IDENTIFYING NOVEL COMPONENTS OF HUMAN TUMOUR SUPPRESSOR NETWORKS

Jarno Drost

ISBN: 978-94-6182-070-9

Layout and printing: Off Page, www.offpage.nl

Copyright © 2012 by J. Drost. All rights reserved.
No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, without prior permission of the author.

IDENTIFYING NOVEL COMPONENTS OF HUMAN TUMOUR SUPPRESSOR NETWORKS

Identificatie van nieuwe componenten van humane tumorsuppressor-netwerken

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 28 maart 2012 om 13.30 uur

door

Jarno Drost
geboren te Nunspeet

PROMOTIECOMMISSIE

Promotor:	Prof.dr. R. Agami
Overige leden:	Prof.dr. L.H.J. Looijenga Prof.dr. H.P.J. te Riele Prof.dr. C.P. Verrijzer
Copromotor:	Dr. P.M. Voorhoeve

CONTENTS

1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

CANCER

When one takes into account that the human body consists of approximately 100 trillion (10^{14}) cells, it is frightening to imagine that the majority of these cells have the intrinsic capacity to become a cancer cell that divides uncontrollably and develops into a malignant tumour. Fortunately, cells have defence mechanisms that protect them from becoming malignant. Still, cancer is a common disease worldwide, indicating that things go wrong despite these mechanisms. Although many types of cancer exist, it is thought that the vast majority originate through the manifestation of several distinct alterations[1]. First, cells have to self-sustain their growth indefinitely and, second, they have to become insensitive to anti-growth signals. Third, they have to evade the induction of programmed cell death, a process called apoptosis. Fourth, cells have to acquire limitless replicative potential. Fifth, in order to progress to a larger size, tumours must develop blood vessels to ensure sustained oxygen and nutrient supply (angiogenesis). Sixth, tumour cells have to acquire the capability to invade neighbouring tissue and gain the potential to settle at distant sites in the organism (metastasis). Additionally, in recent years it has become apparent that development of certain cancers also requires a switch in the energy metabolism and the ability to evade destruction by the host immune system[2]. This would make a total of at least eight capabilities cells have to acquire to be able to develop into a malignant tumour.

MAKING CANCER CELLS *IN VITRO*

The cancer-causing alterations described above originate from errors in the pathways that normally govern these processes. Genetic alterations (mutations) as well as changes in gene expression devoid of alterations in the genetic code (the so-called epigenetic changes) are the cause of these errors. In healthy cells, mutations are repaired by cellular DNA repair systems. However, the genomes of cancer cells are in general unstable, thereby increasing the frequency of gaining alterations in pivotal pathways.

The (epi-) genetic alterations underlying cancer development are found in so-called tumour suppressor genes (cellular "brake" genes that inhibit growth) and oncogenes (cellular "accelerator" genes that support growth) resulting in the respective inactivation and activation of these genes. Examples of genes that have a high inactivating mutation frequency in human cancer are the retinoblastoma (pRB) and p53 tumour suppressor genes. Ras (Ras^{V12}; glycine 12 to valine) and B-Raf ($B\text{-}Raf^{E600}$; valine 600 to glutamine) are examples of proto-oncogenes in which activating mutations are frequently found.

In 1999 Hahn et al. described an *in vitro* system to make tumour cells from normal (primary) human cells by expression of defined genetic elements (Fig. 1). Expression of telomerase reverse transcriptase (hTERT), the early region of simian virus 40 (SV40), and oncogenic Ras was shown to be sufficient to fully transform human primary cells into cells having all the characteristics of tumour cells[3]. HTERT is the enzymatic component of the human telomerase enzyme.

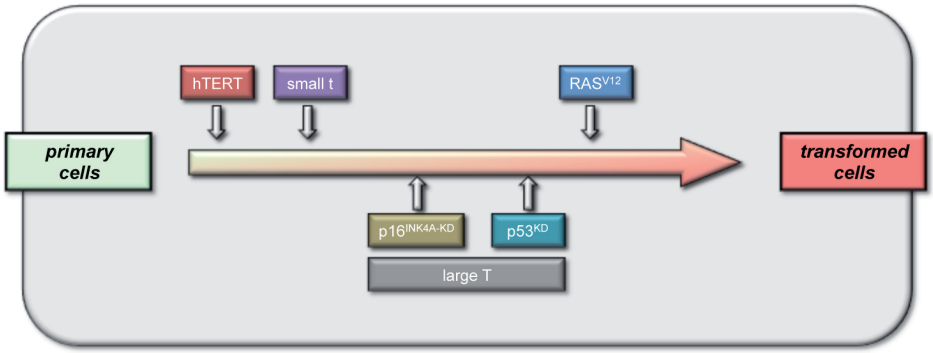


Figure 1. Schematic model for the *in vitro* transformation of human primary cells. Overexpression of hTERT, small t, and Ras^{V12} in combination with inhibition of the p53 and $p16^{INK4A}$/pRB tumour suppressor pathways – either by short interfering RNAs (KD: knockdown) or expression of viral antigens (e.g. large T) – results in the full neoplastic transformation of human primary cells.

Normal cells have a limited lifespan due to erosion of the telomeres (the ends of chromosomes) by repeated cell division. During tumorigenesis, cells have to find a way to prevent that telomere length decreases to an extent that would make them stop proliferating. HTERT expression immortalizes cells by enabling them to sustain telomere length[4]. Telomerase activity is only present in certain types of stem and germ cells, and not in normal human cells. Cancer cells are also able to sustain their telomeres either by expression of hTERT[5] or by a recombination-based mechanism called ALT (alternative lengthening of telomeres)[6]. The early region of SV40 encodes for the small and large T antigens. Small t is thought to participate in the transformation process through interference with the function of protein phosphatase 2A[7]. Large T expression can be substituted by expression of short interfering RNAs (siRNAs) targeting components of the pRB and p53 tumour suppressor pathways[8] (Fig. 1), indicating that the effect of large T mainly encompasses inactivation of these two pathways. Lastly, the expression of oncogenic Ras provides cells with the sustained mitogenic trigger needed to proliferate without being dependent on external growth signals. In this thesis we utilized this well-defined system to identify novel components of the involved pathways and to get a better understanding of how normal cells can transform into tumour cells.

SENESCENCE: A MECHANISM AGAINST TRANSFORMATION

Senescence

Senescence is an irreversible growth arrest that can be induced in response to certain cellular stresses (Fig. 2). One of the first observations describing this phenomenon was that *in vitro* propagation ultimately drives primary human cells into an irreversible growth arrest[9,10]. It was termed replicative senescence and is

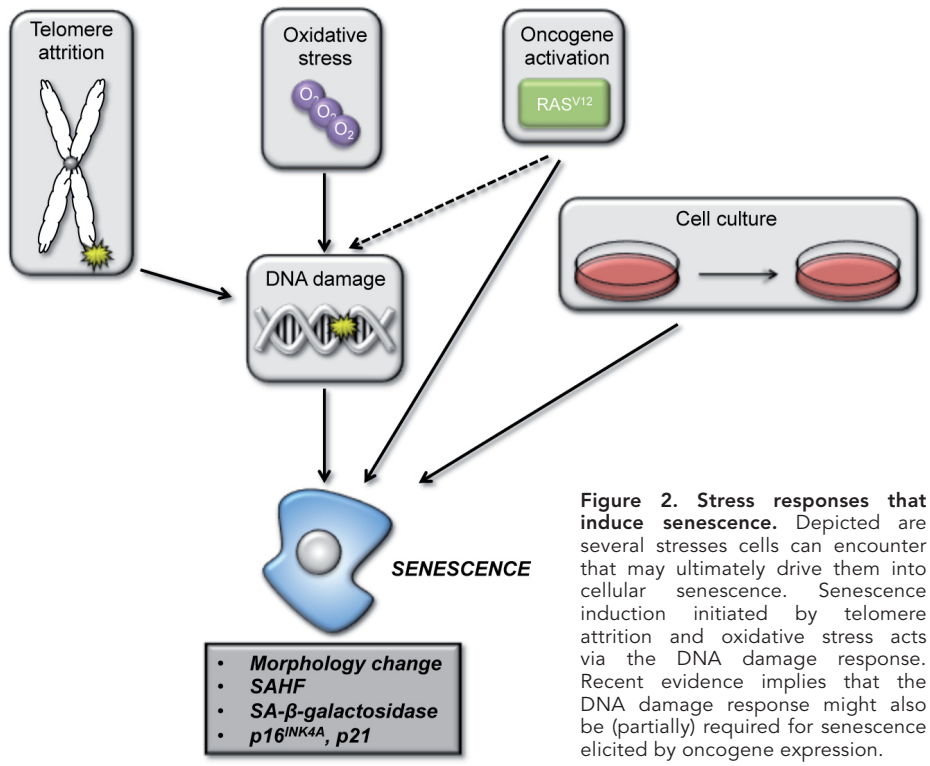


Figure 2. Stress responses that induce senescence. Depicted are several stresses cells can encounter that may ultimately drive them into cellular senescence. Senescence induction initiated by telomere attrition and oxidative stress acts via the DNA damage response. Recent evidence implies that the DNA damage response might also be (partially) required for senescence elicited by oncogene expression.

caused by progressive shortening of telomeres[11-13]. The DNA damage response plays a central role in replicative senescence. When telomeres reach their critical minimal length a DNA damage response is initiated. This stress response can drive cells into a reversible cell cycle arrest. Within the timeframe of the arrest cells can repair their damage and re-enter the cell cycle. When the damage exceeds a certain limit, cells can either go into apoptosis or enter replicative senescence. Maintaining telomere length through activation of telomerase prevents that cells go into replicative senescence[12,13].

Oncogene-induced senescence

Cells can also enter senescence independently of telomere attrition (Fig. 2). In primary cells, for example, excessive mitogenic signalling elicits a stress response that drives them into in an irreversible cell cycle arrest. The arrest phenotypically resembles replicative senescence and was therefore named oncogene-induced senescence (OIS)[14]. *In vitro*, OIS can be induced by expression of oncogenes, such as Ras^{V12}. Although OIS and the DNA damage response are known as separate cellular stress responses, recent reports suggest that they actually might be causally linked. This model proposes that the initial hyper-replication phase upon oncogene expression leads to DNA replication stress and the activation of a DNA damage response, which ultimately drives cells into senescence[15,16].

1

Importantly, OIS was found to play a pivotal role in the protection against tumour development *in vivo*[17-20].

Markers of senescence

Several markers have been established that can be used to identify senescent cells (reviewed in ref. 21) (Fig. 2). First of all, senescent cells are characterized by their typical morphological appearance. The acquired morphology might differ depending on the stress responsible for the initiation of senescence. Cells become big and flat in the case of Ras^{V12}-induced senescence. Several general markers have been found that are widely used to study this important tumour suppressor mechanism. Increased protein levels of certain components of the p53 and pRB tumour suppressor pathways – the two main pathways involved in the establishment of senescence – are found in senescent cells. High levels of $p16^{INK4A}$, its relative $p15^{INK4B}$, and the p53 target gene p21 can be detected[21]. Senescence is, at least *in vitro*, not always accompanied with increased p53 levels. The oncogenic trigger can also result in post-translational modification of p53 that activate, rather than stabilize, the protein[8]. Other proteins are highly expressed in senescent cells as well, but their functional involvement is not very well established. Two examples of this class of markers are DCR2 and DEC1 (ref. 21). Another feature of senescent cells is the activity of senescence-associated β-galactosidase. The enzymatic activity is derived from the increased lysosomal content of senescent cells, which can be detected both *in vitro* and *in vivo*[22].

Finally, chromatin-dense foci are present in senescent cells that can be visualized by staining of the DNA with DNA dyes, like DAPI[23]. Chromatin is an organized combination of DNA and protein that is used to deal with the large amount of DNA that is present in every nucleus of a cell. Winding of every 147 basepairs of the DNA helix around eight histone proteins (histone octamer) creates the so-called nucleosome. It consists of two copies of the four core histone proteins: H2A, H2B, H3, and H4. Linked by histone H1, the nucleosomes coil into the 30 nm fibre. The packaging of DNA in chromatin also serves as an additional layer of transcriptional regulation. Transcriptionally silent domains are in densely packed chromatin (heterochromatin), whereas transcriptionally active chromatin (euchromatin) appears to be open and thereby accessible for proteins that regulate transcription. Posttranslational modifications of histones are an important factor in regulation of chromatin states. Active and inactive chromatin domains are marked by specific histone modifications. The foci in senescent cells bear features of transcriptionally silent chromatin and are therefore named senescence-associated heterochromatin foci (SAHF)[23]. Initially it was thought that heterochromatin formation during the establishment of senescence stably silences genes required for proliferation (like target genes of the transcription factor E2F). However, a recent report describes that SAHF are also present in oncogene-expressing cells that bypassed OIS, implying that they are not required for the proliferation arrest[24]. They point to a role of SAHF in inhibiting the replication stress-induced DNA damage response[15], thereby driving the cells

into senescence rather than apoptosis. This would mean that SAHF are not true markers of senescent cells. In fact, none of the above-described features solely mark senescent cells. Therefore, a combination of several markers has to be used to detect them.

THE P53 TUMOUR SUPPRESSOR PATHWAY

The p53 gene is the most frequently mutated gene in human cancer as mutations appear in more than 50% of tumours. In the case that tumours harbour the wild-type p53 gene, it is thought that alterations in other p53 pathway components are responsible for its inactivation[25]. The p53 pathway plays a central role in senescence and its tumour suppressive function mainly relies on this effect[26]. Nonetheless, it has been well documented that p53 is also essential for other stress responses, including induction of apoptosis. The p53 gene encodes a transcription factor that can activate and repress transcription of target genes. Although other functions have been ascribed to p53, the observation that the inactivating mutations found in cancer are mainly in its sequence-specific DNA binding domain implies that regulating transcription is its main function in tumour suppression[25].

It is not surprising that p53 expression and activity are tightly regulated, as its function forms an important barrier to malignant transformation. The p53 protein is highly unstable due to the activity of the ubiquitin ligase MDM2. MDM2 itself is a p53 target gene, thereby creating a negative feedback loop. MDM2 activity is counteracted by the $p14^{ARF}$ protein, which can bind and sequester MDM2, thereby stabilizing p53. In addition, posttranslational modifications of p53 can interfere with the p53/MDM2 interaction, like N-terminal phosphorylation[27,28]. Besides phosphorylation and ubiquitination, p53 protein stability and activity are regulated by many other posttranslational modifications, including acetylation, sumoylation and methylation[29]. For example, acetylation of p53's lysine residues is a requisite for its activation[30].

As a transcription factor, p53 regulates the transcription of dozens of target genes. Selective regulation of distinct target gene sets is responsible for the different responses that p53 activation can elicit. Certain posttranslational modifications enable p53 to transcriptionally activate specific subsets of target genes[31-34]. Furthermore, quantitative and qualitative differences in stress input, cell-type specific variations in the availability of cofactors, as well as differences in the affinity of p53 and its cofactors for target gene promoters appear to be involved in regulating specific target gene sets and driving cells into a certain response (ref. 35-39) (Fig. 3). In general, p53 has a higher affinity for promoters of target genes required for the induction of a cell cycle arrest than for promoters of pro-apoptotic target genes. Depending on the strength or nature of the stress response, p53 can be stabilized and activated in different degrees. In response to mild stress, relatively low levels of p53 will preferentially activate high affinity

1

pro-cell cycle arrest genes whereas massive stress will lead to higher levels of active p53, which can then also bind and activate the low affinity promoters of pro-apoptotic target genes (ref. 25) (Fig. 3). The bromodomain-containing protein BRD7 is specifically required for p53 binding to the high affinity target gene promoters and is essential for recruitment of histone acetyltransferases to these promoters, thereby keeping the chromatin in an open and active state[39,40]. BRD7 is one of the factors of which inhibition can neutralize the p53 pathway without affecting the p53 gene itself. Indeed, BRD7 loss is found in a substantial amount of human breast tumours harbouring wild-type p53 (ref. 39).

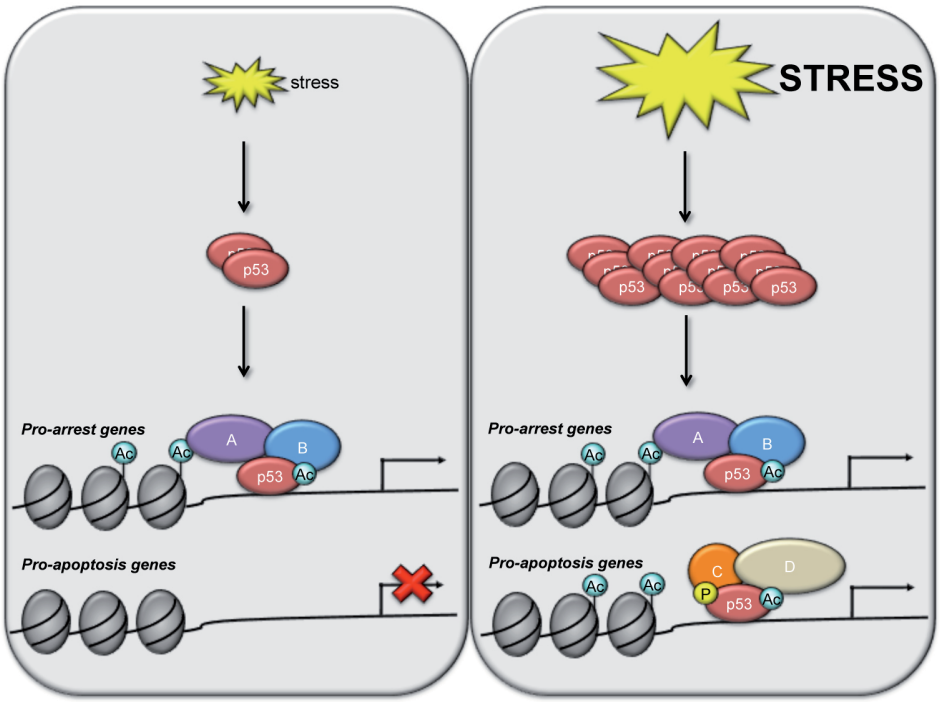


Figure 3. Triggering the p53 pathway. Activation of the p53 tumour suppressor pathway can result in several cellular outcomes, including cell cycle arrest, senescence and apoptosis. This depends on the nature of the stress, the availability of cofactors and the affinity of p53 and/or its cofactors for their target genes. Upon mild stress, relatively low levels of p53 bind preferentially to high affinity pro-cell cycle arrest gene promoters and recruit cofactors (depicted as A and B) to efficiently activate transcription. Severe stress results in high levels of p53, which can now also bind to low affinity pro-apoptotic target gene promoters and recruit different factors to activate transcription (depicted as C and D). Additionally, posttranslational modifications of p53 are involved in regulating specific subsets of target genes as well. For convenience only acetylation (Ac) and phosphorylation (P) are depicted. The p53 protein can be modified at multiple residues. A combination of modifications is required for its proper activation.

NON-CODING RNAS IN THE P53 PATHWAY

The p53 tumour suppressor network consists of – and regulates – many genes. Until recently this complex network only contained protein-coding genes. The discovery of non-coding RNAs initiated the search for a possible role of this class of regulatory molecules in the p53 pathway.

MicroRNAs

MicroRNAs (miRNAs) were the first class of non-coding RNAs shown to play a role in the p53 pathway. MiRNAs are small endogenous RNAs that silence gene expression posttranscriptionally. This mainly occurs by binding of the mature (processed) miRNA to the 3'-untranslated region (3'UTR) of its target genes, resulting in translational inhibition and/or transcript decay. Nucleotides 2 – 8 from the 5'-end of the mature miRNA, the so-called seed sequence, appear to be important for binding to its target sequences. A role for miRNAs in tumorigenesis is well established, acting both as tumour suppressor genes and oncogenes.

The miR-34 family is a direct transcriptional target of p53 and is required for proper p53 function[41-45]. As a downstream effector of the p53 pathway, this miRNA family functions as a tumour suppressor gene. On the other hand, the miR-372&373 cluster has oncogenic properties. Expression of this cluster bypasses OIS, partially via neutralization of downstream signalling of p53 (ref. 46). Furthermore, expression of miR-373 promotes tumour invasion and metastasis[47]. Another oncogenic miRNA cluster implicated in the p53 pathway is the miR-17-92 cluster. This miRNA cluster contains six miRNAs, namely miR-17, miR-18, miR-19a, miR-19b, miR-20 and miR-92. Targeting of the p53 target gene p21 and the E2F transcription factors by miR-17&20 partially explains the oncogenicity of the cluster[48-52]. In addition, miR-19a&b have been shown to target the tumour suppressor PTEN, an important inhibitor of the PI3K/AKT signalling pathway that regulates cell survival, cell cycle progression and cellular growth[53,54]. Finally, the pro-apoptosis factor BIM is targeted by multiple miRNAs of the miR-17-92 cluster, including miR-92 (ref. 55-58). Therefore, it seems that the miR-17-92 cluster reaches its optimal oncogenic activity through all its miRNA components. This is confirmed by several recent reports showing that expression of the complete miRNA cluster is required for the highest oncogenic potential in the oncogene-induced stress response (ref. 49, Drost et al., this thesis) and most potent inhibition of the TGF-β pathway in neuroblastoma[59].

At present, many more miRNAs have been described that target either components of the p53 pathway, or even p53 itself (reviewed in ref. 60). However, the picture appears to be even more complex as miRNAs are not the only non-coding RNAs that are implicated in the p53 pathway.

Long intergenic non-coding RNAs (lincRNAs)

Recently, over a thousand long intergenic non-coding RNAs (lincRNAs) were identified, using genome-wide chromatin-state maps[61]. Besides the presence

1

and/or absence of specific histone modifications, the location – outside known protein-coding genes – and ultra-conservation are other features of potential lincRNA-encoding regions. Interestingly, several lincRNAs are transcribed in a p53-dependent manner[61,62]. One of these lincRNAs, termed lincRNA-p21, is specifically required for p53-dependent repression of target genes and induction of apoptosis[62]. The mechanism whereby lincRNA-p21 is repressing transcription is not well understood. However, several possibilities exist based on observations for other lincRNAs. HOTAIR, for example, was shown to interact with the Polycomb Repressive Complex 2 (PRC2). This complex keeps chromatin in a transcriptionally silent state. HOTAIR is required for transcriptional silencing of the HOXD locus and for PRC2-dependent histone modifications at this locus[63]. In addition, HOTAIR can also interact with other transcription regulation complexes[64]. Essentially, 20% of the thus-far known lincRNAs was shown to bind the PRC2 complex[65]. This indicates that non-coding RNA-mediated recruitment of histone modifying complexes is an important mechanism by which lincRNAs can regulate gene expression.

Another possible mechanism by which non-coding RNAs could regulate transcription was proposed with the discovery of enhancer RNAs (eRNAs)[66]. Enhancers are regulatory elements that enhance transcription of genes at distant sites. A genome-wide search for enhancer domains identified a subset of enhancers that were bound by RNA polymerase II and produced transcripts bi-directionally. The transcription of these so-called eRNAs positively correlated with the level of mRNA production of neighbouring genes. However, the functional relevance of eRNAs is still unclear. One proposed mechanism is that eRNAs affect chromatin modifications at enhancer domains that may facilitate loop formation between promoter and enhancer regions. In this thesis we describe the identification of several regions that are bound by p53 with high affinity. These regions carry the characteristics of eRNA-producing enhancer domains and produce transcripts (eRNAs) in a p53-dependent fashion. We further demonstrate that the eRNAs produced from the identified enhancer regions are required for efficient transcription of target genes and a p53-dependent cell cycle arrest. Finally, we show that p53 is required for the enhancer activity and that intra-chromosomal interactions take place between the enhancers and surrounding genes. These results imply that p53 may control transcription of multiple genes via the regulation of enhancer activity.

