in human tumor cell lines. *Oncogene*, **27**, 5303-5314.

101. Laurie,N.A., Donovan,S.L., Shih,C.S., Zhang,J., Mills,N., Fuller,C., Teunisse,A., Lam,S., Ramos,Y., Mohan,A., Johnson,D., Wilson,M., Rodriguez-Galindo,C., Quarto,M., Francoz,S., Mendrysa,S.M., Guy,R.K., Marine,J.C., Jochemsen,A.G., and Dyer,M.A. (2006) Inactivation of the p53 pathway in retinoblastoma. *Nature*, **444**, 61-66.

102. Coll-Mulet,L., Iglesias-Serret,D., Santidrian,A.F., Cosialls,A.M., de Frias,M., Castano,E., Campas,C., Barragan,M., de Sevilla,A.F., Domingo,A., Vassilev,L.T., Pons,G., and Gil,J. (2006) MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. *Blood*, **107**, 4109-4114.

103. Lam,S., Lodder,K., Teunisse,A.F., Rabelink,M.J., Schutte,M., and Jochemsen,A.G. (2010) Role of Mdm4 in drug sensitivity of breast cancer cells. *Oncogene*.

104. Kojima,K., Konopleva,M., McQueen,T., O'Brien,S., Plunkett,W., and Andreeff,M. (2006) Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. *Blood*, **108**, 993-1000.

105. Barbieri,E., Mehta,P., Chen,Z., Zhang,L., Slack,A., Berg,S., and Shohet,J.M. (2006) MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death. *Mol.Cancer Ther.*, **5**, 2358-2365.

106. Grasberger,B.L., Lu,T., Schubert,C., Parks,D.J., Carver,T.E., Koblish,H.K., Cummings,M.D., LaFrance,L.V., Milkiewicz,K.L., Calvo,R.R., Maguire,D., Lattanze,J., Franks,C.F., Zhao,S., Ramachandren,K., Bylebyl,G.R., Zhang,M., Manthey,C.L., Petrella,E.C., Pantoliano,M.W., Deckman,I.C., Spurlino,J.C., Maroney,A.C., Tomczuk,B.E., Molloy,C.J., and Bone,R.F. (2005) Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells. *J.Med.Chem.*, **48**, 909-912.

107. Ding,K., Lu,Y., Nikolovska-Coleska,Z., Wang,G., Qiu,S., Shangary,S., Gao,W., Qin,D., Stuckey,J., Krajewski,K., Roller,P.P., and Wang,S. (2006) Structure-based design of spiro-

oxindoles as potent, specific small-molecule inhibitors of the MDM2-p53 interaction. *J.Med.Chem.*, **49**, 3432-3435.

108. Reed,D., Shen,Y., Shelat,A.A., Arnold,L.A., Ferreira,A.M., Zhu,F., Mills,N., Smithson,D.C., Regni,C.A., Bashford,D., Cicero,S.A., Schulman,B.A., Jochemsen,A.G., Guy,R.K., and Dyer,M.A. (2010) Identification and characterization of the first small molecule inhibitor of MDMX. *J.Biol.Chem.*, **285**, 10786-10796.

109. Bernal,F., Wade,M., Godes,M., Davis,T.N., Whitehead,D.G., Kung,A.L., Wahl,G.M., and Walensky,L.D. (2010) A stapled p53 helix overcomes HDMX-mediated suppression of p53. *Cancer Cell*, **18**, 411-422.

110. Wang,H. and Yan,C. (2011) A Small-Molecule p53 Activator Induces Apoptosis through Inhibiting MDMX Expression in Breast Cancer Cells. *Neoplasia.*, **13**, 611-619.

111. Issaeva,N., Bozko,P., Enge,M., Protopopova,M., Verhoef,L.G., Masucci,M., Pramanik,A., and Selivanova,G. (2004) Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat.Med.*, **10**, 1321-1328.

112. Enge,M., Bao,W., Hedstrom,E., Jackson,S.P., Moumen,A., and Selivanova,G. (2009) MDM2-dependent downregulation of p21 and hnRNP K provides a switch between apoptosis and growth arrest induced by pharmacologically activated p53. *Cancer Cell*, **15**, 171-183.

113. Kivela,T. (2009) The epidemiological challenge of the most frequent eye cancer: retinoblastoma, an issue of birth and death. *Br.J.Ophthalmol.*, **93**, 1129-1131.

114. Dyer,M.A. and Bremner,R. (2005) The search for the retinoblastoma cell of origin. *Nat.Rev.Cancer*, **5**, 91-101.

115. Chang,A.E., Karnell,L.H., and Menck,H.R. (1998) The National Cancer Data Base report on cutaneous and noncutaneous melanoma: a summary of 84,836 cases from the past decade. The American College of Surgeons Commission on Cancer and the American Cancer Society. *Cancer*, **83**, 1664-1678.

116. Virgili,G., Gatta,G., Ciccolallo,L., Capocaccia,R., Biggeri,A., Crocetti,E., Lutz,J.M., and Paci,E. (2007) Incidence of uveal melanoma in Europe. *Ophthalmology*, **114**, 2309-2315.

117. Bedikian,A.Y., Kantarjian,H., Young,S.E., and Bodey,G.P. (1981) Prognosis in metastatic choroidal melanoma. *South.Med.J.*, **74**, 574-577.

118. Char,D.H. (1978) Metastatic choroidal melanoma. *Am.J.Ophthalmol.*, **86**, 76-80.

119. Lorigan,J.G., Wallace,S., and Mavligit,G.M. (1991) The prevalence and location of metastases from ocular melanoma: imaging study in 110 patients. *AJR Am.J.Roentgenol.*, **157**, 1279-1281.

120. Kivela,T., Eskelin,S., and Kujala,E. (2006) Metastatic uveal melanoma. *Int.Ophthalmol.Clin.*, **46**, 133-149.

121. Augsburger,J.J., Correa,Z.M., and Shaikh,A.H. (2009) Effectiveness of treatments for metastatic uveal melanoma. *Am J Ophthalmol.*, **148**, 119-127.

122. Brantley,M.A., Jr. and Harbour,J.W. (2000) Deregulation of the Rb and p53 pathways in uveal melanoma. *Am J Pathol.*, **157**, 1795-1801.

123. Chana,J.S., Wilson,G.D., Cree,I.A., Alexander,R.A., Myatt,N., Neale,M., Foss,A.J., and Hungerford,J.L. (1999) c-myc, p53, and Bcl-2 expression and clinical outcome in uveal melanoma. *Br.J Ophthalmol.*, **83**, 110-114.

124. Hussein,M.R. (2005) The relationships between p53 protein expression and the clinicopathological features in the uveal melanomas. *Cancer Biol.Ther.*, **4**, 57-59.

125. Sun,Y., Tran,B.N., Worley,L.A., Delston,R.B., and Harbour,J.W. (2005) Functional analysis of the p53 pathway in response to ionizing radiation in uveal melanoma. *Invest Ophthalmol.Vis.Sci.*, **46**, 1561-1564.