Altogether, non-coding RNAs are an important class of regulatory molecules in the p53 pathway. With the on-going discovery of ultra-conserved RNA species it would not be surprising if many more non-coding RNAs play pivotal roles in this tumour suppressor pathway.

OUTLINE OF THE THESIS

The p53 pathway plays a central role in protecting cells from becoming tumorigenic. Reactivation of the pathway is under extensive study in the development of anti-cancer drugs (reviewed by Mandinova and Lee, Sci. Transl. Med. 2011). Although a lot is known about the pathway, the way it is inactivated in human tumours harbouring the wild-type p53 gene is in many cases unknown. This raises the question if we indeed know all the components of this important tumour suppressor pathway. In this thesis, we aimed to identify novel components of the p53 tumour suppressor network. Besides focussing on protein-coding genes, we also set out to identify non-coding gene components. To reach these goals, we made use of genetic screens and genome-wide p53 binding analysis. Ultimately, newly identified members might be used as novel targets for anti-cancer drugs.

Chapter 1 is a general introduction giving an overview of the processes, pathways and molecules that are being discussed in this thesis.

Chapter 2 describes a screen to find novel tumour suppressor genes in the transformation process. We identified BRD7 as an important cofactor of p53 that is required for p53-dependent transcription of a subset of target genes and oncogene-induced senescence. We suggest that BRD7 loss is a way to neutralize the p53 pathway in human tumours that retain wild-type p53.

Chapter 3 gives an extra view on the results described in chapter 2 and links our findings to published data about related topics.

In chapter 4 we analysed genome-wide p53 binding patterns to identify non-coding transcripts as transcriptional targets of the p53 pathway. We describe the identification of several enhancer domains that are bound by p53 with high affinity and produce eRNAs in a p53-dependent manner. In addition, the activity of these enhancers requires p53. We suggest that p53 can regulate target gene transcription by producing eRNAs and affecting the activity of enhancer domains.

In chapter 5 a screen is described that was performed to identify oncogenic miRNAs. We show that expression of the miR-17-19 cluster – containing miR-17, miR-18, miR-19a, miR-20 and miR-19b – interferes with the pRB pathway, thereby contributing to the full neoplastic transformation of human primary cells.

Chapter 6 discusses a recent paper describing the role of a positive feedback loop consisting of IL-6, NF-κB, miRNA let-7 and LIN28B in oncogene-induced transformation. A transient oncogenic trigger appears to be sufficient to fully transform certain cell types. The status of the pRB and p53 pathways might be decisive whether or not cells are driven into this feedback loop.

Chapter 7 is a general discussion about the findings presented in this thesis.

1

REFERENCES

1. Hanahan, D. and Weinberg, R.A. The hallmarks of cancer. Cell **100,** 57 – 70 (2000).
2. Hanahan, D. and Weinberg, R.A. Hallmarks of cancer: the next generation. Cell **144,** 646 – 674 (2011).
3. Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. Creation of human tumour cells with defined genetic elements. Nature **400,** 464 – 468 (1999).
4. Lustig, A.J. Crisis intervention: The role of telomerase. Proc. Natl. Acad. Sci. USA **96,** 3339 – 3341 (1999).
5. Shay, J.W. and Bacchetti, S. A survey of telomerase activity in human cancer. Eur. J. Cancer **33,** 787 – 791 (1997).
6. Muntoni, A. and Reddel, R.R. The first molecular details of ALT in human tumor cells. Hum. Mol. Genet. **14,** R191 – R196 (2005).
7. Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and Weinberg, R.A. Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol. Cell. Biol. **22,** 2111 – 2123 (2002).
8. Voorhoeve, P.M. and Agami R. The tumor-suppressive functions of the human INK4A locus. Cancer Cell **4,** 311 – 319 (2003).
9. Hayflick, L. and Moorhead, P.S. The serial cultivation of human diploid cell strains. Exp. Cell Res. **25,** 585 – 621 (1961).
10. Hayflick, L. The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. **37,** 614 – 636 (1965).
11. Harley, C.B., Futcher, A.B., and Greider, C.W. Telomeres shorten during ageing of human fibroblasts. Nature **345,** 458 – 460 (1990).
12. Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. Extension of life-span by introduction of telomerase into normal human cells. Science **279,** 349 – 352 (1998).
13. Vaziri, H., and Benchimol, S. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. Curr. Biol. **8,** 279 –282 (1998).
14. Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell **88,** 595 – 602 (1997).
15. Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre', M., Nuciforo, P.G., Bensimon, A., Maestro, R., Pelicci, P.G., and d'Adda di Fagagna, F. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature **444,** 638 – 642 (2006).
16. Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C.L., Dyrskjot, L., Ørntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T.D., Bartek, J., and Gorgoulis, V.G. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature **444,** 633 – 637 (2006).
17. Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H., Schlegelberger, B., Stein, H., Dörken, B., Jenuwein, T., and Schmitt, C.A. Oncogene-induced senescence as an initial barrier in lymphoma development. Nature **436,** 660 – 665 (2005).
18. Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., Cordon-Cardo, C., and Pandolfi, P.P. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumourigenesis. Nature **436,** 725-730 (2005).
19. Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguría, A., Zaballos, A., Flores, J.M., Barbacid, M., Beach, D., and Serrano, M. Tumour biology: senescence in premalignant tumours. Nature **436,** 642 (2005).
20. Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S. BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature **436,** 720-724 (2005).
21. Collado, M. and Serrano, M. The power and the promise of oncogene-induced senescence markers. Nat. Rev. Cancer **6,** 472 – 476 (2006).
22. Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., and Campisi, J. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA **92,** 9363 – 9367 (1995).
23. Narita, M., Nũnez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell **113,** 703 – 716 (2003).
24. Di Micco, R., Sulli, G., Dobreva, M., Liontos, M., Botrugno, O.A., Gargiulo, G., dal Zuffo, R., Matti, V., d'Ario, G., Montani, E., Mercurio, C., Hahn, W.C., Gorgoulis, V., Minucci, S., and d'Adda di Fagagna, F. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat. Cell Biol. **13,** 292 – 302 (2011).

25. Vousden, K.H., and Lu, X. Live or let die: the cell's response to p53. Nat. Rev. Cancer **2,** 594-604 (2002).
26. Christophorou, M.A., Ringshausen, I., Finch, A.J., Swigart, L.B., and Evan, G.I. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature **443,** 214-217 (2006).
27. Michael, D., and Oren, M. The p53-Mdm2 module and the ubiquitin system. Semin. Cancer Biol. **13,** 49 – 58 (2003).
28. Harris, S.L. and Levine, A.J. The p53 pathway: positive and negative feedback loops. Oncogene **24,** 2899 – 2908 (2005).
29. Kruse, J.P. and Gu, W. SnapShot: p53 post-translational modifications. Cell **133,** 930 – 930e1 (2008).
30. Tang, Y., Zhao, W., Chen, Y., Zhao, Y., and Gu, W. Acetylation is indispensable for p53 activation. Cell **133,** 612 – 626 (2008).
31. Sykes, S.M., Mellert, H.S., Holbert, M.A., Li, K., Marmorstein, R., Lane, W.S., and McMahon, S.B. Acetylation of the p53 DNA-binding domain regulates apoptosis induction. Mol. Cell **24,** 841 – 851 (2006).
32. Tang, Y., Luo, J., Zhang, W., and Gu, W. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. Mol. Cell **24,** 827 – 839 (2006).
33. Knights, C.D., Catania, J., Di Giovanni, S., Muratoglu, S., Perez, R., Swartzbeck, A., Quong, A.A., Zhang, X., Beerman, T., Pestell, R.G., and Avantaggiati, M.L. Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate. J. Cell Biol. **173,** 533 – 544 (2006).
34. Zhao, Y., Lu, S., Wu, L., Chai, G., Wang, H., Chen, Y., Sun, J., Yu, Y., Zhou, W., Zheng, Q., Wu, M., Otterson, G.A., and Zhu, W.G. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). Mol. Cell. Biol. **26,** 2782 – 2790 (2006).
35. Lahav, G., Rosenfeld, N., Sigal, A., Geva-Zatorsky, N., Levine, A.J., Elowitz, M.B., and Alon, U. Dynamics of the p53-Mdm2 feedback loop in individual cells. Nat. Genetics **36,** 147 – 150 (2004).
36. Mantovani, F., Tocco, F., Girardini, J., Smith, P., Gasco, M., Lu, X., Crook, T., and Del Sal, G. The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. Nat. Struct. Mol. Biol. **14,** 912 – 920 (2007).
37. Das, S., Raj, L., Zhao, B., Kimura, Y., Bernstein, A., Aaronson, S.A., and Lee, S.W. Hzf Determines cell survival upon genotoxic stress by modulating p53 transactivation. Cell **130,** 624 – 637 (2007).
38. Tanaka, T., Ohkubo, S., Tatsuno, I., and Prives, C. hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. Cell **130,** 638 – 650 (2007).
39. Drost, J., Mantovani, F., Tocco, F., Elkon, R., Comel, A., Holstege, H., Kerkhoven, R., Jonkers, J., Voorhoeve, P.M., Agami, R., and Del Sal, G. BRD7 is a candidate tumour suppressor gene required for p53 function. Nat. Cell Biol. **12,** 380 – 389 (2010).
40. Burrows, A.E., Smogorzewska, A., and Elledge, S.J. Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence. Proc. Natl. Acad. Sci. USA **107,** 14280 – 14285 (2010).
41. Bommer, G.T., Gerin, I., Feng, Y., Kaczorowski, A.J., Kuick, R., Love, R.E., Zhai, Y., Giordano, T.J., Qin, Z.S., Moore, B.B., MacDougald, O.A., Cho, K.R., and Fearon, E.R. p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr. Biol. **17,** 1298 – 1307 (2007).
42. Chang, T.C., Wentzel, E.A., Kent, O.A., Ramachandran, K., Mullendore, M., Lee, K.H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J., Arking, D.E., Beer, M.A., Maitra, A., and Mendell, J.T. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol. Cell **26,** 745 – 752 (2007).
43. He, L., He, X., Lim, L.P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., Jackson, A.L., Linsley, P.S., Chen, C., Lowe, S.W., Cleary, M.A., and Hannon, G.J. A microRNA component of the p53 tumour suppressor network. Nature **447,** 1130 – 1134 (2007).
44. Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z., and Oren, M. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol. Cell **26,** 731 – 743 (2007).
45. Tarasov, V., Jung, P., Verdoodt, B., Lodygin, D., Epanchintsev, A., Menssen, A., Meister, G., and Hermeking, H. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle **6,** 1586 – 1593 (2007).
46. Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A.J., Stoop, H., Nagel, R., Liu, Y.P., van Duijse, J., Drost, J., Griekspoor, A., Zlotorynski, E., Yabuta, N., De Vita, G., Nojima, H., Looijenga, L.H., and Agami, R. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumours. Cell **124,** 1169-1181 (2006).
47. Huang, Q., Gumireddy, K., Schrier, M., le Sage, C., Nagel, R., Nair, S., Egan, D.A., Li, A., Huang, G., Klein-Szanto, A.J., Gimotty, P.A., Katsaros, D., Coukos, G., Zhang, L., Puré, E., and Agami, R. The microRNAs miR-373 and miR-520c promote tumour

invasion and metastasis. Nat. Cell Biol. **10,** 202 – 210 (2008).

48. Ivanovska, I., Ball, A.S., Diaz, R.L., Magnus, J.F., Kibukawa, M., Schelter, J.M., Kobayashi, S.V., Lim, L., Burchard, J., Jackson, A.L., Linsley, P.S., and Cleary, M.A. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol. Cell. Biol. **28,** 2167 – 2174 (2009).
49. Hong, L., Lai, M., Chen, M., Xie, C., Liao, R., Kang, Y.J., Xiao, C., Hu, W.Y., Han, J., and Sun, P. The miR-17-92 cluster of microRNAs confers tumorigenicity by inhibiting oncogene-induced senescence. Cancer Res. **70,** 8547 – 8557 (2010).
50. O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. c-Myc-regulated microRNAs modulate E2F1 expression. Nature **435,** 839 – 843 (2005).
51. Sylvestre, Y., De Guire, V., Querido, E., Mukhopadhyay, U.K., Bourdeau, V., Major, F., Ferbeyre, G., and Chartrand, P. An E2F/miR-20a autoregulatory feedback loop. J. Biol. Chem. **282,** 2135 – 2143 (2007).
52. Woods, K., Thomson, J.M., and Hammond, S.M. Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. J. Biol. Chem. **282,** 2130 – 2134 (2007).
53. Olive, V., Bennett, M.J., Walker, J.C., Ma, C., Jiang, I., Cordon-Cardo, C., Li, Q.J., Lowe, S.W., Hannon, G.J., and He, L. miR-19 is a key oncogenic component of mir-17-92. Genes Dev. **23,** 2839 – 2849 (2009).
54. Mu, P., Han, Y.C., Betel, D., Yao, E., Squatrito, M., Ogrodowski, P., de Stanchina, E., D'Andrea, A., Sander, C., and Ventura, A. Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas. Genes Dev. **23,** 2806 – 2811 (2009).
55. Koralov, S.B., Muljo, S.A., Galler, G.R., Krek, A., Chakraborty, T., Kanellopoulou, C., Jensen, K., Cobb, B.S., Merkenschlager, M., Rajewsky, N., and Rajewsky, K. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. Cell **132,** 860 – 874 (2008).
56. Petrocca, F., Vecchione, A., and Croce, C.M. Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor beta signaling. Cancer Res. **68,** 8191 – 8194 (2008).
57. Ventura, A., Young, A.G., Winslow, M.M., Lintault, L., Meissner, A., Erkeland, S.J., Newman, J., Bronson, R.T., Crowley, D., Stone, J.R., Jaenisch, R., Sharp, P.A., and Jacks, T. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell **132,** 875 – 886 (2008).
58. Xiao, C., Srinivasan, L., Calado, D.P., Patterson, H.C., Zhang, B., Wang, J., Henderson, J.M., Kutok, J.L., and Rajewsky, K. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. Nat. Immunology **9,** 405 – 414 (2008).
59. Mestdagh, P., Boström, A.K., Impens, F., Fredlund, E., Van Peer, G., De Antonellis, P., von Stedingk, K., Ghesquière, B., Schulte, S., Dews, M., Thomas-Tikhonenko, A., Schulte, J.H., Zollo, M., Schramm, A., Gevaert, K., Axelson, H., Speleman, F., and Vandesompele, J. The miR-17-92 microRNA cluster regulates multiple components of the TGF-β pathway in neuroblastoma. Mol. Cell **40,** 762 – 773 (2010).
60. Shi, M., Liu, D., Shen, B., and Guo, N. Helpers of the cellular gatekeeper-miRNAs dance in P53 network. Biochim. Biophys. Acta. **1805,** 218 – 225 (2010).
61. Guttman, M., Amit, I., Garber, M., French, C., Lin, M.F., Feldser, D., Huarte, M., Zuk, O., Carey, B.W., Cassady, J.P., Cabili, M.N., Jaenisch, R., Mikkelsen, T.S., Jacks, T., Hacohen, N., Bernstein, B.E., Kellis, M., Regev, A., Rinn, J.L., and Lander, E.S. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature **458,** 223 – 227 (2009).
62. Huarte, M., Guttman, M., Feldser, D., Garber, M., Koziol, M.J., Kenzelmann-Broz, D., Khalil, A.M., Zuk, O., Amit, I., Rabani, M., Attardi, L.D., Regev, A., Lander, E.S., Jacks, T., and Rinn, J.L. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. Cell **142,** 409 – 419 (2010).
63. Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., and Chang, H.Y. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell **129,** 1311 – 1323 (2007).
64. Tsai, M.C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. Long noncoding RNA as modular scaffold of histone modification complexes. Science **329,** 689 – 693 (2010).
65. Khalil, A.M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., Thomas, K., Presser, A., Bernstein, B.E., van Oudenaarden, A., Regev, A., Lander, E.S., and Rinn, J.L. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc. Natl. Acad. Sci. USA **106,** 11667 – 11672 (2009).
66. Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S., Markenscoff-Papadimitriou, E., Kuhl, D., Bito, H., Worley, P.F., Kreiman, G., and Greenberg, M.E. Widespread transcription at neuronal activity-regulated enhancers. Nature **465,** 182 – 187 (2010).

BRD7 IS A CANDIDATE TUMOUR SUPPRESSOR GENE REQUIRED FOR P53 FUNCTION

Jarno Drost*, Fiamma Mantovani*, Francesca Tocco, Ran Elkon, Anna Comel, Henne Holstege, Ron Kerkhoven, Jos Jonkers, P. Mathijs Voorhoeve, Reuven Agami and Giannino Del Sal

*These authors contributed equally to this work

Nature Cell Biology 2010; 12: 380-389

2

ABSTRACT

Oncogene-induced senescence is a p53-dependent defence mechanism against uncontrolled proliferation. Consequently, many human tumours harbour *p53* mutations and others show a dysfunctional p53 pathway, frequently by unknown mechanisms. Here we identify BRD7 (bromodomain-containing 7) as a protein whose inhibition allows full neoplastic transformation in the presence of wild-type *p53*. In human breast tumours harbouring wild-type, but not mutant, *p53* the *BRD7* gene locus was frequently deleted and low BRD7 expression was found in a subgroup of tumours. Functionally, BRD7 is required for efficient p53-mediated transcription of a subset of target genes. BRD7 interacts with p53 and p300 and is recruited to target gene promoters, affecting histone acetylation, p53 acetylation and promoter activity. Thus, BRD7 suppresses tumorigenicity by serving as a p53 cofactor required for the efficient induction of p53-dependent oncogene-induced senescence.

Jarno Drost[1,8], Fiamma Mantovani[2,3,8], Francesca Tocco[2,3], Ran Elkon[1], Anna Comel[2,3], Henne Holstege[4], Ron Kerkhoven[5], Jos Jonkers[4], P. Mathijs Voorhoeve[1,7,9], Reuven Agami[1,6,9] and Giannino Del Sal[2,3,9]

[1]The Netherlands Cancer Institute, Division of Gene Regulation, Plesmanlaan 121, 1066CX, Amsterdam, The Netherlands. [2]Laboratorio Nazionale CIB, Area Science Park, Padriciano 99, 34012 Trieste, Italy. [3]Dipartimento di Scienze della Vita, Università di Trieste, 34100 Trieste, Italy. [4]The Netherlands Cancer Institute, Division of Molecular Biology, Plesmanlaan 121, 1066CX, Amsterdam, The Netherlands. [5]The Netherlands Cancer Institute, Central Microarray Facility, Plesmanlaan 121, 1066CX, Amsterdam, The Netherlands. [6]Center for Biomedical Genetics, The Netherlands. [7]Present address: Duke-NUS Graduate Medical School, Division of Cancer and Stem Cell Biology, 8 College Road, Singapore 169857. [8]These authors contributed equally to this work. [9]Correspondence should be addressed to P.M.V., R.A. or G.D.S. (e-mail: mathijs.voorhoeve@duke-nus.edu.sg; r.agami@nki.nl; delsal@lncib.it).

INTRODUCTION

The p53 tumour suppressor is an essential mediator of the cellular response triggered by oncogene activation, which causes a permanent cell-cycle arrest termed oncogene-induced senescence (OIS). OIS provides an important barrier to tumour development in vivo[1–3], and work in animal models suggests that this may represent the major mechanism of the tumour suppressive activity of p53 (ref. 4). Thus, interference with OIS by inhibiting the p53 tumour suppressor pathway allows cells to continue to proliferate in the presence of active oncogenes, leading to an increased frequency of tumour formation. This is reflected in the appearance of mutations in *p53* in as many as 50% of human tumours[5]. In most other cases, in which the *p53* gene is wild-type, genetic lesions in components of the p53 pathway are found interfering with its activation. A classical example is the increased turnover or sequestration of the p53 protein itself through, for example, overexpression of HDM2 (ref. 5). Other examples involve the loss of gene products that are required for full activation of the p53 protein, or inhibition of p53 function downstream of its activation[6,7]. However, for many tumours that retain wild-type *p53* the genetic alterations leading to functional impairment of the tumour suppressive response are not defined.

RESULTS

A loss of function screen for genes required for p53-dependent OIS identifies BRD7

To identify genes in the p53 pathway that are required for OIS, we performed a loss-of-function screen in primary human BJ fibroblasts expressing hTERT and 4-OH-tamoxifen (4-OHT)-inducible oncogenic H-Ras^{V12} (BJ/ET/Ras^{V12}ER cells[7]). We have shown previously that loss of p53 in primary human fibroblasts accelerates cell proliferation and contributes to cellular transformation[8]. Whereas the acceleration of cell proliferation was mediated through p14^{ARF}, cellular transformation was independent of it. Therefore, to focus on genes acting in the transformation process, we performed our screen in BJ/ET/Ras^{V12}ER cells in which p14^{ARF} was knocked down (Fig. 1a). Nineteen outliers were selected (Supplementary Information, Table S1). Validation with additional knockdowns targeting these genes identified several that conferred a growth advantage on BJ/ET cells expressing Ras^{V12}, all targeting the same gene, *BRD7* (Fig 1b; Supplementary Information, Fig. S1a, b). There was a correlation between BRD7 knockdown efficiency and functionality (Fig. 1b), and no changes in p53 and Ras^{V12} expression levels were observed (Fig. 1c). These results suggest that inhibition of BRD7 expression enables primary human BJ cells to continue proliferating in the presence of oncogenic Ras^{V12}, implying that BRD7 is involved in OIS.

Next, we characterized the effect of BRD7 loss on several well-established markers of OIS, including flat cell morphology, expression of senescence-associated β-galactosidase, and formation of senescence-associated heterochromatin foci. In comparison with control cells, a marked decrease in all senescence markers was observed in Ras^{V12}-expressing BRD7-knockdown (BRD7^{KD}) and p53-knockdown

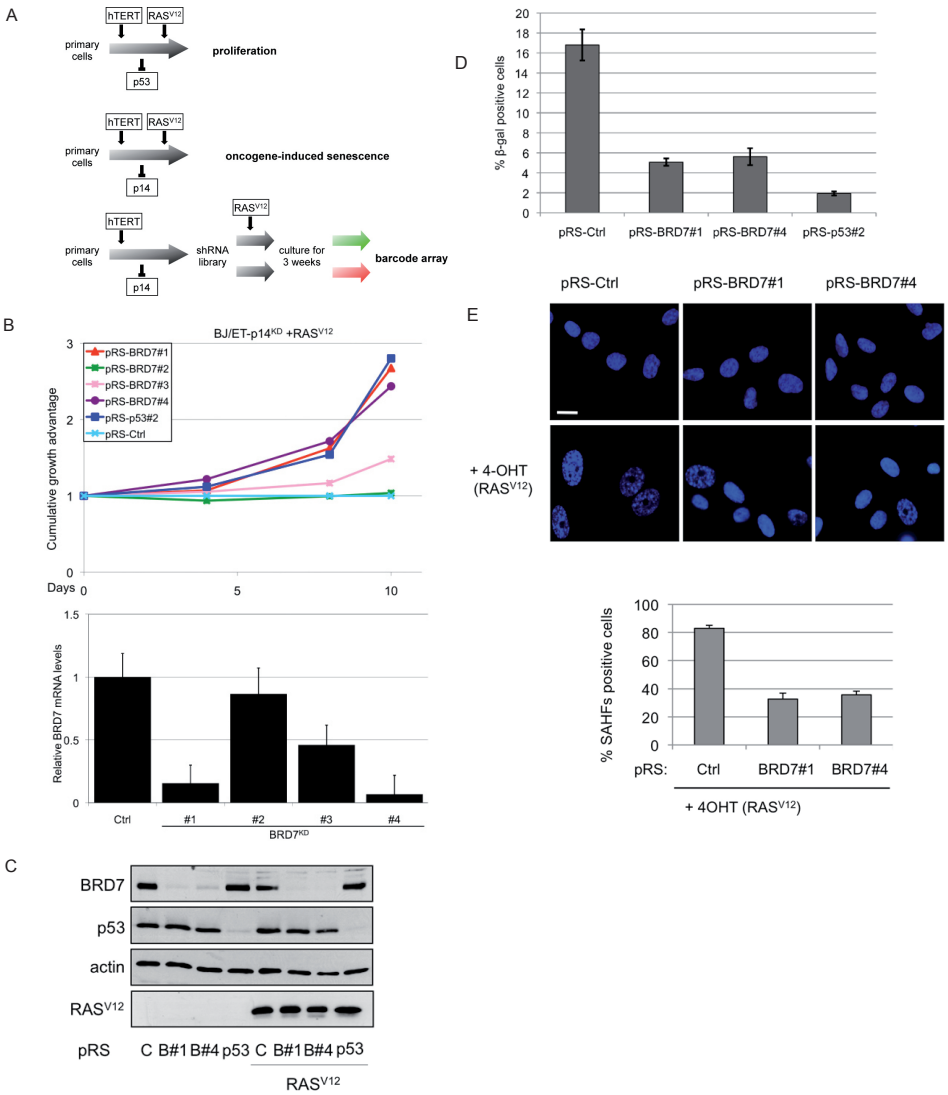


Figure 1. A genetic screen for genes required for OIS identifies BRD7. A) Schematic representation of OIS and the setup of the loss-of-function screen. B) GFP-growth competition assays of BJ/ET/p14$^{ARF\text{-}KD}$/Ras^{V12}ER cells transduced with the indicated pRetrosuper (pRS) knockdown vectors and cultured in the presence of 4-OHT (+Ras^{V12}). An accompanying quantitative-RT-PCR for *BRD7* mRNA expression shows the functionality of the BRD7 knockdown vectors. Graphs represent mean and s.d. for three independent experiments. C) BJ/ET/Ras^{V12}ER cells were transduced with the indicated knockdown vectors and cultured in the absence or presence of 4-OHT for 3 days and then harvested; western blot analysis was performed to detect BRD7, p53, Ras^{V12}, and actin (loading control). Uncropped images of blots are shown in Supplementary Information, Fig. S15. D) Cells from (C) were cultured in the presence of 4-OHT for 10 days and stained to detect cells that were positive for SA-β-galactosidase. Graphs show means and s.d. for three independent experiments. E) Cells from (C) were cultured in the presence of 4-OHT for 10 days and senescence-associated heterochromatin foci were revealed by Hoechst staining. Scale bars, 20 μM. Graphs show means and s.d. for three independent experiments.

(p53^{KD}) cells (Fig. 1d, e; Supplementary Information, Fig. S2a). No significant changes in *BRD7* messenger RNA and protein levels were observed on expression of Ras^{V12} (data not shown; Fig. 1c), and in addition the nuclear localization of BRD7 did not change (Supplementary Information, Fig. S3b). Further, BRD7 did not co-localize with senescence-associated heterochromatin foci (Supplementary Information, Fig. S3b). No change in appearance or persistence of γ-H2AX foci during recovery from DNA damage was observed in BJ/ET cells expressing BRD7^{KD} (Supplementary Information, Fig. S4), implying that BRD7 does not directly influence the DNA damage response. Re-expression of short hairpin RNA (shRNA)-resistant *BRD7* in Ras^{V12}-expressing BJ/ET-BRD7^{KD} cells significantly inhibited cell growth, showing that BRD7 is indeed the functional factor in these assays (Supplementary Information, Fig. S1c, d). Finally, the requirement of BRD7 for OIS is not specific to Ras^{V12}-induced senescence, because cells expressing BRD7^{KD} were also less efficient in inducing senescence on overexpression of c-Myc (ref. 9) (Supplementary Information, Fig. S2b–d). Taken together, these results indicate that BRD7 is required for p53-dependent OIS.

Genetic interaction of BRD7 with the p53 pathway

The endogenous expression of p53 limits the cellular proliferation of primary human cells under normal culture conditions, as observed by the growth advantage induced by knocking down p53 in primary human fibroblasts and in primary mammary epithelial cells[8,10]. To investigate whether a genetic interaction exists between BRD7 and the p53 pathway, we performed green fluorescent protein (GFP)-growth competition assays in primary human fibroblasts expressing p53^{KD}. Whereas BRD7^{KD} conferred a significant growth advantage comparable to that of p53^{KD} on cells with normal p53 levels, the effect was greatly diminished in cells with decreased p53 levels (Fig. 2a, c). As controls we used p53^{KD} and p14$^{ARF\text{-}KD}$, which only conferred a growth advantage on cells with functional p53 levels, and p16$^{INK4A\text{-}KD}$, which confers a growth advantage independently of p53. The same results were obtained in cells expressing Ras^{V12} (Fig. 2b, d). Intriguingly, the growth advantage conferred by p53^{KD} was greatly decreased in cells deficient in BRD7 (Fig. 2e, f). These observations show that proper function of p53 in controlling proliferation and OIS requires BRD7, and vice versa.

BRD7 interacts physically with p53

Confirming the genetic and functional interaction between BRD7 and p53, we observed that the two proteins interact physically. Initially, a yeast two-hybrid screen led to the identification of BRD7 as a new interaction partner of p53, specifically with the carboxy-terminal domain of p53 (Supplementary Information, Fig. S5). The interaction between p53 and BRD7 was validated by co-immunoprecipitation and co-localization assays in BJ/ET cells (Fig. 3a; Supplementary Information, Fig. S3c). The expression of Ras^{V12} increased the interaction between the two proteins (Fig. 3a).

In vitro binding assays with a maltose-binding protein (MBP)–BRD7 fusion protein and several deletion mutants of p53 mapped the region of binding of

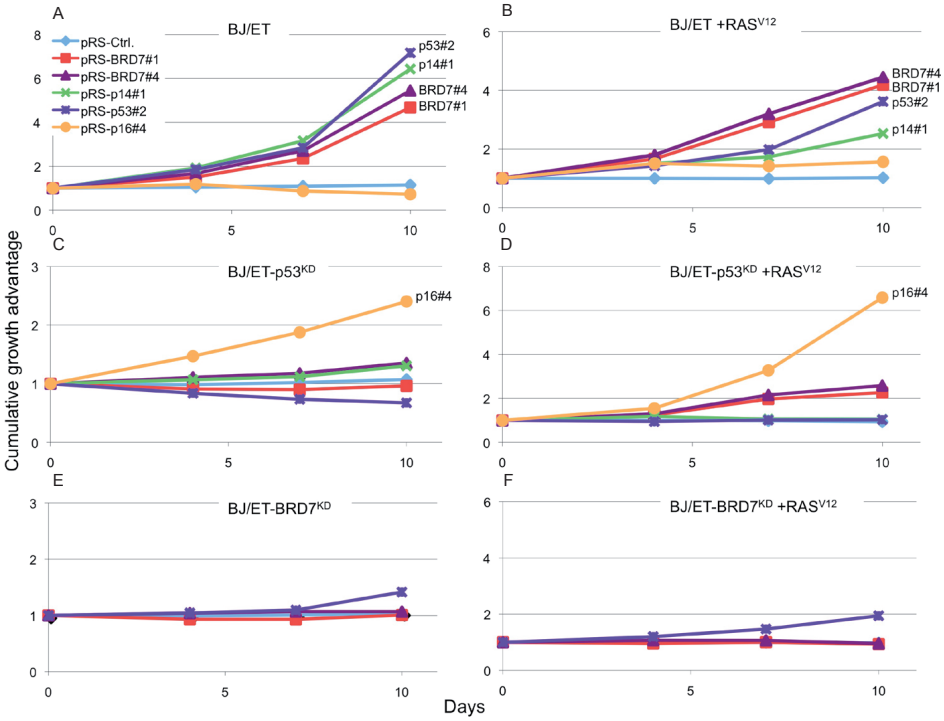


Figure 2. Genetic interaction of BRD7 with the p53 pathway. (A – F) GFP growth competition assays of BJ/ET/Ras^{V12}ER cells with the indicated genetic backgrounds and expressing the indicated pRS-GFP vectors cultured in the absence (A, C, E) or presence (B, D, F) of 4-OHT: (A) BJ/ET, (B) BJ/ET +Ras^{V12}, (C) BJ/ET-p53^{KD}, (D) BJ/ET-p53^{KD} +Ras^{V12}, (E) BJ/ET-BRD7^{KD}, (F) BJ/ET-BRD7^{KD} +Ras^{V12}.

p53 to BRD7 to its C-terminal domain (residues 363–393; Fig. 3b). Pull-down assays indicated that p53 interacts with the amino-terminal part of BRD7 (residues 1–128) upstream of its bromodomain (residues 128–238; Fig. 3c). Indeed, BRD7 and BRD7ΔBD (a mutant lacking the bromodomain) bound equally well to wild-type p53 and p53-9KR[11] (Fig. 3d), indicating that neither the bromodomain of BRD7 nor the C-terminal lysine residues of p53 are required for this interaction.

Re-expression of an shRNA-resistant BRD7 mutant lacking residues 1–128 in Ras^{V12}-expressing BJ/ET-BRD7^{KD} cells inhibited cell growth less efficiently than re-expression of wild-type BRD7, indicating that the p53–BRD7 interaction is indeed required for at least part of the growth-inhibitory effects of BRD7 (Supplementary Information, Fig. S1c, d).

BRD7 is required for transcriptional activation of a subset of p53 target genes

BRD7 is known to bind acetylated lysine residues in histones[12,13] and to be a component of a chromatin-remodelling complex[14], suggesting a possible role in the regulation of transcription. We therefore examined whether BRD7 is required for the transcriptional activation of p53 target genes. We assessed the expression

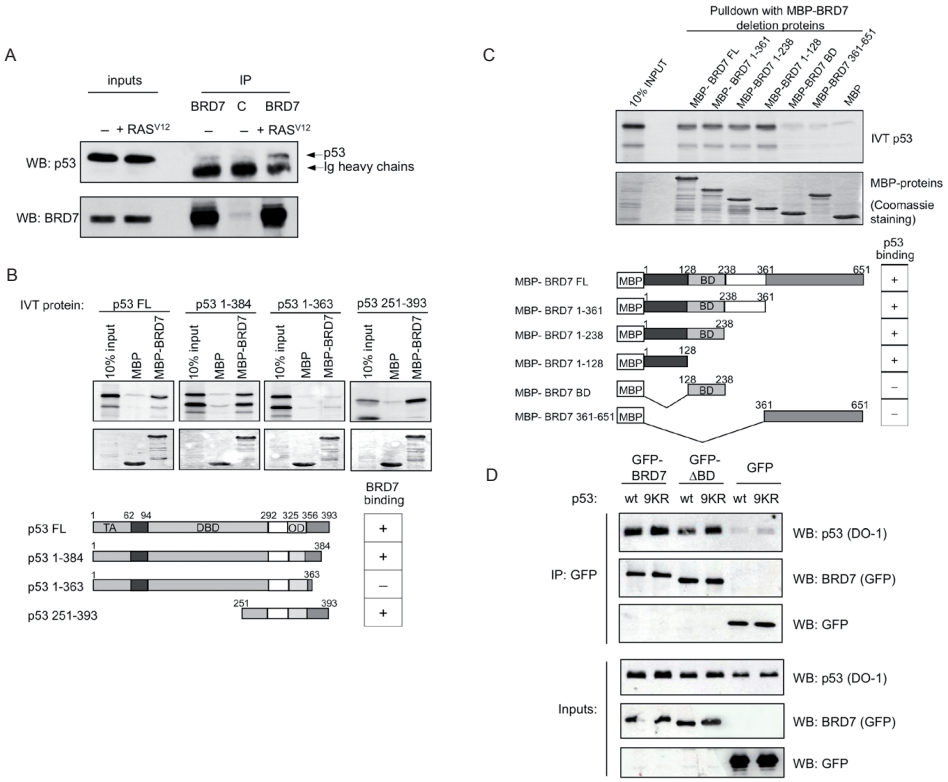


Figure 3. BRD7 interacts physically with p53. A) Co-immunoprecipitation of endogenous BRD7 and p53 in BJ/ET-Ras^{V12}ER cells cultured in the absence (–) or presence (+Ras^{V12}) of 4-OHT. Lysates were immunoprecipitated (IP) with anti-BRD7 antibody. Control immunoprecipitation (C) was performed with anti-haemagglutinin antibody. WB, western blot. B) *In vitro* binding of p53 deletion mutants with full-length (FL) BRD7. Top: p53 proteins were produced by *in vitro* translation (IVT) and incubated with bacterially expressed MBP-BRD7 protein or with MBP protein as control. Bottom: schematic representation of the p53 proteins used. TA, transactivation domain; DBD, DNA-binding domain; OD, oligomerization domain. C) *In vitro* binding of BRD7 deletion mutants with full-length p53. Full-length p53 protein was translated *in vitro* and incubated with bacterially expressed MBP-BRD7 deletion proteins (depicted in the scheme below), or with MBP protein as control. BD, bromodomain. D) H1299 cells (p53-null) were transiently transfected with either p53 wild-type (WT) or p53-9KR expression vector, together with GFP-tagged full-length BRD7, bromodomain-deleted BRD7 (ΔBD) or empty EGFP control. The interaction was analysed by immunoprecipitating GFP and subsequent western blot analysis to detect bound p53. Uncropped images of the western blots are shown in Supplementary Information, Fig. S15.

levels of several known p53 targets in normally growing and Ras^{V12}-expressing BJ/ET cells containing either a control, p53^{KD} or BRD7^{KD} vector. Figure 4a and Supplementary Information, Figure S6, show that Ras^{V12} induces transcription of all these genes in a p53-dependent manner. BRD7^{KD} greatly affected the expression of *P21* (*CDKN1A*), *HDM2* (*MDM2*), *TIGAR* (*C12orf5*) and *PAI1* (*SERPINE1*), whereas no significant effect was observed for *BAX*, *PUMA* (*BBC3*), *PIG3* (*TP53I3*), *FAS* (*CD95*) and *NOXA* (*PMAIP1*).

To get an overview of which p53 target genes require BRD7 for their transcription, we performed mRNA-expression array analysis comparing Ras^{V12}-expressing BJ/ET cells containing either one of two different $BRD7^{KD}$ vectors, or $p53^{KD}$ vector or control vector (Fig. 4b). Clustering analysis of genes whose expression was decreased in knockdown cells revealed three groups: $p53^{KD}$-specific genes, $BRD7^{KD}$-specific genes, and genes shared by $p53^{KD}$ and $BRD7^{KD}$ (Supplementary Information, Fig. S7a–c). Gene ontology (GO) analysis revealed a significant enrichment for pro-apoptotic genes in the cluster of genes

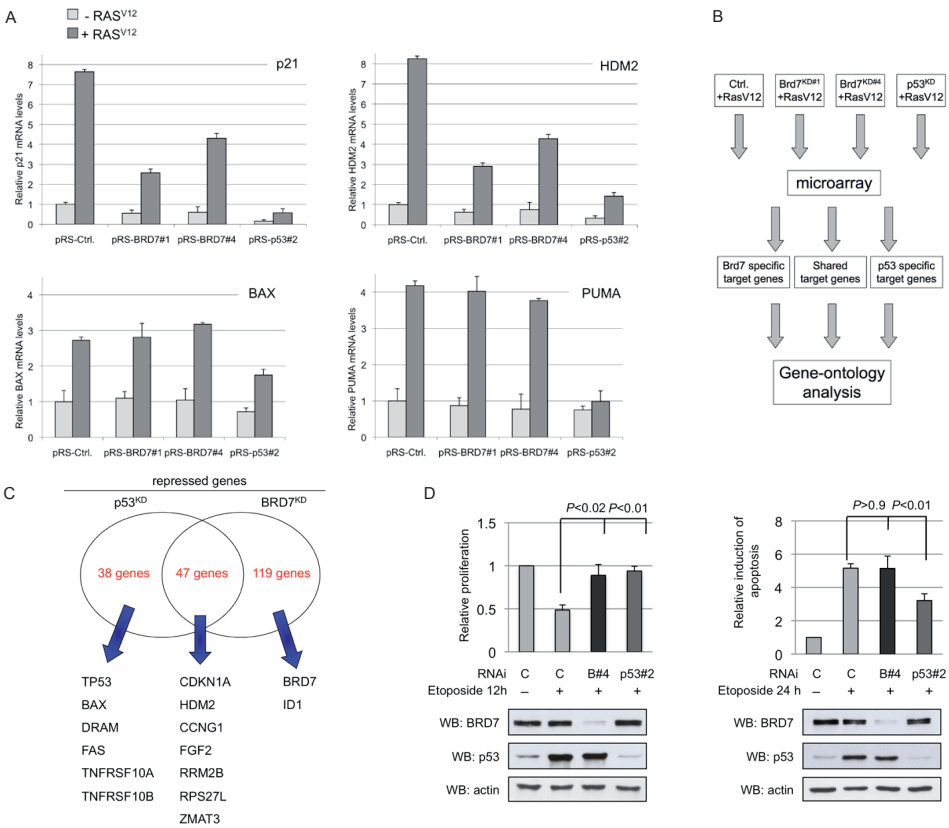


Figure 4. BRD7 is required for efficient transcriptional activation of a specific subset of p53 target genes. A) BJ/ET/Ras^{V12}ER cells containing control vector, one of two different $BRD7^{KD}$ vectors, or $p53^{KD}$ vector were cultured for 10 days in the absence (grey bars) or presence (black bars) of 4-OHT. mRNA levels of the indicated genes were determined by quantitative RT-PCR. Graphs show means and s.d. for three independent experiments. B) Schematic overview of the mRNA-expression array analysis performed on RNA extracted from cells from (A) cultured in the presence of 4-OHT. C) Representation of genes downregulated specifically in $p53^{KD}$ or $BRD7^{KD}$ cells, or downregulated genes shared by the two genetic backgrounds. Numbers represent the amount of genes present in that GO cluster. D) U2-OS cells (wild-type p53), transfected with the indicated siRNAs, were treated with etoposide (+) or DMSO (–) for 12 h (left) or 24 h (right). The effects of $BRD7^{KD}$ on S-phase entry were evaluated by incorporation of bromodeoxyuridine (left); the effects on apoptosis were evaluated by analysis of DNA content by staining with propidium iodide. Graphs show means and s.d. for three independent experiments. *P* values were calculated with two-tailed unpaired *t*-tests. Western blot analysis was performed to detect BRD7, p53 and actin (loading control).

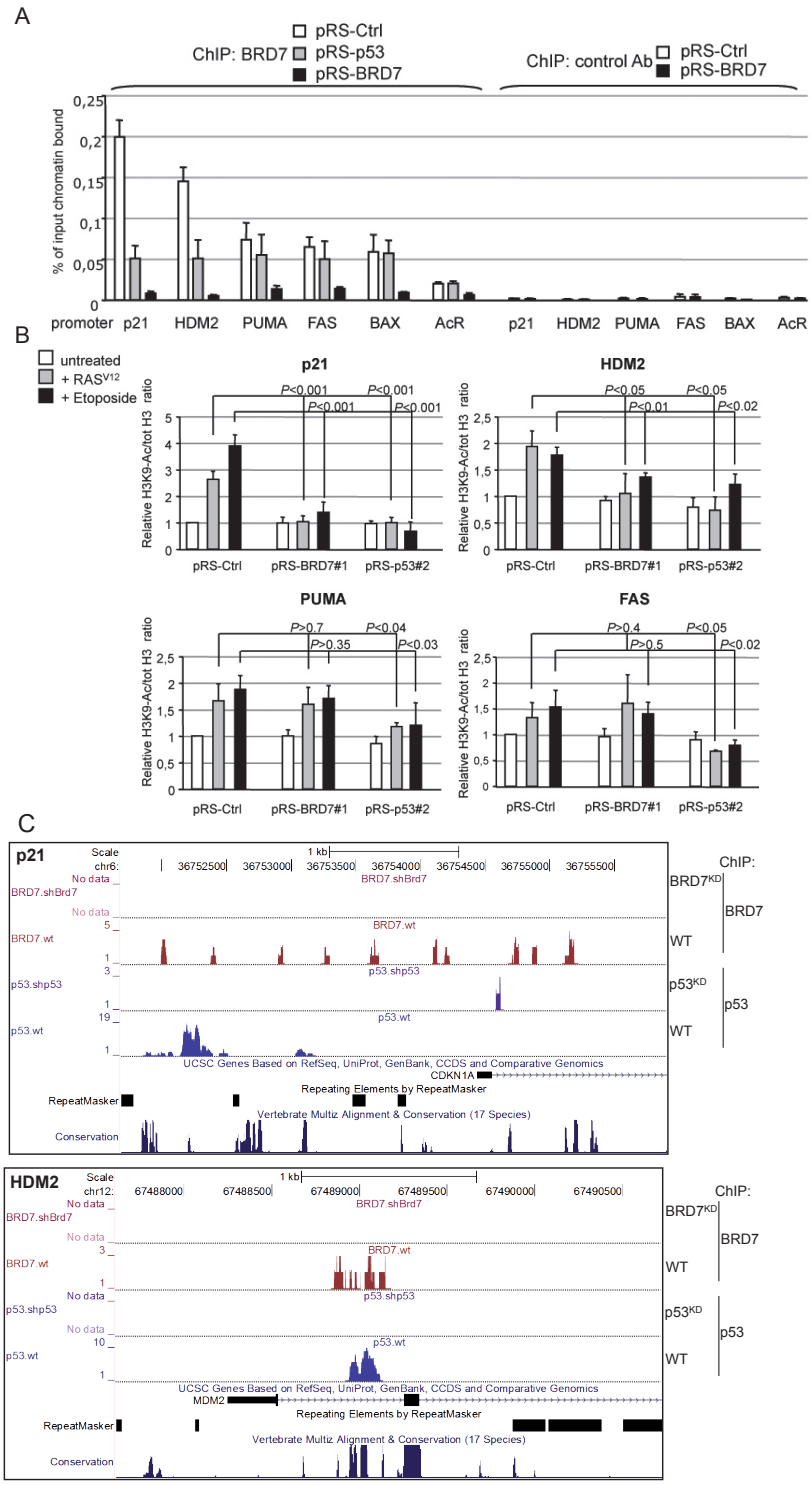
downregulated in $p53^{KD}$ cells but not in $BRD7^{KD}$ cells ($P < 0.002$; hypergeometric distribution, after correction for multiple testing). This included, as expected, *p53* and *BAX*, but also *FAS*, *TNFRSF10A*, *TNFRSF10B* and *DRAM1* (*FLJ11259*) (Fig. 4c). $BRD7^{KD}$ significantly affected the expression of several p53 target genes, among them *P21*, *HDM2*, *cyclin G1* (*CCNG1*), *RRM2B*, *ZMAT3* (*WIG1*) and *FGF2* (Fig. 4c). There was also a subset of genes specifically repressed in $BRD7^{KD}$ cells but not in $p53^{KD}$ cells (Supplementary Information, Fig. S7c), suggesting that p53 is not the only transcriptional partner of BRD7. As no clear GO appeared in this group, the acting factors remain unknown. Thus, BRD7 is required for the transcriptional activation of a subset of p53 target genes.

Furthermore, in U2-OS cells (p53 wild-type), loss of BRD7 expression resulted in lower basal *P21* mRNA levels and impaired induction of *p21* after treatment with etoposide, whereas *PUMA* mRNA remained unchanged (Supplementary Information, Fig. S8). Cell proliferation assays did indeed show that $BRD7^{KD}$ cells bypass a DNA damage-induced growth arrest, whereas apoptosis is still normally induced by etoposide or ultraviolet irradiation (Fig. 4d; Supplementary Information, Fig. S8). This effect of BRD7 is highly relevant to OIS, because the main feature of OIS is the induction of an irreversible growth arrest.

BRD7 binds to a subset of p53 target gene promoters and affects histone acetylation

Our results suggest that BRD7 acts as a transcriptional cofactor of p53 for a subset of target genes. We therefore determined whether BRD7 is found within transcriptional complexes on the p53 cognate sites in these promoters. Chromatin immunoprecipitation (ChIP) for BRD7 from BJ/ET cells expressing Ras^{V12} showed specific binding of BRD7 at the upstream (−2.3 kilobases (kb)) p53-binding site in the *P21* promoter, and within the p53-binding region of the *HDM2* promoter (Fig. 5a), which both require BRD7 for efficient transcriptional activation (Fig. 4a). In contrast, much less binding of BRD7 was detected on the promoters of the p53 target genes that do not require BRD7 for their transcription, including *PUMA*, *FAS* and *BAX*, although it was higher than that to the promoter of a non-transcribed gene (Fig. 5a). BRD7 binding to the *P21* and *HDM2* promoters (but not *PUMA*, *FAS* or *BAX*) was compromised in $p53^{KD}$ cells (Fig. 5a). These results suggest that the activation of several target gene promoters by p53 involves the recruitment of BRD7.

Seeking wide-scale delineation of the cooperation between p53 and BRD7 in the transcriptional regulation of target genes, we performed a ChIP-sequencing analysis on these proteins (Fig. 5c). BRD7 showed much broader chromatin binding than p53, and its peaks were sharply concentrated near the transcriptional start sites (TSSs) of target genes (Supplementary Information, Fig. S9). Binding peaks of BRD7 were identified near TSSs of 4,422 genes, whereas p53 binding peaks were identified upstream of or within 52 genes. The overlap between genes bound by BRD7 and p53 was statistically significant (26 genes; $P = 1.6 \times 10-4$; Supplementary Information, Table S2a). Notably, integration of ChIP-sequencing and mRNA-expression microarray results showed that the binding peaks for both p53 and BRD7 were enriched in the group of genes downregulated in both $p53^{KD}$ and $BRD7^{KD}$ cells but not in the group of genes downregulated only in $p53^{KD}$ cells,


A
ChIP: BRD7
pRS-Ctrl
pRS-p53
pRS-BRD7
ChIP: control Ab
pRS-Ctrl
pRS-BRD7
% of input chromatin bound
promoter p21 HDM2 PUMA FAS BAX AcR p21 HDM2 PUMA FAS BAX AcR
B
untreated
+ RASV12
+ Etoposide
p21
HDM2
PUMA
FAS
Relative H3K9-Ac/tot H3 ratio
pRS-Ctrl
pRS-BRD7#1
pRS-p53#2
C
p21
HDM2
Scale
chr6:
chr12:
No data
BRD7.shBrd7
BRD7.wt
p53.shp53
p53.wt
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
CDKN1A
MDM2
Repeating Elements by RepeatMasker
RepeatMasker
Vertebrate Multiz Alignment & Conservation (17 Species)
Conservation
ChIP:
BRD7KD
BRD7
WT
p53KD
p53

which contains the p53 pro-apoptotic targets (Supplementary Information, Table S2b). This observation is in agreement with previous studies reporting that p53 binds pro-apoptotic targets with lower affinity[5] (Supplementary Information, Table S2c) and indicates that BRD7 cooperates with p53 mainly on its high-affinity, non-apoptotic targets.

To investigate the effect of BRD7 on histone modifications, we determined the acetylation status of histone 3 at lysine 9 (H3K9), a marker of active chromatin[15]. ChIP analysis revealed that BRD7^{KD} results in a great decrease in H3K9 acetylation around p53-binding sites in the *P21* and *HDM2* promoters induced by either Ras^{V12} or etoposide treatment (Fig. 5b). No significant effect was observed on basal H3K9 acetylation. In addition, H3K9 acetylation on the *PUMA* and *FAS* promoters was induced by oncogene expression and etoposide-induced DNA damage. However, BRD7^{KD} did not affect this induction. This is in agreement with our observation that BRD7 is not required for transcription of these genes (Fig. 4a; Supplementary Information, Fig. S6).

Next we examined the promoter activity of reporter constructs containing the *P21*, *HDM2* or *FAS* promoter in MCF7 cells (expressing BRD7 and wild-type p53). As expected, p53^{KD} greatly decreased the promoter activity of all reporter constructs (Supplementary Information, Fig. S10). However, BRD7^{KD} only significantly affected the activity of the *P21* and *HDM2* reporters ($P < 0.01$) but not that of *FAS* ($P > 0.15$). Taken together, these results show a p53-dependent recruitment of BRD7 to several p53 target gene promoters, resulting in an induction of histone acetylation and promoter activity.

BRD7 interacts with p300 and controls acetylation of the p53 C-terminus at target gene promoters

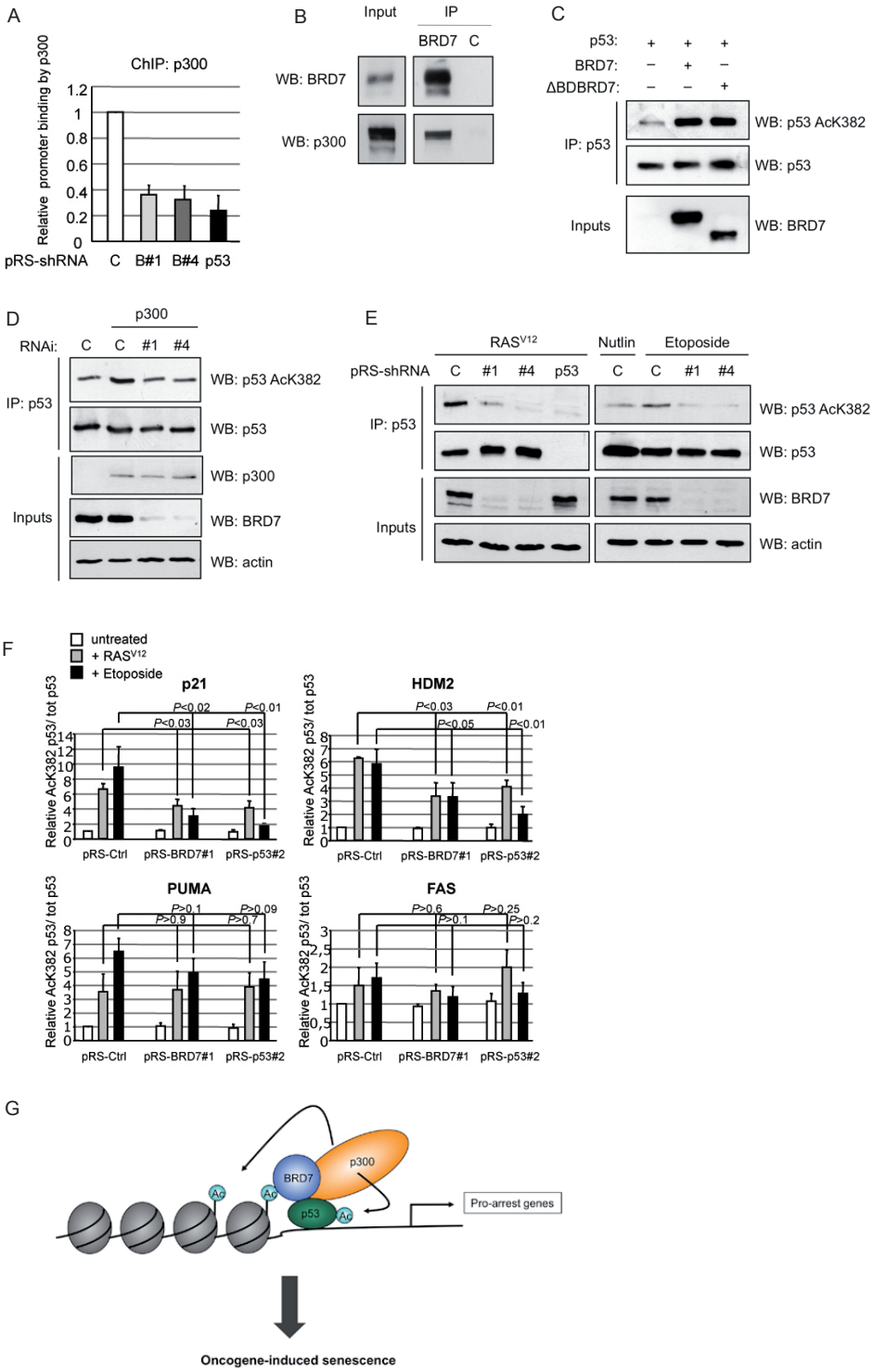
Activated p53 induces the *P21* promoter by recruiting p300, as well as other histone-acetyl transferases, to modify histone tails[16–18]. Because BRD7 affects histone acetylation, we investigated its requirement for the recruitment of p300 to the *P21* promoter. BRD7^{KD} resulted in a marked decrease in p300 on the *P21* promoter in Ras^{V12}-expressing BJ/ET cells (Fig. 6a). Moreover, endogenous BRD7

◀ **Figure 5.** BRD7 binds to selected p53 target gene promoters in a p53-dependent fashion and affects histone acetylation and promoter activity. A) BJ/ET/Ras^{V12}ER cells containing control, BRD7^{KD} (no. 4), or p53^{KD} vector were cultured for 48 h in the presence of 4-OHT h and subjected to ChIP with either BRD7-specific or unrelated (anti-haemagglutinin) antibody as control. Binding of BRD7 to the indicated genomic regions was quantified by calculating the percentage of input chromatin bound by real-time PCR. Genomic regions amplified were surrounding the upstream p53-binding site (5′ BS) within the *P21* promoter, the p53-binding site within *HDM2*, *PUMA*, *FAS* and *BAX* promoters, and the promoter region of muscarinic acetylcholine receptor (*AcR*). Graphs show means and s.d. for three independent experiments. B) BJ/ET/Ras^{V12}ER cells expressing the indicated knockdowns were cultured in the presence or absence of 4-OHT (+Ras^{V12}) or treated with etoposide, and subjected to ChIP with antibodies against acetylated H3K9 or total H3 histone. The abundance of Ac-H3K9 histone mark within the indicated genomic region (*P21* 5′ BS; p53 BS in *HDM2*, *PUMA* and *FAS* promoters) was calculated as the ratio between Ac-H3K9 and total H3 and is represented as fold difference from values measured in control-transduced, normally growing BJ/ET cells. Graphs show means and s.d. for three independent experiments. *P* values were calculated with two-tailed unpaired *t*-tests. C) BJ/ET/Ras^{V12}ER cells expressing the indicated knockdowns were cultured in the presence of 4-OHT and subjected to ChIP-sequencing for BRD7 and p53. Shown are the binding profiles on the *P21* and *HDM2* promoters.

and p300 proteins are found in complex in these cells (Fig. 6b). This interaction required the C-terminus of BRD7 and seemed to be p53-independent, because it can also be observed in cells lacking p53 (Supplementary Information, Fig. S11). DNA-bound p53 is acetylated at several lysine residues by p300/CREB-binding protein (CBP) and this contributes to induction of the transcription of *P21* (refs 19, 20). Because Ras^{V12} expression induces acetylation of p53 on K382 by means of p300/CBP[20], we analysed the effect of BRD7 on this modification. H1299 cells were co-transfected with p53 and BRD7, and the K382 acetylation status of p53 was analysed. Figure 6c shows that BRD7 expression increases the acetylation of p53, independently of its bromodomain. Furthermore, expression of p300 induced acetylation of p53 on K382, and this effect was dependent on BRD7 (Fig. 6d). Consistently, $BRD7^{KD}$ in BJ/ET cells decreased Ras^{V12}-induced or DNA-damage-induced p53 acetylation on K382 (Fig. 6e). These results imply that BRD7 is important for p300-dependent p53 acetylation.

To determine whether BRD7 affects the amount of acetylated (active) p53 bound to target gene promoters we performed ChIPs for total and K382-acetylated p53 from BJ/ET cells either expressing Ras^{V12} or treated with etoposide. Both types of cellular stress revealed an increase in chromatin-bound acetylated p53 on all target promoters examined (Fig. 6f). This increase was greatly diminished in cells expressing $BRD7^{KD}$ on the promoters that require BRD7 for activation (*P21* and *HDM2*) but not on promoters not requiring BRD7 (*PUMA* and *FAS*), indicating that BRD7 regulates p53 protein activity at specific target promoters. Finally, acetylation of p53 was shown to promote its binding to target promoters[21]. We therefore examined whether BRD7 affected the binding of p53 to the *P21* promoter in BJ/ET cells either expressing Ras^{V12} or treated with etoposide. Indeed, $BRD7^{KD}$

Figure 6. BRD7 controls loading of p300 on the *P21* promoter and affects p53 C-terminal acetylation. ▶ A) BJ/ET/Ras^{V12}ER cells expressing the indicated knockdowns were cultured in the presence of 4-OHT and subjected to ChIP for p300. Binding of p300 to the *P21* promoter (5′ BS) is represented as fold difference from values measured in control cells. Graphs show means and s.d. for three independent experiments. B) Co-immunoprecipitation of endogenous BRD7 and p300 in BJ/ET-Ras^{V12}ER cells, cultured in the presence of 4-OHT. Lysates were immunoprecipitated with anti-BRD7 antibody. Control immunoprecipitation (C) was performed with anti-haemagglutinin antibody. Uncropped images of the western blots are shown in Supplementary Information, Fig. S15. C) H1299 cells were transfected with p53 and either Flag-pCDNA3, Flag-BRD7, or Flag-BRD7-ΔBD. p53 was immunoprecipitated and its K382 acetylation status was evaluated by western blot analysis with anti-p53-AcK382 antibody. D) H1299 cells were transfected with p53 and haemagglutinin-p300 expression constructs as indicated, together with siRNAs targeting BRD7 (#1 and #4) or control (C) siRNA. Acetylation of p53 on K382 acetylation was analysed as in (C). Western blot analysis was performed to evaluate expression levels of BRD7, p53, p300 and actin (loading control). E) BJ/ET/Ras^{V12}ER cells expressing the indicated knockdowns were cultured in the presence of 4-OHT (+Ras^{V12}, left) or treated with etoposide (right). As a control, cells were treated with Nutlin-3a to stabilize p53. Acetylation of p53 on K382 acetylation was analysed as in (C). Western blotting was performed to analyse BRD7 expression, using actin as loading control. Uncropped images of the western blots are shown in Supplementary Information, Fig. S15. F) BJ/ET/Ras^{V12}ER cells expressing the indicated knockdowns were cultured in the absence or presence of 4-OHT (+Ras^{V12}) or treated with etoposide, and subjected to ChIP with anti-p53-AcK382 or total p53 antibodies. Enrichment for acetylated p53 within the indicated genomic region (*P21* 5′ BS; p53 BS on *HDM2*, *PUMA* and *FAS* promoters) was calculated as the ChIP ratio of AcK382-p53 to total p53 and is represented as fold difference from values measured in control-transduced, normally growing BJ/ET cells. *P* values were calculated using two-tailed unpaired *t*-tests. Graphs show means and s.d. for three independent experiments. G) Schematic model showing how BRD7 may serve as a p53 cofactor to regulate the transcriptional activity of p53 target genes.


A
ChIP: p300
Relative promoter binding by p300
1.2
1
0.8
0.6
0.4
0.2
0
pRS-shRNA C B#1 B#4 p53
B
Input
IP
BRD7 C
WB: BRD7
WB: p300
C
p53: + + +
BRD7: − + −
ΔBDBRD7: − − +
IP: p53
WB: p53 AcK382
WB: p53
Inputs
WB: BRD7
D
p300
RNAi: C C #1 #4
IP: p53
WB: p53 AcK382
WB: p53
Inputs
WB: p300
WB: BRD7
WB: actin
E
RASV12
Nutlin
Etoposide
pRS-shRNA C #1 #4 p53 C C #1 #4
IP: p53
WB: p53 AcK382
WB: p53
Inputs
WB: BRD7
WB: actin
F
untreated
+ RASV12
+ Etoposide
p21
HDM2
PUMA
FAS
Relative AcK382 p53/ tot p53
P<0.02
P<0.01
P<0.03
P<0.03
P<0.03
P<0.01
P<0.05
P<0.01
P>0.1
P>0.09
P>0.9
P>0.7
P>0.6
P>0.25
P>0.1
P>0.2
pRS-Ctrl
pRS-BRD7#1
pRS-p53#2
G
p300
BRD7
p53
Ac
Pro-arrest genes
Oncogene-induced senescence

caused a significant decrease in the amount of p53 bound to the *P21* promoter in both conditions (Supplementary Information, Fig. S12). In conclusion, BRD7 is instrumental in promoting p53 activation at target promoters by affecting the acetylation of K382 through p300, loading of p53 and acetylation of histones at these promoters. Therefore BRD7 is required for the efficient induction of p53-dependent OIS (Fig. 6g).

Inhibition of BRD7 cooperates in oncogene-induced transformation

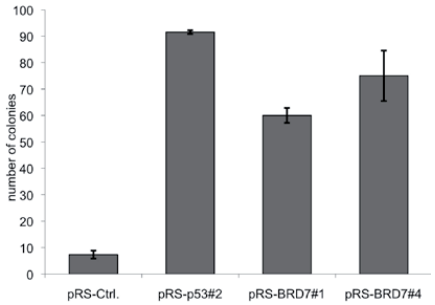
Loss of p53 is an essential step in the full neoplastic transformation of cells, characterized by their acquired ability to grow anchorage-independently in semi-solid medium (soft agar assay) and as tumours in immuno-compromised mice[22]. We therefore tested whether BRD7^{KD} inactivates the p53 pathway sufficiently to induce cellular transformation. As observed previously, BJ/ET cells expressing p14$^{ARF\text{-}KD}$, p16$^{INK4A\text{-}KD}$, small t and Ras^{V12} require effective loss of the p53 pathway to gain a transformed phenotype[8] (Fig. 7a, b). In addition, BRD7^{KD}, by either of two independent shRNAs, resulted in efficient colony formation and tumour growth in immuno-compromised mice (Fig. 7a, b; Supplementary Information, Fig. S13). Similar results were obtained in MCF10A human mammary epithelial cells (Supplementary Information, Fig. S14). Thus, efficient activation of p53 by oncogenes, as a mechanism against transformation, requires BRD7.

Loss of BRD7 in a subset of human breast tumours harbouring wild-type p53

BRD7 is encoded in a locus on chromosome 16q12 that is a well-known hotspot for loss of heterozygosity in breast cancers[23]. However, the tumour suppressor present at that locus has not been unequivocally identified. On the basis of our genetic and biochemical analyses, we predicted that decreased BRD7 expression would be beneficial for pre-tumorigenic cells still containing wild-type *p53*. Decreased BRD7 expression should therefore be observed in a subset of *p53* wild-type, but not in *p53* mutant, tumours.

Figure 7. Inhibition of BRD7 cooperates to transform primary human fibroblasts, and BRD7 expression is lost in a subset of breast tumours containing wild-type p53. A) BJ/ET cells expressing SV40 small t, p14$^{ARF\text{-}KD}$, p16$^{INK4A\text{-}KD}$, Ras^{V12}, and the indicated constructs were plated in soft agar. Colonies were counted after 3 weeks. Graphs show means and s.d. for three independent experiments. B) Cells from (A) were injected subcutaneously in immuno-compromised mice. The numbers of mice per group that developed tumours are shown. C) Normalized *BRD7* mRNA signal (lowest = 0, highest = 1) from 251 breast cancer samples[23] was plotted. *p53* status was assigned as wild-type (WT, left) when there were no mutations found or if the sample showed a *p53* wild-type signature as described[23]. BRD7 level cutoff was determined at the average minus one standard deviation (horizontal line). The χ^2 *P* value is indicated. D) Kernel smoothed estimate (KSE) curves of 31 p53 mutated (blue) and 35 p53 wild-type (grey) sporadic breast tumours. The curves for gains and losses were determined separately by KC-SMART. Significant differences between the two groups were determined by comparative KC-SMART. Genomic regions with a significantly greater gain or loss in the p53 wild-type tumours (compared with the p53 mutant tumours) are depicted with a green bar on top of or below the figure, respectively, and with a green line in the grey KSE curves. The significant changes in p53 mutant tumours (compared with p53 wild-type) are shown in red. The enlargement depicts a detailed view of the two areas of differential loss on chromosome 16q and the genomic location of BRD7 within the first differential loss.

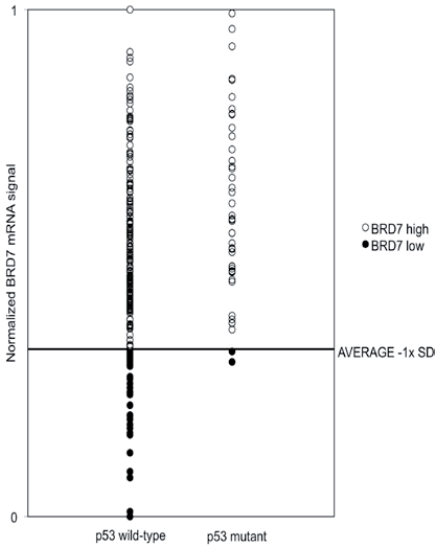
A



B

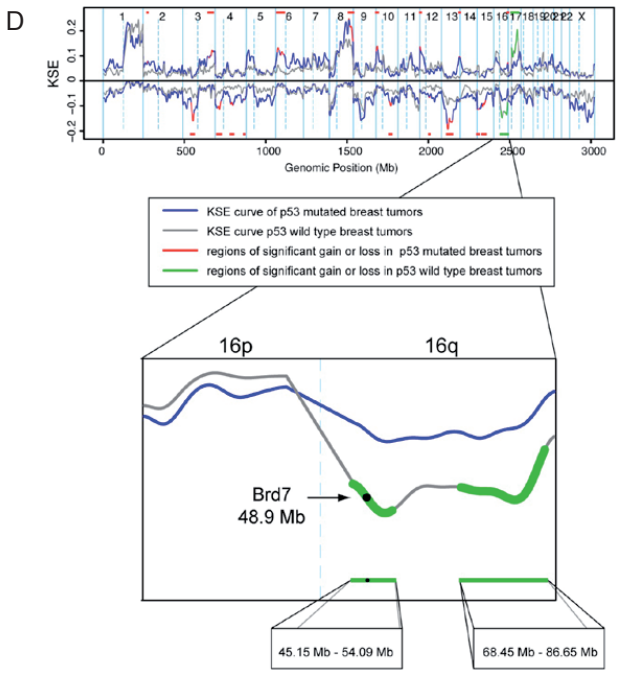
	Tumours in mice
Control	0/12
BRD7^{KD}	5/12
p53^{KD}	6/6

C



P < 0.007	*p53* WT	*p53* mutant	Total
BRD7 low	40	2	42
BRD7 high	165	44	209
Total	205	46	251

D



We monitored BRD7 in a breast cancer expression data set containing 251 tumours that were differentiated for *p53* status by profiling analysis and sequencing[24]. We found a subset (40/205) of *p53* wild-type tumours expressing BRD7 at levels lower than one standard deviation below the mean, whereas this subset was largely absent from *p53* mutant tumours (2/46) ($P < 0.007$; Fig. 7c). A similar subgroup was found in two independent breast cancer data sets whose *p53* status is known from immunohistochemistry[25] (BRD7 was low in 9/52 *p53* wild-type tumours, compared with 0/20 *p53* mutant tumours; $P = 0.04$ (data not shown)) or by applying the Miller profiling analysis[26] (BRD7 was low in 40/211 *p53* wild-type tumours, compared with 6/84 *p53* mutant tumours; $P = 0.0058$ (data not shown)).

To investigate possible changes in BRD7 copy number in *p53* wild-type breast tumours, we analysed comparative genomic hybridization data for a group of 68 sporadic breast tumours[27]. For 66 of these tumours the *p53* status was determined by sequence analysis. This data set was used to compare the profiles of 35 *p53* wild-type and 31 *p53* mutant breast tumours (Fig. 7d). Interestingly, in addition to a gain on chromosome arm 17q, the clearest significant difference was a loss on chromosome arm 16q. This loss comprised two regions: 45.15–54.09 Mb and 68.45–86.65 Mb. The genomic position of BRD7 is at 48.9 Mb, at the centre of the first differential loss. This result, together with our mRNA expression analysis, implies that BRD7 expression drives selection for chromosome 16q loss during tumorigenesis in *p53* wild-type tumours, whereas tumours harbouring *p53* mutations do not have this selection pressure. However, our data do not exclude the possibility that other genes in this region are involved.

DISCUSSION

We have identified BRD7 in a screen for tumour suppressor genes required for p53-dependent OIS and show that BRD7 interacts with p53. This interaction is stimulated by the Ras^{V12} oncogene and is required for the p53-dependent induction of OIS through regulation of a subset of p53 target genes. We have shown that BRD7 interacts with p300 and is critical for its recruitment and for the induction of histone and p53 acetylation at p53 target promoters. It is conceivable that BRD7, which binds acetylated histones through its bromodomain, helps to sustain a proper acetylation status of histones surrounding p53-binding sites during transcription. BRD7 was recently identified as a component of a BRG1-specific SWI/SNF (switch/sucrose non-fermentable) chromatin-remodelling complex[14]. BRG1 knockdown has been shown to impair p21 expression[28,29], partly as a result of decreased binding of p53 to the *P21* promoter[30]. Moreover, p53 has been shown to promote nucleosomal repositioning[31]. It seems conceivable that BRD7 may serve for the recruitment of chromatin-remodelling complexes to p53 target promoters to regulate transcription during OIS.

In addition to p53, BRD7 could also serve as a transcriptional cofactor for other proteins. Recently it was shown, by overexpression studies, that BRD7 transcriptionally regulates components of the Ras/MEK/ERK and Rb/E2F pathways[32,33]. Therefore, besides its important role in the p53 pathway, BRD7 may

also regulate the transcription of other genes involved in cell-cycle regulation. Furthermore, many differentially expressed genes in BRD7^{KD} cells are not p53 target genes. In accordance with this, ChIP-sequencing identified BRD7 binding peaks near TSSs of 4,422 genes, whereas only 26 of these were p53 target genes. This implies that BRD7 is required for the transcriptional regulation of up to hundreds of genes. However, the presence of BRD7 on promoters does not necessarily mean that it regulates gene transcription. Although BRD7 is present, at low levels, on low-affinity p53 target genes, it is not required for their transcriptional activation. However, so far we have no clear indication of what determines the requirement of BRD7 for the transcriptional regulation of the genes it binds to.

Functional impairment of the p53 pathway is instrumental for neoplastic transformation. Our evidence that BRD7 is essential for p53 activity in OIS and that its knockdown cooperates in oncogene-induced transformation would predict that a subset of tumours retaining wild-type *p53* could benefit from its functional loss. Indeed, low levels of BRD7 were found in nasopharyngeal carcinoma through methylation of the *BRD7* gene[34,35], and most nasopharyngeal carcinoma tumours retain wild-type *p53* (ref. 36). Moreover, BRD7 overexpression in a BRD7-negative nasopharyngeal carcinoma cell line induces growth arrest[33]. In accordance with this, we report low levels of BRD7 specifically in breast tumours harbouring wild-type, but not mutant, *p53*. As mentioned above, loss of BRD7 expression can occur through methylation of the *BRD7* gene[34]. Here we show that loss of BRD7 expression in breast tumours can also occur through deletion of its genetic locus. Theoretically, tumours that have inactivated the p53-dependent OIS pathway by loss of BRD7 should still be sensitive to apoptosis. It is therefore likely that these tumours complement BRD7 loss with genetic events affecting the apoptotic response. On chromosome 16q12, next to *BRD7*, is the cylindromatosis (*CYLD*) gene, which encodes a well-known pro-apoptotic factor with tumour suppressive activity. Therefore, deleting 16q12 would endow tumours with the means to interfere with both OIS and induction of apoptosis by the loss of *BRD7* and *CYLD*, respectively. The frequent loss of 16q12 observed in breast cancer and its correlation with the retention of wild-type p53 (ref. 23) (Fig. 7d) support such notion.

METHODS

Cell culture, transfection and retroviral transduction

All cells, except H1299 and MCF10A, were cultured in DMEM medium with 10% FCS and antibiotics. H1299 cells were cultured in RPMI medium with 10% FCS and antibiotics. MCF10A cells were maintained in DMEM:F12 Ham's medium (Sigma) 1:1, supplemented with 5% horse serum (Gibco), insulin (10 μg ml^{-1}; Sigma), hydrocortisone (0.5 μg ml^{-1}) and epidermal growth factor (20 ng ml^{-1}; Peprotech). Transfection of DNA was performed with polyethylenimine for MCF7 cells and Lipofectamine 2000 (Invitrogen) for H1299 and U2-OS cells. Cells were transfected with 50 nM siRNA oligonucleotides with Lipofectamine RNAiMax (Invitrogen). Retroviruses were made by calcium phosphate transfection of

Ecopack 2 cells (Clontech) and harvesting 40 and 64 h later. BJ cells were selected with the proper selection medium 48 h after transduction for at least 1 week. For Ras^{V12}-encoding retroviruses, cells were kept under selection for the entire duration of the experiment.

RNA-interference screen

BJ/ET/$p14^{ARF\text{-}KD}$/Ras^{V12}ER cells were retrovirally transduced in triplicate with the NKI shRNA library[37]. After drug-selection for library-transduced cells, cells were grown in the presence of 100 nM 4-OHT to induce OIS. As control, cells were grown in the absence of 4-OHT (Fig. 1a). Genomic DNA was isolated with a DNeasy tissue kit (Qiagen). shRNA-encoding sequences were recovered by PCR and subjected to hybridization with a DNA microarray containing complementary shRNA probes[37]. To verify the functionality of the identified shRNA vectors, GFP-growth competition assays were performed[8]. Six shRNA vectors conferred a substantial growth advantage to Ras^{V12}-expressing cells undergoing senescence (Supplementary Information, Fig. S1a). To identify on-target effectors, four additional shRNAs were constructed for each of the six genes and GFP-growth competition assays were performed to test their functionality. Two additional shRNA vectors conferred a growth advantage on the cells, both targeting BRD7, implying that the other hits were picked up as a result of off-target effects. This was confirmed by the observations that some of the identified genes are not expressed in BJ fibroblasts and that some of the selected shRNAs did not cause a downregulation of the mRNA to be targeted (data not shown).

Protein production and *in vitro* binding assays

MBP fusion proteins were extracted in 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA pH 8, 10 mM 2-mercaptoethanol and purified with amylose resin (NEB E8021S) in accordance with the manufacturer's instructions (NEB). In vitro translation was performed in accordance with the standard protocol (Promega) in the presence of [^{35}S] methionine. The proteins were diluted in 150 mM Tris-HCl pH 7.5, 1% Nonidet P40 (NP40), 10% glycerol, and incubated with 2.5 µg of MBP–BRD7 fusion constructs or MBP.

GFP growth competition assays

BJ/ET cells were retrovirally transduced with pMSCV-blast-Ras^{V12}ER, drug-selected, and transduced with pRS–yellow fluorescent protein (YFP) or pRS–GFP constructs expressing the respective shRNA. Efficiency of transduction was determined by flow cytometry (FL1) and cells were plated in medium with and without 100 nM 4-OHT. Cells were propagated, and the percentage of GFP-positive or YFP-positive cells was measured twice a week. The relative growth advantage was calculated as described[8].

Immunoprecipitation

Co-immunoprecipitation of BRD7 and p53 was performed in 50 mM Tris-HCl pH 8, 100 mM NaCl, 1% NP40, 1 mM EDTA, 5% glycerol with protease inhibitor cocktail (Sigma), 1 mM phenylmethylsulphonyl fluoride, 5 mM NaF, 1 mM

Na_3VO_4, 1 μM trichostatin A (TSA) and 5 μM nicotinamide, with 2 mg of total lysate and 3 μg of anti-BRD7 antibody or anti-haemagglutinin antibody as control. Co-immunoprecipitation of endogenous BRD7 and p300 was performed in 25 mM HEPES/KOH pH 8, 150 mM KCl, 0,5% NP40, 2 mM EDTA, 2% glycerol with protease and deacetylase inhibitors as above, with 2 mg of total lysate and 3 μg of anti-BRD7 antibody or anti-haemagglutinin antibody as control. p53 immunoprecipitation was performed in RIPA buffer with DO-1 antibody. When required, antibodies were covalently bound to Protein A-Sepharose (Amersham) by using 5 mg ml^{-1} dimethylpimelimidate (Pierce).

Tumour growth assays

BJ/EHT/$p14^{ARF\text{-}KD}$/$p16^{INK4A\text{-}KD}$/st cells were transduced with pRS-control, pRS-$BRD7^{KD}$ or pRS-$p53^{KD}$, drug-selected, and transduced with pBabe-puro-Ras^{V12}. After 1 week, 5 x 10^4 cells were plated in triplicate in soft agar (30 mm dishes). After 3 weeks of growth, macroscopically visible colonies were counted. To assay in vivo tumorigenesis, 10^6 cells were injected subcutaneously into immuno-compromised BALB/c mice.

Quantitative RT–PCR and mRNA expression array analysis

Total RNA was extracted with Trizol (Invitrogen) and hybridized to mRNA expression arrays (HumanWG-6 BeadChip; Illumina) in accordance with the manufacturer's instructions (http://microarrays.nki.nl). cDNA was transcribed with Superscript III (Invitrogen) in accordance with the manufacturer's protocol. To determine mRNA expression levels, primers were designed with Primer Express v. 3.0 Software. Real-time PCR was performed with SYBR Green PCR master mix (Applied Biosystems) and Chromo 4 Real Time PCR Detector (Bio-Rad Laboratories). Primers sequences are shown in Supplementary Information, Table S3a. Probes that were not detected (detection $P > 0.05$) in all samples were filtered out. Data were normalized with quantile normalization and further analysed with the EXPANDER package[38]. Expression data were submitted to GEO (accession number GSE20076).

Constructs and antibodies

The NKI shRNA library was described previously[37]. Additional information can be found on the Screeninc website (http://screeninc.nki.nl). pRetrosuper (pRS), pRS-blast, pRS-hygro, pRS-YFP and pRS-GFP, pBabepuro, pBabe-puro-Ras^{V12}, pBabe-puro-$Ras^{V12}ER^{TAM}$, pMSCV-blast, pMSCV-blast-$Ras^{V12}ER^{TAM}$, pMSCV-GFP-st and pBabe-H2B-GFP were described previously[8,39–41]. pRS-p53 2, pRS-p16 4 and pRS-p14 1 shRNA constructs were described previously[8]. BRD7 shRNA sequences were cloned into pRS-YFP, pRS-hygro or pRS-blast. Targeting sequences: no. 312, 5′-CCAGATGGAACAACGACGT-3′; no. 313, 5′-GAAGCTTTGAATCAACTGA-3′; no. 1, 5′-GTACTAATGCCATGATTTA-3′; no. 2, 5′-GCAAGTAACTCCAGGTGAT-3′; no. 3, 5′-CCAGAGACCATTTATTATA-3′; no. 4, 5′-GCACGTATGGAGTTCGAAA-3′. pcDNA3-Flag–BRD7 and pcDNA3-Flag–BRD7ΔBD have been described previously[42]. From these, pEGFP–BRD7 and pEGFP–BRD7ΔBD were obtained by subcloning. pMSCV–BRD7 was obtained by PCR amplification, using pcDNA3-Flag–BRD7

as a template, and cloned into pMSCV-blast (BRD7 forward, 5′-GCATCTCGAG-GCCACCATGGGCAAGAAGCACAAGAAGC-3′; BRD7 reverse, 5′-GCATCTC-GAGTCAACTTCCACCAGGTCCAC-3′). This was used as a template to obtain pMSCV–BRD7Δ1/128 (Δ1/128 forward,5′-GAAGATCTGCCACCATGCCTCCAAA AAAGAAGAGAAAGGTAGCTGAAGAAGTAGAACAGACACCCCTTCAAG-3′) and pMSCV–BRD7Δ361/651 (Δ361/651 reverse, 5′-GCATCTCGAGTCAAATGGGATC-CACAGGATGGAG-3′). The plasmids for bacterial expression of MBP–BRD7 fusion proteins were all obtained by PCR amplification of the entire BRD7 open reading frame or its portions, using pcDNA3-Flag–BRD7 as a template, and cloning in frame with the MBP gene of pMAL-C2X vector (NEB). p21-luc, containing 2.4 kb of the human P21 promoter and upstream region, pGL3-HDM2-luc, containing 0.3 kb of the HDM2 promoter, and pGL3-FAS-luc, containing 1.7 kb of the human FAS promoter, were described previously[43,44].

Antibodies against p53 (DO1, 1:1000 for WB, 1:50 for immunofluorescence; and FL-393, 1 mg for ChIP), p21 (F5, 1:500 for WB), RAS (F235, 1:1000 for WB), c-myc (C33, 1:1000 for WB), CDK4 (C22, 1:1000 for WB) and HA (Y11, 1 mg for ChIP) were purchased from Santa Cruz Biotechnology; anti-BRD7 rabbit polyclonal serum was raised against the C-terminal portion (aa 361-651) of human BRD7 expressed in bacteria, affinity purified by standard procedures, and used diluting a 200 ng ml^{-1} solution 1:1000 for WB, and 1 mg for ChIP. Anti-GFP rabbit polyclonal serum was raised against GST-GFP fusion protein expressed in bacteria, affinity purified and used 1:1000 for WB. Other antibodies were anti– acetyl-Lys382-p53 (Cell Signaling Technology and Abcam, 1:1000 for WB; 1 mg for ChIP), anti-p300 (NM11; BD-Pharmingen, 1:500 for WB, 1 mg for ChIP), anti-acetyl-Histone-H3 (Lys 9) (#07-352, Millipore, 1 mg for ChIP), anti-Histone- H3 (ab1791, Abcam, 1 mg for ChIP), anti-γ-H2AX (JBW301, Millipore).

Senescence-associated β-galactosidase assay

BJ/EHT/Ras^{V12}ER cells were transduced with pRS-blast constructs and drug selected. Cells were plated in triplicate and cultured for 10 days in the presence of 100 nM 4-OHT. Senescence-associated β-galactosidase activity was determined as described[40].

Luciferase assays

MCF7 cells were co-transfected with 400 ng of pRS, pRS-$BRD7^{KD}$ or pRS-$p53^{KD}$ in combination with 100 ng luciferase reporter and 0.5 ng renilla plasmid. Three days after transfection, luciferase assays were performed in accordance with the manufacturer's instructions (Dual Luciferase system; Promega).

Chromatin immunoprecipitation assay

ChIP experiments were performed within the first 48 h on Ras^{V12} induction, when the differences in proliferation rate between control and knockdown cells are minimal (Supplementary Information, Fig. S1b). Cells were crosslinked for 15 min with 1% formaldehyde, neutralized with 125 mM glycine pH 2.5 and washed in PBS. For BRD7 ChIP, formaldehyde crosslinking was preceded by protein–protein

crosslinking with 2 mM disuccinimidyl glutarate for 30 min. Preparation and lysis of nuclei, chromatin sonication to 500–1,000 base pairs (bp) average fragment size, immunoprecipitation and DNA extraction were performed as described previously[45]. A negative control for immunoprecipitation was performed in the presence of isotype-specific unrelated antibody. Purified DNA was resuspended in water and a one-tenth aliquot was used for quantification. Real-time PCR was performed on a StepOne Plus cycler (Applied Biosystems), using SYBR Green Universal PCR Master Mix (Applied Biosystems). Primer sequences are shown in Supplementary Information, Table S3b. Promoter occupancy was calculated as the percentage of input chromatin immunoprecipitated with the $2^{-\Delta\Delta Ct}$ method[46].

Apoptosis assay

At 48 h after transfection, cells were treated with 50 μM etoposide (Sigma) or left untreated; 24 h later, cells (adherent and floating) were harvested and resuspended in PBS with 0.1% NP40 and 2 μg ml^{-1} RNaseA, adding 10 μg ml^{-1} propidium iodide, and were then analysed by flow cytometry. At least 1.5 x 10^4 cells were analysed in each acquisition, and the percentage of cells with a DNA content less than 2n was calculated.

Proliferation assay

Cells were treated for 12 h with 50 μM etoposide and pulsed for 3 h with 30 μM bromodeoxyuridine (BrdU; Sigma). Cells were then fixed, permeabilized and treated with NaOH to denature DNA. BrdU incorporation was measured by immunofluorescence with an anti-BrdU antibody (GE Healthcare), and the nuclei were stained with Hoechst. At least 300 cells were scored for BrdU incorporation.

Immunofluorescence assay

Cells seeded on polylysine-coated coverslips were incubated for 5 min with CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100) at 4°C, fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. After incubation with primary antibodies, anti-mouse tetramethylrhodamine B isothiocyanate and anti-rabbit fluorescein isothiocyanate-conjugated secondary antibodies (Sigma) were used. Images were obtained with a Leica DM4000B epifluorescence microscope or a Leica DM IRE2 confocal microscope where indicated.

Yeast two-hybrid screening

The yeast strains and yeast two-hybrid screening procedures used were described previously[47]. The primary screening was performed by using a LexA-p53 wild-type construct lacking the p53 transactivation domain (Δ11–69) and a human fetal brain cDNA library cloned into the galactose-inducible expression vector pJG4–5 described (ref. 48). At least 2 x 10^6 primary clones were analysed, and positive interactions were further confirmed in a secondary two-hybrid assay that also included LexA-p53 constructs with deletions of the proline-rich domain ΔPro: 62–92) and at the carboxy end (LexAp53/74–298) as bait.

ChIP sequencing

DNA was purified by SDS–PAGE to obtain 100–300-bp fragments and sequenced on an Illumina 1G sequencer. In brief, size-fractionated DNA was extracted and a single adenosine was added with Klenow exo− (3´and 5´ exo minus; Illumina). Illumina adaptors were then added and DNA was subjected to 20 cycles of PCR in accordance with the manufacturer's instructions. DNA was purified and sequenced on the Illumina Genome Analyzer II platform to generate 36-bp reads, in accordance with the manufacturer's instructions. Sequence reads were mapped to the NCBI Build 36 (hg18) reference human genome by using Eland (Illumina), allowing up to two mismatches (Supplementary Information, Table S2d). Peaks were detected with the CisGenome tool[49], comparing reads data from the p53-immunoprecipitation and BRD7-immunoprecipitation samples with their matching controls derived from cells in which these factors had been knocked down. In all, 24,921 and 133 peaks were identified for BRD7 and p53, respectively (false discovery rate 10%). Peaks were mapped to the nearest gene (up to 20 kb) with the CisGenome utility. BRD7 peaks were significantly concentrated near the TSS of target genes (Supplementary Information, Fig. S9); in subsequent analyses we therefore considered only BRD7 peaks that fell within 800 bp of the TSS (4,914 peaks near the TSS of 4,422 genes). A total of 56 p53 peaks were located near or within 52 genes. The significance of the overlap between genes containing p53 and BRD7 peaks (26 genes) was estimated with a hypergeometric test performed with respect to a background set of 13,276 genes expressed in BJ cells (according to results obtained by expression microarray profiling). ChIP-sequencing Data were submitted to GEO (accession number GSE20076).

ACKNOWLEDGEMENTS

We thank Licio Collavin for discussion; Mauro Giacca, Marina Lusic and Lucia Pattarini for access to ICGEB facilities; Thomas Dobner for providing BRD7 expression constructs; Bruno Amati for p300 expression plasmid; Roderick Bijersbergen for assistance in the RNA-mediated interference array experiment; and Joachim Oude-Vrielink and Mariette Schrier for technical assistance. This work was supported by grants from the Dutch Cancer Society (KWF) to J.D., P.M.V and R.A., a European Young Investigator (EURYI) award to R.A. and by the Centre for Biomedical Genetics (CBG) (R.A.), the European Research Council (ERC) (R.A.), the Associazione Italiana per la Ricerca sul Cancro, Ministero dell'Università e della Ricerca (G.D.S.) and by EC FP6 (contracts 503576 and 502963).

AUTHOR CONTRIBUTIONS

J.D., F.M. and F.T. performed most of the experimental work. J.D., F.M., R.A. and G.D.S wrote the manuscript. P.M.V., R.A. and G.D.S supervised the project. R.E. performed bioinformatical analyses regarding ChIP-sequencing and mRNA expression experiments. A.C. conducted the *in vitro* pull-down assays. H.H. and J.J. performed the comparative genomic hybridization analysis. R.K. conducted

the ChIP-sequencing procedure. P.M.V. contributed to analyses of mRNA expression data sets of human breast tumours.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturecellbiology

Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

REFERENCES

1. Michaloglou, C. *et al.* BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436,** 720 – 724 (2005).
2. Chen, Z. *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436,** 725 – 730 (2005).
3. Collado, M. *et al.* Tumour biology: senescence in premalignant tumours. *Nature* **436,** 642 (2005).
4. Christophorou, M. A., Ringshausen, I., Finch, A. J., Swigart, L. B. & Evan, G. I. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature* **443,** 214 – 217 (2006).
5. Vousden, K. H. & Lu, X. Live or let die: the cell's response to p53. *Nature Rev. Cancer* **2,** 594 – 604 (2002).
6. Bartkova, J. *et al.* DNA damage response mediators MDC1 and 53BP1: constitutive activation and aberrant loss in breast and lung cancer, but not in testicular germ cell tumours. *Oncogene* **26,** 7414 – 7422 (2007).
7. Voorhoeve, P. M. *et al.* A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* **124,** 1169 – 1181 (2006).
8. Voorhoeve, P. M. & Agami, R. The tumor-suppressive functions of the human INK4A locus. *Cancer Cell* **4,** 311 – 319 (2003).
9. Robinson, K., Asawachaicharn, N., Galloway, D. A., Grandori, C. c-Myc accelerates S-phase and requires WRN to avoid replication stress. *Plos ONE* **4,** e5951 (2009).
10. Godar, S. *et al.* growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* **134,** 62 – 73 (2008).
11. Ard, P. G. *et al.* Transcriptional regulation of the *mdm2* oncogene by p53 requires TRRAP acetyltransferase complexes. *Mol. Cell. Biol.* **22,** 5650 – 5661 (2002).
12. Peng, C. *et al.* The transcriptional regulation role of BRD7 by binding to acetylated histone through bromodomain. *J. Cell Biol.* **97,** 882 – 892 (2006).
13. Sun, H. *et al.* Solution structure of BRD7 bromodomain and its interaction with acetylated peptides from histone H3 and H4. *Biochem. Biophys. Res. Commun.* **358,** 435 – 441 (2007).
14. Kaeser, M. D., Aslanian, A., Dong, M. Q., Yates, J. R. III & Emerson, B. M. BRD7, a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells. *J. Biol. Chem.* **283,** 32254 – 32263 (2008).
15. Kouzarides, T. Chromatin modifications and their function. *Cell* **128,** 693 – 705 (2007).
16. Zhao, Y. *et al.* Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21 (Waf1/Cip1). *Mol. Cell. Biol.* **26,** 2782 – 2790 (2006).
17. Barlev, N. A. *et al.* Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Mol. Cell* **8,** 1243 – 1254 (2001).
18. Espinosa, J. M., Verdun, R. E. & Emerson, B. M. p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage. *Mol. Cell* **12,** 1015 – 1027 (2003).
19. Dornan, D., Shimizu, H., Perkins, H. D. & Hupp, T. R. DNA-dependent acetylation of p53 by the transcription coactivator p300. *J. Biol. Chem.* **278,** 13431 – 13441 (2003).
20. Pearson, M. *et al.* PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. *Nature* **406,** 207 – 210 (2000).

21. Luo, J. *et al.* Acetylation of p53 augments its site-specific DNA binding both *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA* **101**, 2259 – 2264 (2004).
22. Hahn, W. C. *et al.* Creation of human tumour cells with defined genetic elements. *Nature* **400**, 464 – 468 (1999).
23. Argos, M. *et al.* Genomewide scan for loss of heterozygosity and chromosomal amplification in breast carcinoma using single-nucleotide polymorphism arrays. *Cancer Genet. Cytogenet.* **182**, 69 – 74 (2008).
24. Miller, L. D. *et al.* An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc. Natl. Acad. Sci. USA* **102**, 13550 – 13555 (2005).
25. Chin, K. *et al.* Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. *Cancer Cell* **10**, 529 – 541 (2006).
26. Van de Vijver, M. J. *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N. Engl. J. Med.* **347**, 1999 – 2009 (2002).
27. Roepman, P. *et al.* Microarray-based determination of estrogen receptor, progesterone receptor, and HER2 receptor status in breast cancer. *Clin. Cancer Res.* **15**, 7003 – 7011.
28. Hendricks, K. B., Shanahan, F. & Lees, E. Role for BRG1 in cell cycle control and tumor suppression. *Mol. Cell. Biol.* **24**, 362 – 376 (2004).
29. Kang, H., Cui, K. & Zhao, K. BRG1 controls the activity of the retinoblastoma protein via regulation of p21CIP1/WAF1/SDI. *Mol. Cell. Biol.* **24**, 1188 – 1199 (2004).
30. Xu, Y., Zhang, J. & Chen X. The activity of p53 is differentially regulated by Brm and Brg1-containing SWI/SNF chromatin remodeling complexes. *J. Biol. Chem.* **282**, 37429 – 37435 (2007).
31. Gévry, N., Chan, H. M., Laflamme, L., Livingston, D. M. & Gaudreau, L. *p21* transcription is regulated by differential localization of histone H2A.Z. *Genes Dev.* **21**, 1869 – 1881.
32. Zhou, J. *et al.* BRD**7**, a novel bromodomain gene, inhibits G1-S progression by transcriptionally regulating some important molecules involved in ras/MEK/ERK and Rb/E2F pathways. *J. Cell Physiol.* **200**, 89 – 98 (2004).
33. Peng, C. *et al.* BRD7 suppresses the growth of nasopharyngeal carcinoma cells (HNE1) through negatively regulating β-catenin and ERK pathways. *Mol. Cell. Biochem.* **303**, 141 – 149 (2007).
34. Liu, H. *et al.* Promoter methylation inhibits BRD7 expression in human nasopharyngeal carcinoma cells. *BMC Cancer* **8**, 253 (2008).
35. Yu, Y. *et al.* Analysis and molecular cloning of differentially expressing genes in nasopharyngeal carcinoma [in Chinese]. *Acta Biochim. Biophys. Sin.* **32**, 327 – 332 (2000).
36. Spruck, C. H. III *et al.* Absence of p53 mutations in primary nasopharyngeal carcinomas. *Cancer Res.* **52**, 4787 – 4790 (1992).
37. Berns, K. *et al.* A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* **428**, 431 – 437 (2004).
38. Shamir, R. *et al.* EXPANDER-an integrative program suite for microarray data analysis. *BMC Bioinformatics* **6**, 232 (2005).
39. Brummelkamp, T. R., Bernards, R. & Agami, R. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2**, 243 – 247 (2002).
40. Kolfschoten, I. G. *et al.* A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. *Cell* **121**, 849 – 858 (2005).
41. De Vita, G. *et al.* Dose-dependent inhibition of thyroid differentiation by RAS oncogenes. *Mol. Endocrinol.* **19**, 76 – 89 (2005).
42. Kzhyshkowska, J., Rusch, A., Wolf, H. & Dobner, T. Regulation of transcription by the heterogeneous nuclear ribonucleoprotein E1B-AP5 is mediated by complex formation with the novel bromodomain-containing protein BRD7. *Biochem. J.* **371**, 385 – 393 (2003).
43. el-Deiry, W. S. *et al.* WAF**1**, a potential mediator of p53 tumor suppression. *Cell* **75**, 817 – 825 (1993).
44. Müller, M. *et al.* P53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J. Exp. Med.* **188**, 2033 – 2045 (1998).
45. Mantovani, F. *et al.* The prolyl-isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. *Nature Struct. Mol. Biol.* **14**, 921 – 920 (2007).
46. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25**, 402 – 408 (2001).
47. Gostissa, M. *et al.* The transcriptional repressor hDaxx potentiates p53-dependent apoptosis. *J. Biol. Chem.* **279**, 48013 – 48023 (2004).
48. Gyuris, J., Golemis, E., Chertkov, H. & Brent, R. Cdi**1**, a human G1 and S phase protein phophatase that associates with Cdk2. *Cell* **75**, 791 – 803 (1993).
49. Ji, H. *et al.* An integrated software system for analyzing ChIP-chip and ChIP-seq data. *Nature Biotechnol.* **26**, 1293 – 1300 (2008).

SUPPLEMENTARY TABLES AND FIGURES

Table S1. Selected outliers from the loss of function screen.

Gene Symbol	Accession	Gene Name
FLJ10986	NM_018291	HYPOTHETICAL PROTEIN FLJ10986
PP2R2A	NM_002717	PROTEIN PHOSPHATASE 2 (FORMERLY 2A), REGULATORY SUBUNIT B (PR 52), ALPHA ISOFORM
EGR1	NM_001964	EARLY GROWTH RESPONSE 1
PANK1	NM_148977	PANTOTHENATE KINASE 1
AATK	NM_001080395	APOPTOSIS-ASSOCIATED TYROSINE KINASE
VDRIP	NM_014166	VITAMIN D RECEPTOR INTERACTING PROTEIN
SHOX	NM_000451	SHORT STATURE HOMEOBOX
CDC5L	NM_001253	CDC5 CELL DIVISION CYCLE 5-LIKE (S. POMBE)
PCDH15	NM_033056	PROTOCADHERIN 15
MEST	NM_002402	MESODERM SPECIFIC TRANSCRIPT HOMOLOG (MOUSE)
NEK9	NM_033116	NIMA (NEVER IN MITOSIS GENE A)-RELATED KINASE 9
KCNJ5	NM_000890	POTASSIUM INWARDLY-RECTIFYING CHANNEL, SUBFAMILY J, MEMBER 5
TEF	NM_003216	THYROTROPHIC EMBRYONIC FACTOR
RNUT1	NM_005701	RNA, U TRANSPORTER 1
GALR2	NM_003857	GALANIN RECEPTOR 2
BRD7	NM_013263	BROMODOMAIN CONTAINING 7
NCOA2	NM_006540	NUCLEAR RECEPTOR COACTIVATOR 2
DKFZP586M0622	AL117396	DKFZP586M0622 PROTEIN
IPMK	NM_152230	INOSITOL POLYPHOSPHATE MULTIKINASE

A

Gene Symbol	GeneID	Expression Cluster#
BCL2L1	598	2
CDKN1A	1026	2
MDM2	4193	2
RPS27L	51065	2
RRM2B	50484	2
TRIM55	84675	2
ZMAT3	64393	2
SPG3A	51062	4
ANKRD11	29123	-
ATF3	467	-
ATP2A2	488	-
DDB2	1643	-
FAM46A	55603	-
GADD45A	1647	-
GAS6	2621	-
ISG20L1	64782	-
MCCC2	64087	-
NSMCE2	286053	-
PDGFC	56034	-
PLK2	10769	-
PLK3	1263	-
PRDM1	639	-
PRKCE	5581	-
PSMD3	5709	-
SCHIP1	29970	-
SHROOM3	57619	-

Table S2. ChIP-sequencing to uncover genome-wide p53 and BRD7 binding. Overlap between genes bound by BRD7 (peak +/- 800 bp. from TSS) and p53 (peak within or 20 kb upstream target genes). #Cluster #2: genes downregulated in both $p53^{KD}$ and $BRD7^{KD}$ cells. Cluster #4: genes downregulated only in $BRD7^{KD}$ cells. *Enrichment for factor: ratio between the prevalence of genes with peaks of the factor (BRD7, p53) in the cluster and in the background set of all genes expressed in the cells. *Expression cluster - cluster #1: genes downregulated only in $p53^{KD}$ cells; cluster #2: genes downregulated in both $p53^{KD}$ and $BRD7^{KD}$ cells. #None of the peaks in cluster 1 reached statistical significance. (D) Number of reads ChIP-sequencing analysis.

C

Gene	Expression cluster*	p53 peak height#
BAX	1	4
DRAM	1	3
FAS	1	3
TNFRSF10A	1	3
TNFRSF10B	1	5
BCL2L1	2	17
CDKN1A	2	19
GDF15	2	8
MDM2	2	10
RPS27L	2	8
RRM2B	2	15
TRIM55	2	6
ZMAT3	2	19

B

Cluster	Expression pattern	# of genes in cluster	Factor	Number of genes with factor's peak	Enrichment for factor*	P value
1	Down in $p53^{KD}$ but not in $BRD7^{KD}$	38	BRD7	15	1.32	0.14
			p53	0	-	-
2	Down in both $p53^{KD}$ and $BRD7^{KD}$	47	BRD7	23	1.63	0.005
			p53	8	49.1	$2.3*10^{-13}$

D

Sample	Number of reads	Number of reads uniquely mapped to the genome
BRD7	12329360	3492990
BRD7.shBRD7	13042650	3364562
p53	13006478	2773414
p53.shp53	12837856	2920460

Table S3. Sequences of primers used for Q-RT-PCR (A) and ChIP (B).

A

Gene	Forward	Reverse
Brd7	CTGGAGATGCCGAAGCACAC	TGGGATCCACAGGATGGAGA
p21	TACCCTTGTGCCTCGCTCAG	GAGAAGATCAGCCGGCGTTT
Hdm2	GAATCTACAGGGACGCCATC	TCCTGATCCAACCAATCACC
Bax	CCGCCGTGGACACAGAC	CAGAAAACATGTCAGCTGCCA
Puma	TGTGAATCCTGTGCTCTGCC	TTCCGGTATCTACAGCAGCG
Tigar	AAAGAAGCGGATCAAAAAGAACA	CTGCCAAAGAAGTTTCCAGACA
PIG3	GGTGAAGTCCTCCTGAAG	GTCCAGATGCCTCAAGTC
PAI1	TGAAGATCGAGGTGAACGAGAGT	TGCGGGCTGAGACTATGACA
Noxa	GTGTGCTACTCAACTCAG	ATTCCTCTCAATTACAATGC
Fas	CACACTCACCAGCAACACCAA	GCTTTCTGCATGTTTTCTGTACTTCC

B

Promoter	Forward	Reverse
p21	AGCAGGCTGTGGCTCTGATT	CAAAATAGCCACCAGCCTCTTCT
Hdm2	GGGCTATTTAAACCATGCATTTTC	GTCCGTGCCCACAGGTCTA
Puma	GCGAGACTGTGGCCTTGTGT	CGTTCCAGGGTCCACAAAGT
Fas	ACAGGAATTGAAGCGGAAGTCT	GAGTTCCGCTCCTCTCTCCAA
Bax	TAATCCCAGCGCTTTGGAAG	TGCAGAGACCTGGATCTAGCAA
AcHR	CAACCAAAGCCCATGTCCTC	AGGCACGCTACAGGGCTTC

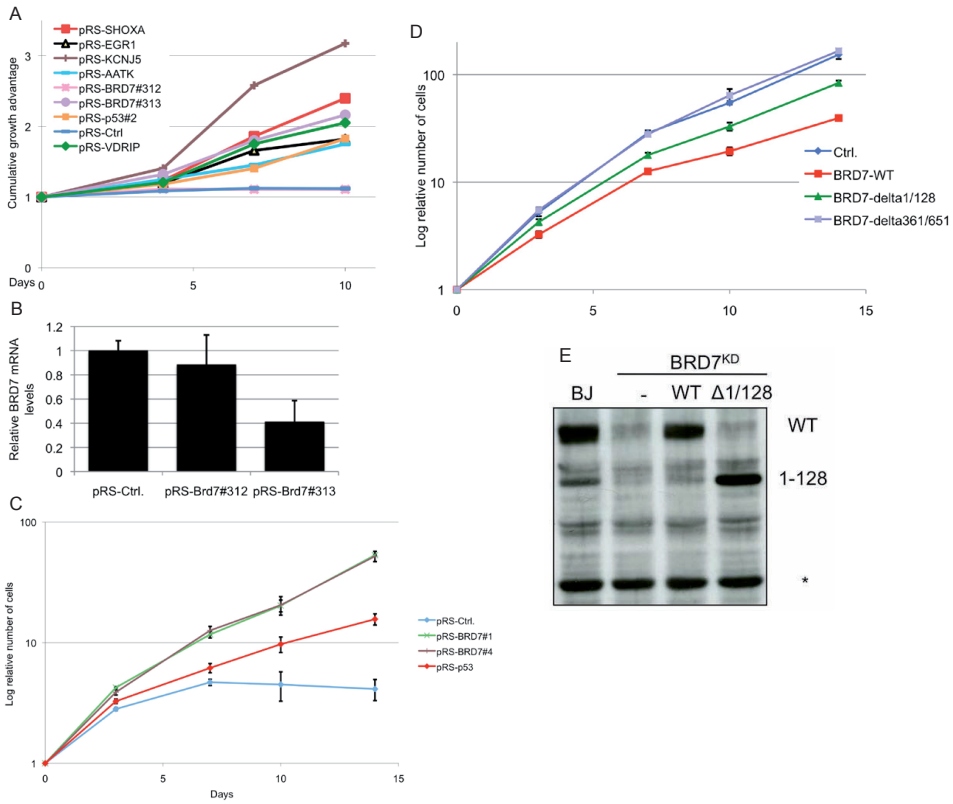


Figure S1. Identification of genes required for OIS. GFP-growth competition assays of the six hits that were validated. Q-RT-PCR for *BRD7* mRNA expression shows the efficiency of the BRD7 knockdown vectors used in (A). Cells from Figure 1D were subjected to a growth assay in the presence of 4-OHT. Standard deviations from three independent experiments are shown. BJ/ET-Ras^{V12}ER-BRD7$^{KD\#4}$ cells expressing the indicated shRNA-resistant BRD7 proteins were subjected to a growth assay in the presence of 4-OHT. An artificial NLS was attached to the Δ1/128 mutant to ensure proper localization. Cells from (C) were harvested and expression levels of re-expressed BRD7 proteins were analysed by western blotting. Background band is used as loading control (*). The BRD7-Δ361/651 mutant cannot be detected by the BRD7 antibody and was not included in the figure.

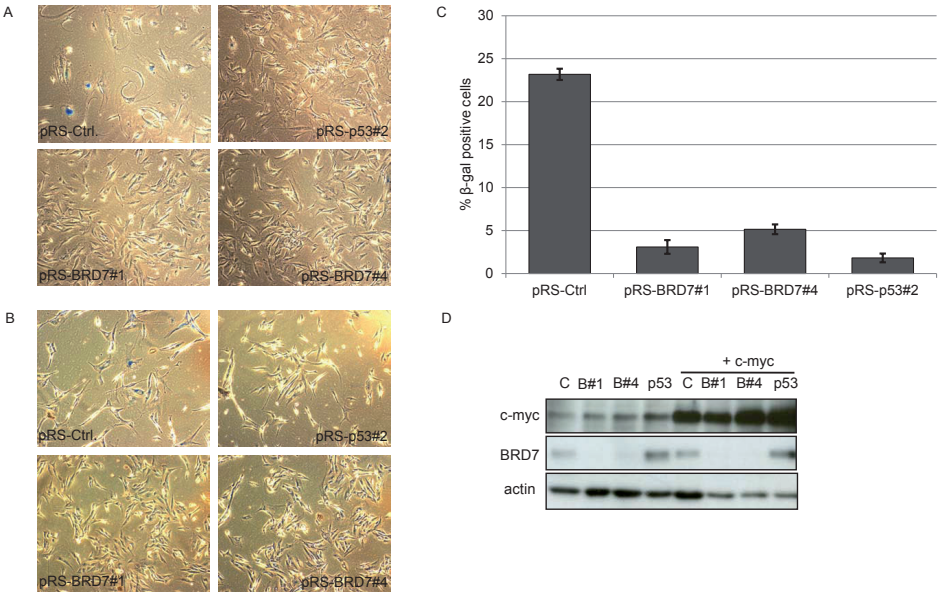


Figure S2. BRD7 is required for p53-dependent OIS. BJ/ET/Ras^{V12}ER cells expressing the indicated knockdowns were cultured in the presence of 4-OHT for 10 days and stained to detect SA-β-galactosidase positive cells. Scale bar, 100 μM. BJ/ET cells expressing the indicated knockdowns were transduced with a c-Myc-expression vector and selected. After 8 days cells were stained to detect SA-β-galactosidase positive cells. Scale bar, 100 μM. Quantification of the experiment described in (B). Graphs represent mean and s.d. from three independent experiments. Cells from (B) were harvested and western blot analysis was performed to detect c-Myc, BRD7, and actin (loading control).

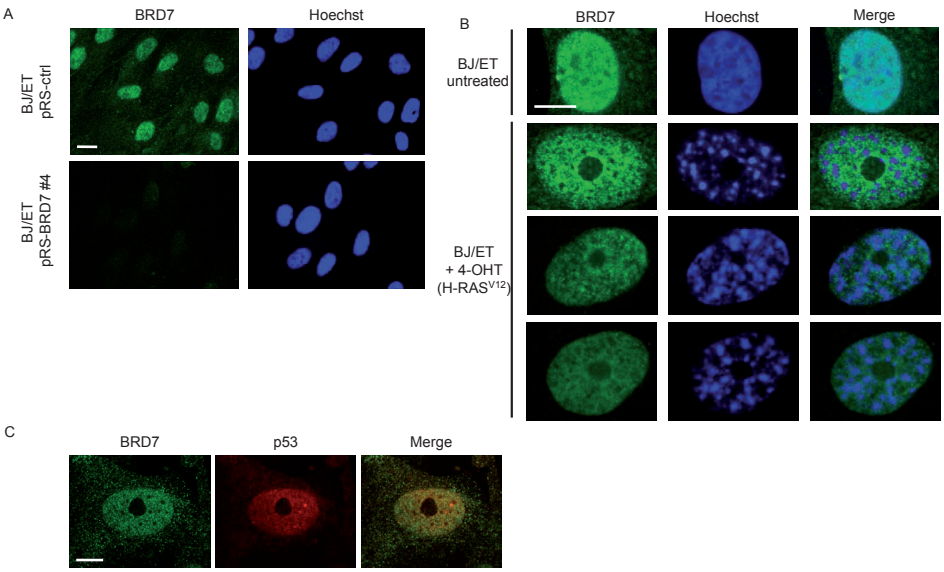


Figure S3. Analysis of BRD7 nuclear localization upon induction of Ras^{V12}. BJ/ET/Ras^{V12}ER cells cultured in the presence or absence of 4-OHT for 9 days were stained with the indicated antibodies and with Hoechst to visualize DNA. As a control for specificity of the BRD7 antibody, BRD7 was silenced by transduction with pRS-$BRD7^{KD}$. SAHFs are evident in the uneven distribution of DNA through the nucleus. In panel C confocal microscopy was performed and yellow staining results from co-localization of BRD7 and p53. Scale bars, 20 μM (A) and 10 μM (B, C).

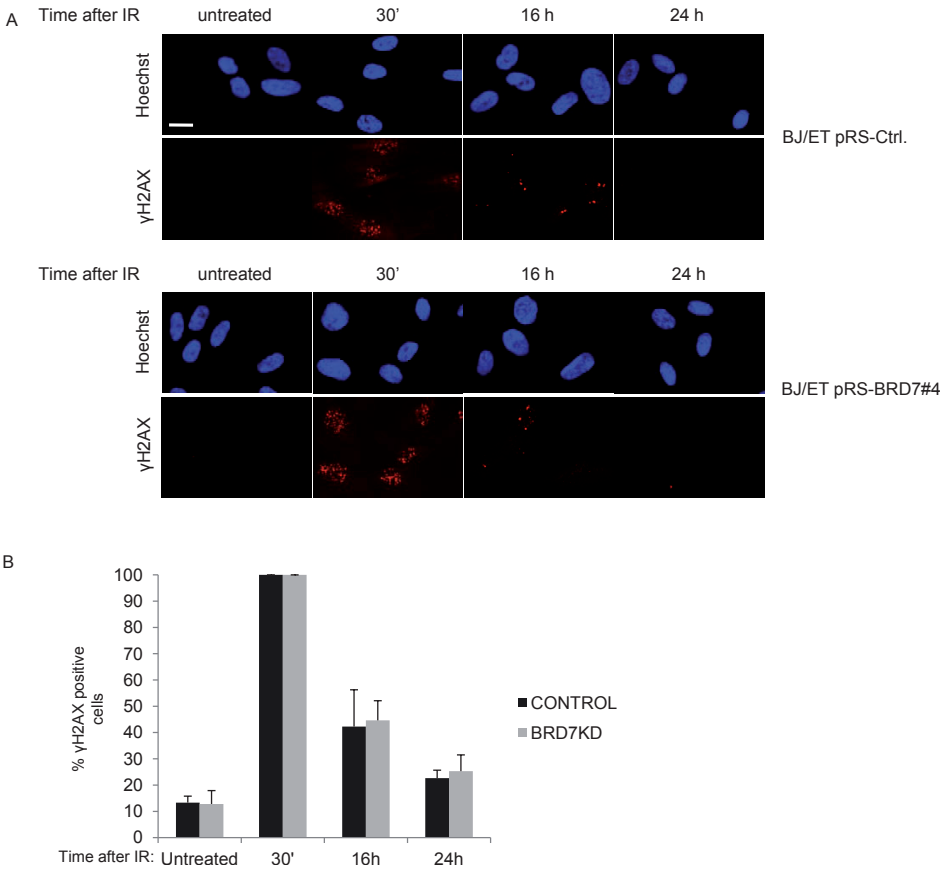


Figure S4. BRD7 does not affect the persistence of DNA damage signals. (A) BJ/ET cells transduced with either pRS-Ctrl or pRS-BRD7#4 were either left untreated or were irradiated with 2 Gy and allowed to recover for 0.5 h, 16 h and 24 h before staining for γH2AX. Representative figures are shown. Scale bars, 20 μM. (B) Quantification of the experiment described in (A). Graphs show means and s.d. for three independent experiments.

A

Screening	Primary	Secondary		
Bait construct	Δ 11-69	Δ 11-69	Δ 95	74-298
Interaction with clone 9 (Brd7)	+ (4 x isolated)	+ (4 x isolated)	+ (4 x isolated)	– (0 x isolated)

B

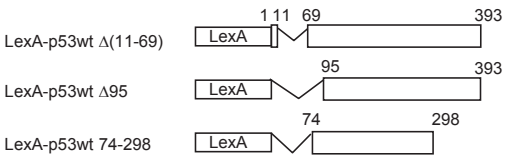


Figure S5. Yeast-two-hybrid screen for p53 binding partners. Summary of the results obtained in the yeast-two-hybrid screening. The screen was performed using a human fetal brain cDNA library and a transactivation domain-deleted human p53 fused to the DNA binding domain of LexA as bait. Schematic representation of the bait constructs used in the screen.

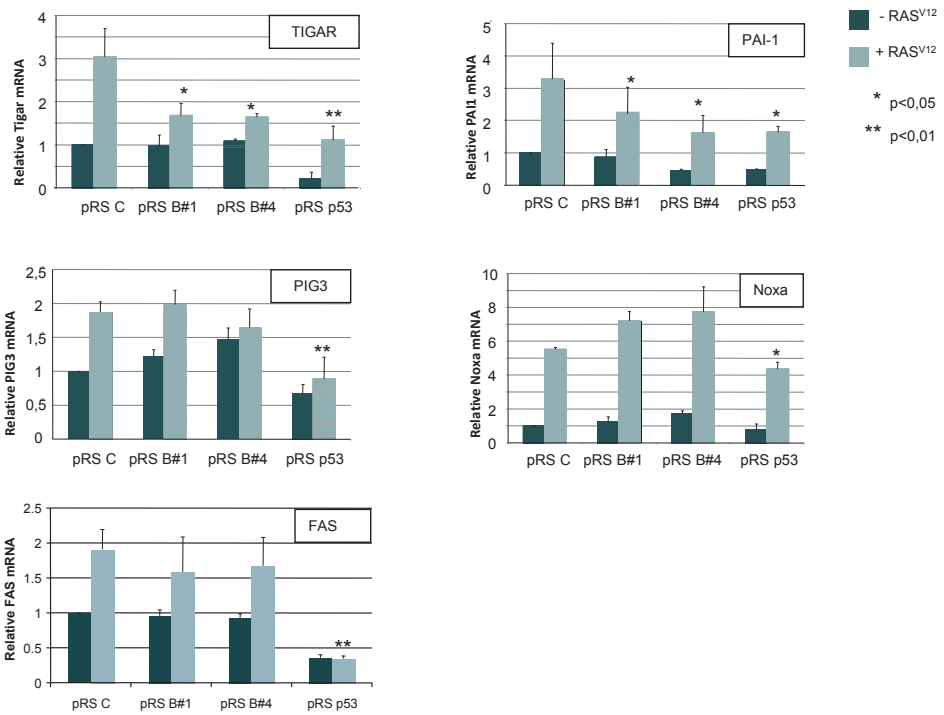


Figure S6. BRD7 is required for the efficient transcriptional activation of a specific subset of p53 target genes. BJ/ET/Ras^{V12}ER cells containing control vector, one of two different BRD7^{KD} vectors, or p53^{KD} vector were cultured in the absence (-Ras^{V12}) or presence (+Ras^{V12}) of 4-OHT for 10 days. mRNA levels of the indicated genes were determined by Q-RT-PCR. Graphs show means and s.d. for three independent experiments. *P* values were calculated using *t*-test.

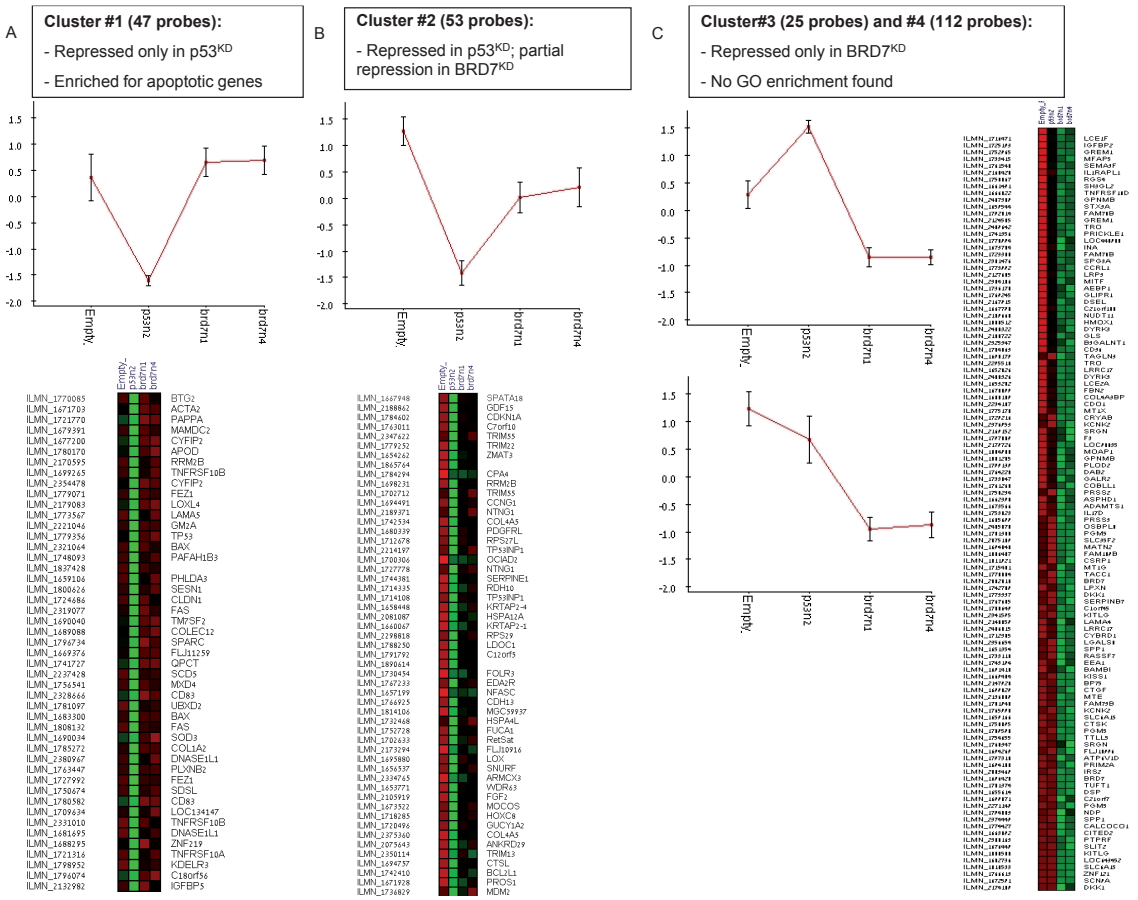


Figure S7. mRNA expression and GO-analysis. Listed are genes significantly repressed in p53^{KD} (A), both p53^{KD} and BRD7^{KD} (B), and BRD7^{KD} (C) BJ/ET/Ras^{V12}ER cells cultured in the presence of 4-OHT for 10 days. No gene name means that the probe was not mapped to any known gene.

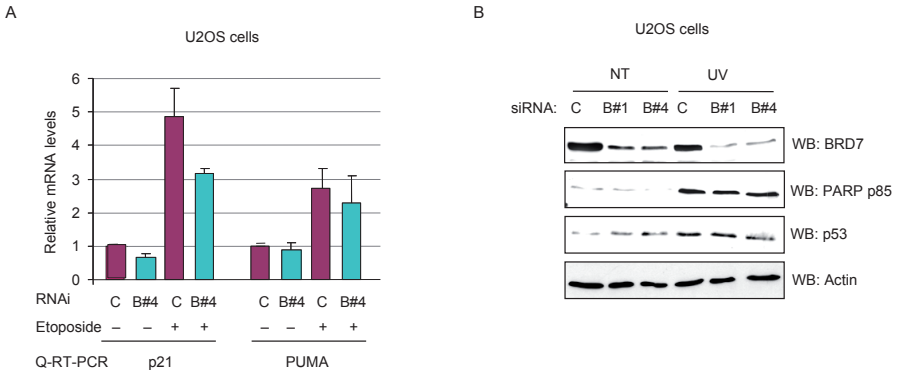


Figure S8. BRD7 is required for stress-dependent transcriptional induction of p21 but not PUMA in U2-OS cells. U2-OS cells were transfected with siRNA against BRD7 (B#4) or control siRNA (C) and treated with etoposide for 18 h. Induction of p21 and PUMA was then evaluated by Q-RT-PCR. Graphs show means and s.d. for three independent experiments. U2-OS cells were transfected with siRNA against BRD7 (B#1 and B#4) or control siRNA (C) and then treated with UV (20 J*m^2) 36 h before harvesting or left untreated (NT). The effect on apoptosis induction was evaluated by western blot analysis of PARP cleavage. Full scans of the western blots are shown in Figure S15.

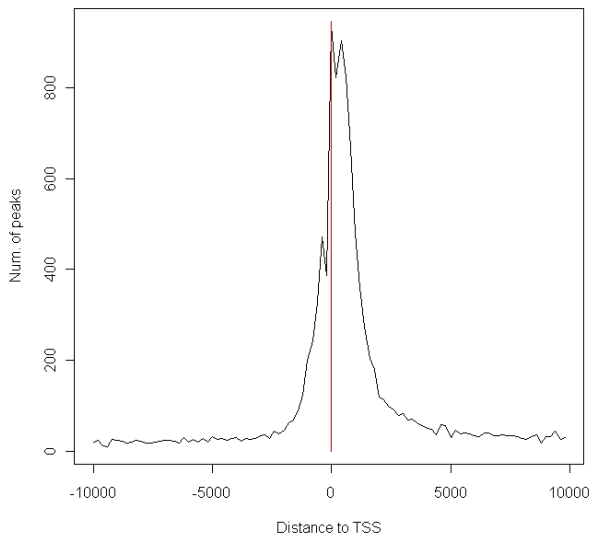


Figure S9. BRD7 binding pattern analysis. Distribution of BRD7 binding peaks as a function of their distance to the TSS of the nearest gene. The distribution is highly concentrated towards TSSs.

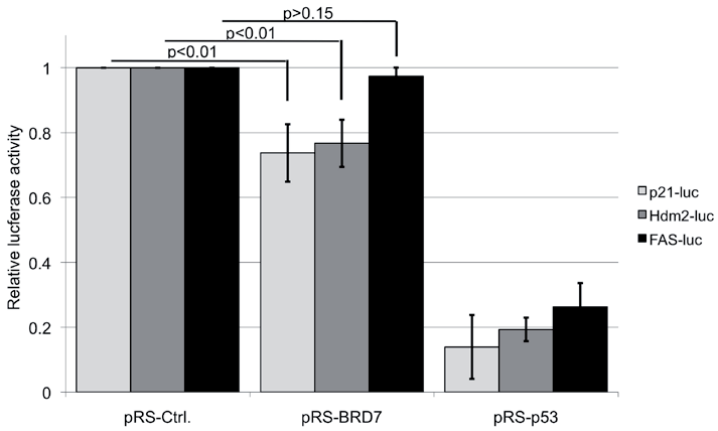


Figure S10. BRD7 affects promoter activity of specific p53 target genes. MCF7 cells were co-transfected with the indicated p53 reporter constructs and knockdown vectors. The relative firefly luciferase/renilla activity was determined and compared to the control vector. Graphs represent mean and s.d. from three independent experiments. *P* values were calculated using *t*-test.

A

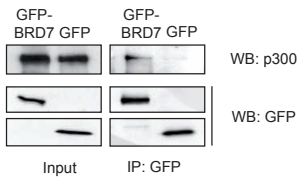
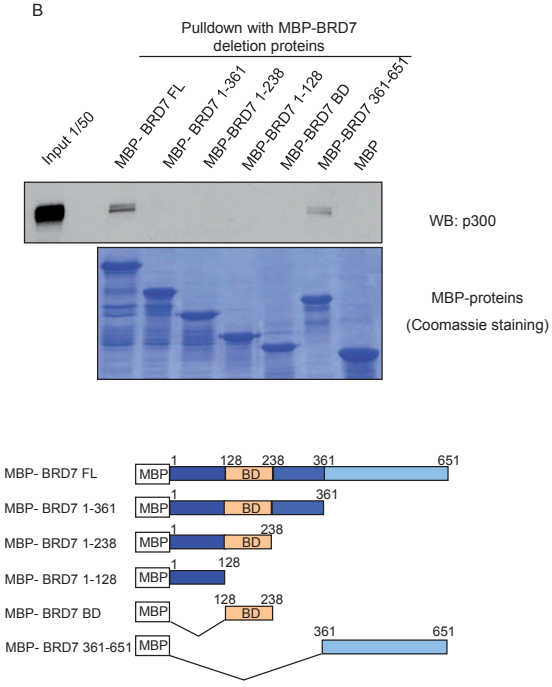


Figure S11. BRD7 and p300 interact independently of p53. H1299 cells (p53-null) were transfected with p300 and either pGFP-BRD7 or pGFP. The interaction between BRD7 and p300 was evaluated by immunoprecipitating with anti-GFP antibody and detecting bound p300 by western blot. Binding of HA-p300 to BRD7 upon overexpression in H1299 cells and pull-down with bacterially expressed MBP-BRD7 deletion proteins. MBP protein was used as control. Binding reactions were compared with 1/50 of protein inputs by western blot analysis. Full scans of the western blots are shown in Figure S15.

B



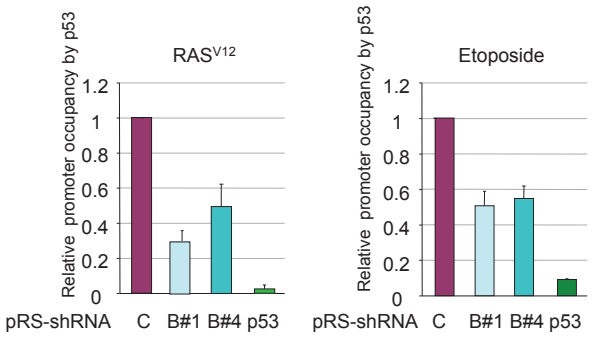


Figure S12. BRD7 is required for p53 loading on target gene promoters. BJ/ET/Ras^{V12}ER cells from Figure 6E were subjected to ChIP for p53. Values are represented as fold change compared to control cells. Graphs represent mean and s.d. from three independent experiments.

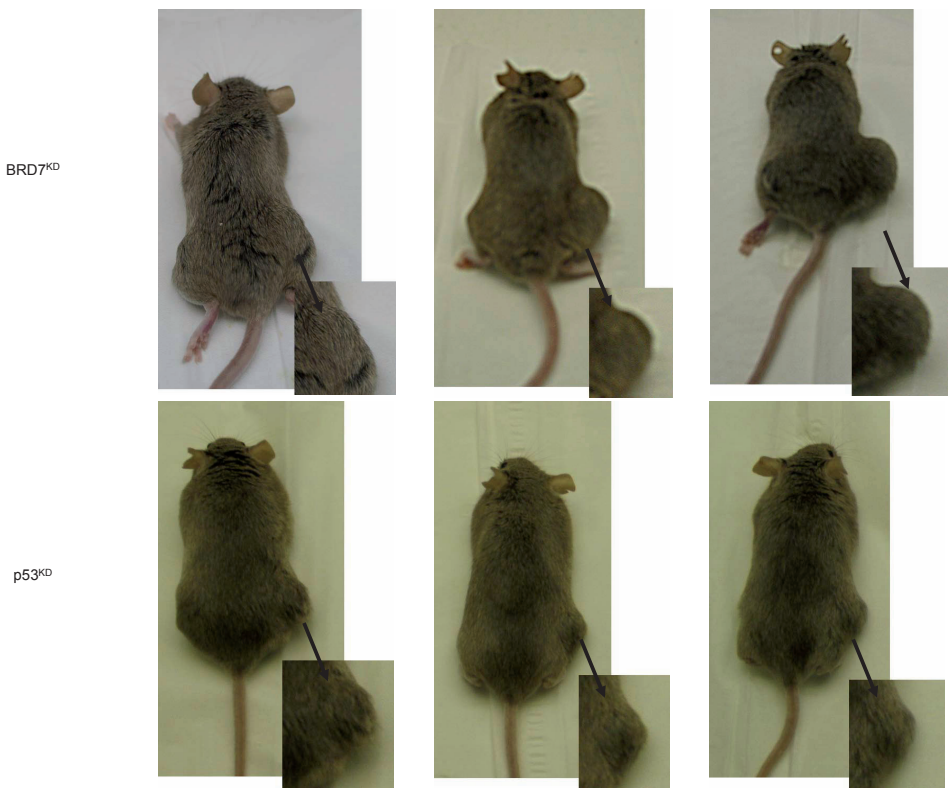
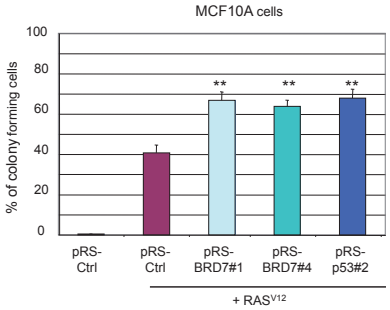
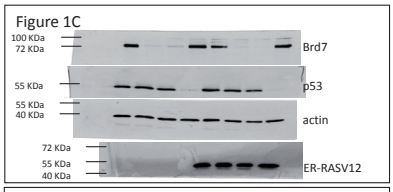


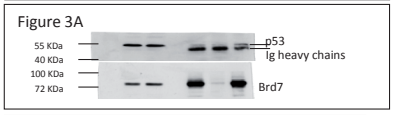
Figure S13. *In vivo* tumour growth. Pictures of several mice that developed tumours in the experiment described in Figure 7A and 7B.

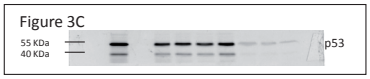


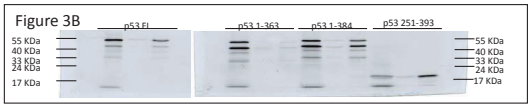
** p<0,01

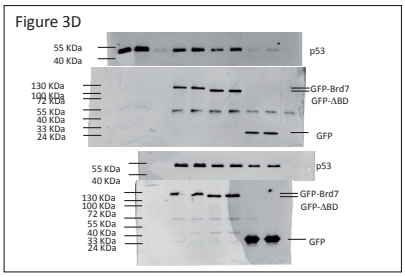
Figure S14. BRD7 enhances oncogene-induced transformation of human mammary epithelial cells. Normal MCF10A breast epithelial cells stably expressing the indicated knockdowns were transduced with pLPC-puro-RAS^{V12} or with empty vector. After selection, cells were seeded in soft agar and colonies were counted 2 weeks later. Histograms show the percentage of colony-forming cells. Error bars show means ± s.d. for three independent experiments. *P* values were calculated using two-tailed unpaired *t*-tests.

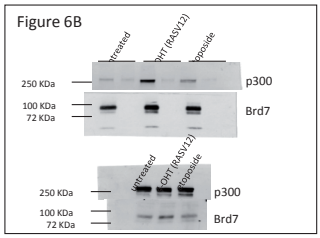


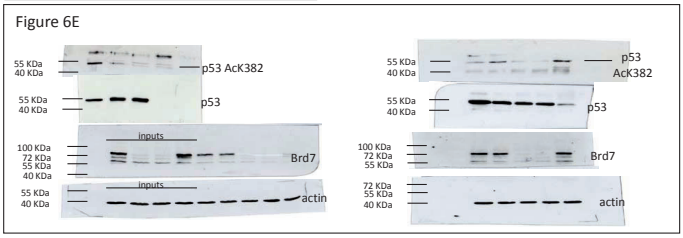


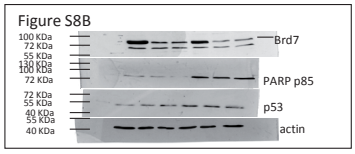












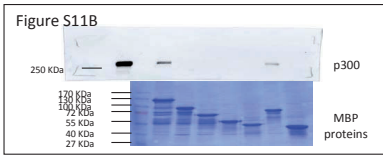


Figure S15. Full scans of key western blots.

3

GENE REGULATION AND TUMOUR SUPPRESSION BY THE BROMODOMAIN-CONTAINING PROTEIN BRD7

Fiamma Mantovani*, Jarno Drost*, P. Mathijs Voorhoeve, Giannino Del Sal and Reuven Agami

*These authors contributed equally to this work

Cell Cycle 2010; 9: 2777-2781

ABSTRACT

Oncogene-induced senescence (OIS) is a cellular defence mechanism against excessive mitogenic signalling and tumorigenesis. One of the major pathways required for OIS is the p53 tumour suppressor pathway. Consequently, many human tumours harbour *p53* mutations while others show a dysfunctional p53 pathway, frequently by unknown mechanisms. We recently identified *BRD7* as a potential tumour suppressor gene acting as a transcriptional cofactor for p53, affecting histone acetylation, p53 acetylation, and promoter activity on a subset of p53 target genes. We further found low BRD7 expression specifically in a subgroup of human breast tumours harbouring wild-type, but not mutant, *p53* and showed that one of the responsible mechanisms is deletion of the *BRD7* gene locus. Here we further discuss the role of BRD7 as a cofactor in transcriptional regulation and highlight its role as a tumour suppressor via association with p53 and other tumour suppressor proteins.

Fiamma Mantovani[1,2,†], Jarno Drost[3,†], P. Mathijs Voorhoeve[4], Giannino Del Sal[1,2,*] and Reuven Agami[3,5,*]

[1]Laboratorio Nazionale CIB; Area Science Park; Trieste, Italy; [2]Dipartimento di Scienze della Vita; Università di Trieste; Trieste, Italy; [3]The Netherlands Cancer Institute; Division of Gene Regulation; Amsterdam, The Netherlands; [4]Duke-NUS Graduate Medical School; Division of Cancer and Stem Cell Biology; Singapore, Singapore; [5]Center for Biomedical Genetics; The Netherlands. *Correspondence to: Giannino Del Sal and Reuven Agami; Email: delsal@lncib.it and r.agami@nki.nl.

[†]These authors contributed equally to this work.

INTRODUCTION

Oncogene-induced senescence (OIS) is an anti-proliferative cellular stress response that serves as an essential intrinsic barrier to tumour development in vivo[1-3]. Although the importance of OIS is acknowledged, the exact mechanism of action is not well understood. Several lines of evidence indicate that oncogene expression, similarly to telomere exhaustion and genotoxic damage, induces the senescence program by activating the DNA damage response (DDR). Specifically, the initial hyper-replication phase fostered by oncogene activity leads to replication stress, which eventually drives cells into senescence[4,5]. Essential for execution of OIS is the p53 tumour suppressor pathway. Activation of this pathway by oncogene expression induces senescence mainly through transcriptional activation of target genes, including the *CDKN1a* gene (encoding the cyclin-dependent kinase inhibitor p21) and *Serpine1* (also known as *PAI-1*, encoding plasminogen activator inhibitor-1; ref. 6). Work with animal models implied that OIS is the major mechanism of tumour suppression by p53 in vivo[7]. Thus, interference with OIS by inhibiting the p53 pathway allows cells to continue to proliferate in the presence of active oncogenes, leading to increased tumorigenicity. Underscoring the therapeutic potential of this pathway, it has been demonstrated that reactivation of p53 in murine tumours causes cellular senescence and associated tumour regression[8,9]. Indeed, DNA-damaging chemotherapeutic agents can induce senescence in cancer cells, which contributes to the anti-tumour effect of these molecules.

Many post-translational modifications have been shown to modulate p53 protein stability, transcriptional activity and selectivity for its target genes. As p53 exerts its multiple functions mainly as a transcription factor, an unresolved issue concerns the routes that lead to specific cellular outcomes by directing p53's choice between different subsets of target genes. For example, it was shown that acetylation of p53 is necessary for its activation[10], and acetylation of specific lysine residues on p53 appears to activate different subsets of p53 target genes. Acetylation of lysine 120 within the DNA binding domain of p53 by the acetyltransferase Tip60 was found to specifically direct p53 towards the induction of apoptotic target genes[11,12], while both acetylation of C-terminal lysine 320 by PCAF, and of lysines 373 and 382 by p300/CBP, have been associated with the induction of p21 (ref. 13,14). Therefore, it seems likely that post-translational modifications of the p53 protein determine its functionality in OIS. However, also cell type-specific variations in the availability of certain p53 cofactors, as well as differences in the affinity of p53 and its cofactors for these promoters, have been documented to play a role in determining the eventual fate of a stressed cell[15].

The central role of the p53 pathway in tumour suppression is reflected in the appearance of *p53* mutations in as many as 50% of human tumours (IARC TP53 Database http://www-p53.iarc.fr/ Statistics.html). When the *p53* gene itself is wild-type, genetic lesions in components of the p53 pathway are often found

to interfere with its activities. Still, in many cases the mechanism behind the inactivation of this major tumour suppressor pathway is unknown.

3 BRD7 IS A TRANSCRIPTIONAL COFACTOR REQUIRED FOR P53-DEPENDENT OIS

We identified Bromodomain-containing 7 (BRD7) as an important transcriptional cofactor of p53 by combining a loss-of-function screen for putative tumour suppressor genes required for p53-dependent OIS and a two-hybrid screen to fish out novel p53-interacting proteins. Through this approach we concomitantly highlighted the genetic and functional interaction of p53 with BRD7 (ref 16). BRD7 is an evolutionarily conserved protein bearing a single bromodomain[17]. The protein has been shown to be a subunit of SWI/SNF chromatin-remodelling complexes and has been implicated in regulation of transcription[18,19].

In human primary fibroblasts, knockdown of BRD7 enabled cells to bypass oncogene-induced senescence in the presence of wild-type p53. BRD7 appeared to be required for efficient transcription of a specific subset of p53 target genes that become induced upon expression of Ras^{V12}, including p21, PAI-1, HDM2, Cyclin G1, p53R2, WIG-1, while apoptotic genes were not dependent on BRD7 (Fig. 1a). Dissecting the mechanism of functional cooperation of p53 and BRD7, we observed that their interaction is stimulated by Ras^{V12} expression. In these conditions, p53 recruits BRD7 to several of its high-affinity target promoters, including *p21* and *HDM2*. While the BRD7 C-terminus is engaged in binding to p53, its N-terminal domain interacts with the histone acetyltransferase p300. We showed that BRD7 is required for the efficient recruitment of p300 to the *p21* promoter, where it activates transcription by facilitating acetylation of both p53 on lysine 382 and histone H3 on lysine 9 within nearby nucleosomes. Histone acetylation may in turn support the spreading of BRD7 throughout a wider chromatin region, by means of its ability to recognize acetylated histones through the bromodomain[17,20]. Spreading of BRD7 across the chromatin surrounding p53-binding sites is confirmed by the larger amplitude of BRD7 binding peaks as compared to those generated by p53, observed by ChIP-sequencing[16]. It is likely that, through continuous recruitment of p300, this process generates a positive loop that amplifies histone acetylation, thereby sustaining promoter activity.

In addition, BRD7 was recently identified as a component of a SWI/SNF chromatin-remodelling complex[18]. This adds further evidence to the previously described interactions of p53 with other SWI/SNF subunits, and of the involvement of SWI/SNF activity in p53-dependent transcription[21,22]. SWI/SNF complexes are targeted to promoters via direct interactions with transcription factors[23]. The ability of p53 to independently bind different SWI/SNF subunits suggests that each may have a specific function. Given its role in OIS, it is conceivable that BRD7 may serve for selective recruitment of chromatin-remodelling complexes to p53 target promoters to regulate transcription during OIS. Strikingly, knockdown of the SWI/

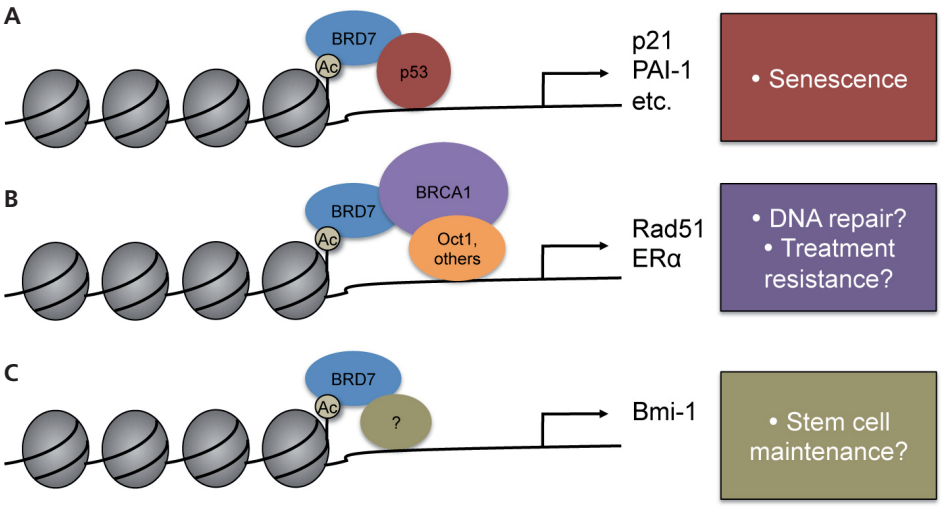


Figure 1. Cell fate determination by BRD7 and mode of action. (A) BRD7 is recruited to p53 target promoters and affects transcription of genes required for oncogene-induced senescence. (B) BRD7-dependent recruitment of Oct1 and BRCA1 to target promoters implies involvement of BRD7 in DNA repair and cancer treatment resistance. (C) A potential role for BRD7 in stem cell self-renewal by regulation of the Bmi1 gene.

SNF core ATPase BRG1 has been shown to impair p21 expression[24,25] and this appeared to partially depend on decreased binding of p53 to the *p21* promoter[26]. BRG1 has also been implicated in restraining p53 activity in the absence of DNA damage by cooperating with the E4 ubiquitin ligase CBP[27]. However, our data do not support a role for BRD7 in regulating p53 stability.

Another level of transcriptional regulation by p53 comes from the recent observation that p53 can cause nucleosome repositioning. In unstressed cells, p53 keeps the *p21* promoter in a poised state by directing the binding of the histone variant H2A.Z to p53-cognate sites within the *p21* promoter, thus inhibiting the onset of senescence. Upon DNA damage, p53-dependent activation of the *p21* promoter then involves eviction of H2A.Z[28]. The establishment of the senescent phenotype is accompanied by extensive changes in chromatin structure. In particular, many senescent cells accumulate specialized domains of facultative heterochromatin, called Senescence Associated Heterochromatin Foci (SAHF). SAHF are thought to repress the expression of proliferation-promoting genes, thereby contributing to the senescence-associated proliferation arrest[29]. SAHF formation is a multistep process that involves chromatin-remodelling events and, at least at late stages, requires intact p53 and retinoblastoma (RB) pathways[30,31]. Although SWI/SNF remodelling complexes are involved in RB-dependent repression of E2F target genes[32,33] and induction of cell differentiation and senescence, it is not known whether they may directly participate in SAHF formation. Clearly, it would be of interest to investigate whether BRD7 also acts

as a cofactor for RB in the onset of senescence. However, our data do not directly implicate BRD7 in SAHF formation, but rather suggest that during OIS BRD7 is excluded from these structures[16]. This observation is consistent with the report that BRD7 is normally associated with actively transcribed chromatin domains[19].

IMPLICATIONS FOR BRD7 IN PROLIFERATION, DNA REPAIR AND STEM CELL BIOLOGY

As a component of SWI/SNF complexes, BRD7 can be expected to function as a transcriptional cofactor for many other proteins. Moreover, SWI/SNF complexes and BRD7 itself can function both as activators and repressors. This suggests that BRD7 may regulate up to thousands of genes. ChIP-sequencing data highlighted binding of BRD7 to thousands of genomic sites, although we have also observed that BRD7 binding to a promoter does not always imply that the gene is transcriptionally regulated by BRD7 (ref. 16). BRD7 targets include proliferation-related genes, such as components of the Ras/MEK/ERK and RB/E2F pathways[34,35], with consequent inhibition of G1/S progression[34].

A role for BRD7 in regulation of genes involved in DNA repair has been highlighted by a recent report showing that BRD7 interacts with the tumour suppressor protein BRCA1[36]. The authors identified a variety of genes co-regulated by BRCA1 and BRD7, including oestrogen receptor α, and the DNA repair factor Rad51 (Fig. 1b). SWI/SNF complexes have been shown to participate directly in DNA repair, an activity that requires prompt alterations of chromatin accessibility to DDR components. In yeast, SWI/SNF complexes are recruited to DNA double strand breaks (DSBs) to facilitate DNA repair, and mammalian SWI/SNF complexes contribute to DSB repair by stimulating the phosphorylation of histone H2A.X at DSB-surrounding chromatin[37]. Notably, this involves a cooperative activation loop among SWI/SNF, γ-H2AX and H3 acetylation[38]. Furthermore, the catalytic SWI/SNF subunit BRG1 facilitates different stages of nucleotide excision repair (NER). It does so by modulating chromatin relaxation, stimulating stabilization of the xeroderma pigmentosum protein (XPG) at the damaged sites and subsequently stimulating the recruitment of downstream NER factors to UV-induced lesions[39]. Our data do not suggest a role for BRD7 in affecting the persistence of DNA damage signals upon acute genotoxic stress[16]. This points towards a restricted role for BRD7 in the executive, rather than the causative, phases of OIS. However, a potential contribution of BRD7 in the activities of BRCA1 and SWI/SNF complexes in DNA repair remains to be explored.

BRCA1 was previously shown to interact with p53 and regulate its stability and transcriptional activity. Intriguingly, this interaction has been proposed to selectively direct p53 towards induction of genes required for DNA repair and growth arrest[40], and shifting p53-mediated cellular outcomes towards senescence[41]. The observation that BRG1 is involved in BRCA1-mediated activation of p21 transcription by p53 (ref. 42), raises the intriguing possibility

that the scaffold protein BRCA1 might contribute to recruit BRD7, and thus SWI/SNF activity, to specific promoters. Based on data of Harte and colleagues, it would be interesting to verify whether BRCA1 is consistently present at promoters co-regulated by p53 and BRD7 (ref. 36).

Finally, an intriguing connection of BRD7 with stem cell biology has been proposed while analysing the consequences of its knockdown on the transcriptional profile of embryonic stem cells[18]. One of the genes induced after BRD7 knockdown was the Bmi-1 oncogene, required for self-renewal properties of stem cells (Fig. 1c). This potential function of BRD7 is in agreement with the suggested contribution of senescence to tissue aging, through exhaustion of renewable tissue stem cell populations[43-45].

BRD7: ANOTHER TUMOUR SUPPRESSOR IN THE SWI/SNF COMPLEX

P53-dependent induction of OIS leads to tumour regression and clearance[8,9]. Our data indicate that impairment of OIS by inhibition of BRD7 allows full neoplastic transformation of both human primary fibroblasts and mammary epithelial cells in the presence of wild-type p53 (ref. 16). Based on this, one could predict that reduced BRD7 expression would be beneficial for pre-tumorigenic cells still containing wild-type p53. Notably, BRD7 is encoded in a locus on chromosome 16q12 that is a well-known hotspot for LOH in breast cancers[46]. Highlighting the relevance of BRD7 for tumour suppression by p53, we found reduced BRD7 expression and deletion of the genomic locus hosting the *BRD7* gene specifically in a subset of breast tumours retaining wild-type *p53*, but not in tumours expressing mutant p53 (ref.16). Loss of BRD7 expression thus provides a means to inactivate the p53 pathway while retaining wild-type *p53*. We are currently analysing BRD7 expression and localization by immunohistochemistry (IHC) in normal breast tissues and in primary breast carcinomas of which the p53 status is known based on direct sequencing.

Further support for a tumour suppressive role for BRD7 in cancer was found in nasopharyngeal carcinoma (NPC). Low levels of BRD7 were found in these tumors[47,48], and the majority of NPC tumours have been shown to retain wild-type *p53* (ref. 49). Ectopic overexpression of BRD7 in NPC cells was associated with a proliferation arrest, and with altered expression of genes belonging to Ras/MEK/ERK and RB/E2F pathways[34]. In addition, a CpG island was recently identified in the *BRD7* gene, which is methylated in a large percentage of NPC tumors[48]. We are currently analysing the methylation status of this CpG island in primary breast tumour datasets, in order to establish whether next to deletion, methylation is also a mechanism to inactivate BRD7 in breast cancers.

Besides allowing pre-tumorigenic cells to grow while expressing activated oncogenes[16], BRD7 loss may have diverse consequences on the development of breast tumours. Transcriptional profiling and ChIP-sequencing of Ras^{V12}-expressing

cells containing BRD7 knockdown suggest that BRD7 regulates genes involved in cell metabolism, DNA repair, as well as putative suppressors of tumour aggressiveness and metastasis[16]. This possibly points to multiple layers of tumour suppression by BRD7. First, BRD7 appeared to be required for the expression of IRS-1 (Insulin receptor substrate 1), a protein that plays a key role in transmitting signals from the insulin and IGF-1 receptors to the PI3K/AKT and Erk/MAP kinase pathways. Suppression of IRS-1 has been reported to promote mammary tumour metastasis in mouse models, and IRS-1 has been frequently found inactivated in human metastatic breast tumors[50]. Second, the involvement of BRD7 in the activities of both p53 and BRCA-1. Somatic loss of BRCA1 and p53 in mice induces mammary tumours with features of human BRCA1-mutated basal-like breast cancer, exhibiting dramatic genomic instability[51]. Third, BRD7 loss could endow breast tumours with resistance to targeted treatments or affect the outcome of chemotherapy. This was recently demonstrated in cells lacking BRD7, where BRCA1-dependent expression of oestrogen receptor α is reduced. Consequently, cells were not affected by the treatment with fulvestrant[36]. In addition, cellular senescence contributes to the anti-tumour effect of some genotoxic drugs used for chemotherapy. Our data imply that BRD7 plays a role in p53-dependent transcription in cells treated with etoposide[16], thereby suggesting that BRD7 may also be involved in p53-dependent senescence in response to drug-induced DNA damage.

Finally, p63 and p73 share many protein partners with p53 (ref. 52), and it is then conceivable that BRD7 might also assist specific tumour suppressive functions of p53 family members[53,54]. In particular, it has been reported that TAp63 isoforms are robust mediators of Ras-induced senescence that prevent tumorigenesis in vivo in p53-nullizygous mice[55], moreover p63 has a crucial role in preventing invasiveness and metastasis of epithelial tumors[56]. It would then be interesting to investigate a possible role of BRD7 in these activities.

In addition to BRD7, several other subunits of chromatin-remodelling complexes have been implicated in human cancer (reviewed in ref. 57). Loss of BRG1, BRM or both was found in 10% to 20% of a range of tumour types, including breast and ovarian tumors[58-60]. SNF5 (Ini1/Baf47/Smarcb1), a core component of the SWI/SNF complex, is a potent tumour suppressor that is consistently lost, mutated or silenced by methylation in pediatric rhabdoid tumours[61,62].

CONCLUDING REMARKS

In this Extra View we discussed the requirement of BRD7 for the tumour suppressive functions of p53, BRCA1, and its role as a component of SWI/SNF chromatin-remodelling complexes. However, many unresolved issues remain. Several of them concern the potential regulation of BRD7 activity by oncogene expression. We have shown that the interaction of BRD7 with p53 increases upon expression of Ras^{V12}. It remains to be determined whether specific oncogene-induced

modifications of p53, BRD7 or both are responsible for this. Although we were not able to observe differences in BRD7 expression, stability or subcellular localization in response to Ras^{V12} expression, it is conceivable to assume that posttranslational modifications are involved in directing this widespread factor towards specific regulation of senescence-inducing targets. Indeed, we have observed that BRD7 can also bind to p53 target promoters that are not involved in OIS, such as some apoptotic promoters. However, it is not required for their expression upon p53 activation[16]. Can BRD7 then be a determinant for specificity? It has been shown that the presence of specific subunits within the BAF complex affects SWI/SNF target genes differentially, in some cases even antagonistically, determining their gene-specific mode of action[18]. It therefore seems likely that SWI/SNF complexes are involved in the regulation of specific p53 target genes in response to different cellular stresses, thereby determining cellular fate.

ACKNOWLEDGEMENTS

We acknowledge support from the Dutch Cancer Society (KWF), the European Young Investigator (EURYI) award, Centre for Biomedical Genetics (CBG) and The European research Council (ERC) to R.A., and from AIRC (Associazione Italiana per la Ricerca sul Cancro) and EC FP6 (contracts 503576 and 502963) to G.D.S.

REFERENCES

1. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature **436,** 720 – 724 (2005).
2. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature **436,** 725 – 730 (2005).
3. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, et al. Tumour biology: senescence in premalignant tumours. Nature **436,** 642 (2005).
4. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature **444,** 638 – 642 (2006).
5. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature **444,** 633 – 637 (2006).
6. Kortlever RM, Higgins PJ, Bernards R. Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. Nat. Cell Biol. **8,** 877 – 884 (2006).
7. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature **443,** 214 – 217 (2006).
8. Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, et al. Restoration of p53 function leads to tumour regression in vivo. Nature **445,** 661 – 665 (2007).
9. Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature **445,** 656 – 660 (2007).
10. Tang Y, Zhao W, Chen Y, Zhao Y, Gu W. Acetylation is indispensable for p53 activation. Cell **133,** 612 – 626 (2008).
11. Sykes SM, Mellert HS, Holbert MA, Li K, Marmorstein R, Lane WS, et al. Acetylation of the p53 DNA-binding domain regulates apoptosis induction. Mol. Cell **24,** 841 – 851 (2006).
12. Tang Y, Luo J, Zhang W, Gu W. Tip60-dependent acetylation of p53 modulates the decision between cell cycle arrest and apoptosis. Mol. Cell **24,** 827 – 839 (2006).
13. Knights CD, Catania J, Di Giovanni S, Muratoglu S, Perez R, Swartzbeck A, et al. Distinct p53 acetylation cassettes differentially influence gene-expression patterns

and cell fate. J. Cell Biol. **173,** 533 – 544 (2006).

14. Zhao Y, Lu S, Wu L, Chai G, Wang H, Chen Y, et al. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). Mol. Cell. Biol. **26,** 2782 – 2790 (2006).
15. Vousden KH, Prives C. Blinded by the Light: The Growing Complexity of p53. Cell **137,** 413 – 431 (2009).
16. Drost J, Mantovani F, Tocco F, Elkon R, Comel A, Holstege H, et al. BRD7 is a candidate tumour suppressor gene required for p53 function. Nat. Cell Biol. **12,** 380 – 389 (2010).
17. Peng C, Zhou J, Liu HY, Zhou M, Wang LL, Zhang QH, et al. The transcriptional regulation role of BRD7 by binding to acetylated histone through bromodomain. J. Cell. Biochem. **97,** 882 – 892 (2006).
18. Kaeser MD, Aslanian A, Dong MQ, Yates JR, 3rd, Emerson BM. BRD**7,** a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells. J. Biol. Chem. **283,** 32254 – 32263 (2008).
19. Staal A, Enserink JM, Stein JL, Stein GS, van Wijnen AJ. Molecular characterization of celtix-**1,** a bromodomain protein interacting with the transcription factor interferon regulatory factor 2. J. Cell. Physiol.**185,** 269 – 279 (2000).
20. Sun H, Liu J, Zhang J, Shen W, Huang H, Xu C, et al. Solution structure of BRD7 bromodomain and its interaction with acetylated peptides from histone H3 and H4. Biochem. Biophys. Res. Commun. **358,** 435 – 441 (2007).
21. Oh J, Sohn DH, Ko M, Chung H, Jeon SH, Seong RH. BAF60a interacts with p53 to recruit the SWI/SNF complex. J. Biol. Chem. **283,** 11924 – 11934 (2008).
22. Lee D, Kim JW, Seo T, Hwang SG, Choi EJ, Choe J. SWI/SNF complex interacts with tumor suppressor p53 and is necessary for the activation of p53-mediated transcription. J. Biol. Chem. **277,** 22330 – 22337 (2002).
23. Peterson CL, Workman JL. Promoter targeting and chromatin remodeling by the SWI/SNF complex. Curr. Opin. Genet. Dev. **10,** 187 – 192 (2000).
24. Hendricks KB, Shanahan F, Lees E. Role for BRG1 in cell cycle control and tumor suppression. Mol. Cell. Biol. **24,** 362 – 376 (2004).
25. Kang H, Cui K, Zhao K. BRG1 controls the activity of the retinoblastoma protein via regulation of p21CIP1/WAF1/SDI. Mol. Cell. Biol. **24,** 1188 – 1199 (2004).
26. Xu Y, Zhang J, Chen X. The activity of p53 is differentially regulated by Brm- and Brg1-containing SWI/SNF chromatin remodeling complexes. J. Biol. Chem. **282,** 37429 – 37435 (2007).
27. Naidu SR, Love IM, Imbalzano AN, Grossman SR, Androphy EJ. The SWI/SNF chromatin remodeling subunit BRG1 is a critical regulator of p53 necessary for proliferation of malignant cells. Oncogene **28,** 2492 – 2501 (2009).
28. Gevry N, Chan HM, Laflamme L, Livingston DM, Gaudreau L. p21 transcription is regulated by differential localization of histone H2A.Z. Genes Dev. **21,** 1869 – 1881 (2007).
29. Adams PD. Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. Gene **397,** 84 – 93 (2007).
30. Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, et al. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell **113,** 703 – 716 (2003).
31. Ye X, Zerlanko B, Zhang R, Somaiah N, Lipinski M, Salomoni P, et al. Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci. Mol. Cell. Biol. **27,** 2452 – 2465 (2007).
32. Strobeck MW, Knudsen KE, Fribourg AF, DeCristofaro MF, Weissman BE, Imbalzano AN, et al. BRG-1 is required for RB-mediated cell cycle arrest. Proc. Natl. Acad. Sci. USA **97,** 7748 – 7753 (2000).
33. Zhang HS, Gavin M, Dahiya A, Postigo AA, Ma D, Luo RX, et al. Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell **101,** 79 – 89 (2000).
34. Zhou J, Ma J, Zhang BC, Li XL, Shen SR, Zhu SG, et al. BRD**7,** a novel bromodomain gene, inhibits G1-S progression by transcriptionally regulating some important molecules involved in ras/MEK/ERK and Rb/E2F pathways. J. Cell. Physiol. **200,** 89 – 98 (2004).
35. Peng C, Liu HY, Zhou M, Zhang LM, Li XL, Shen SR, et al. BRD7 suppresses the growth of Nasopharyngeal Carcinoma cells (HNE1) through negatively regulating beta-catenin and ERK pathways. Mol. Cell. Biochem. **303,** 141 – 149 (2007).
36. Harte MT, O'Brien GJ, Ryan NM, Gorski JJ, Savage KI, Crawford NT, et al. BRD**7,** a subunit of SWI/SNF complexes, binds directly to BRCA1 and regulates BRCA1-dependent transcription. Cancer Res. **70,** 2538 – 2547 (2010).
37. Park JH, Park EJ, Lee HS, Kim SJ, Hur SK, Imbalzano AN, et al. Mammalian SWI/SNF complexes facilitate DNA double-strand break repair by promoting gamma-H2AX induction. EMBO J. **25,** 3986 – 3997 (2006).
38. Lee HS, Park JH, Kim SJ, Kwon SJ, Kwon J. A cooperative activation loop among SWI/

SNF, gamma-H2AX and H3 acetylation for DNA double-strand break repair. EMBO J. **29,** 1434 – 1445 (2010).
39. Zhao Q, Wang QE, Ray A, Wani G, Han C, Milum K, et al. Modulation of nucleotide excision repair by mammalian SWI/SNF chromatin-remodeling complex. J. Biol. Chem. **284,** 30424 – 30432 (2009).
40. MacLachlan TK, Takimoto R, El-Deiry WS. BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets. Mol. Cell. Biol. **22,** 4280 – 4292 (2002).
41. Ongusaha PP, Ouchi T, Kim KT, Nytko E, Kwak JC, Duda RB, et al. BRCA1 shifts p53-mediated cellular outcomes towards irreversible growth arrest. Oncogene **22,** 3749 – 3758 (2003).
42. Bochar DA, Wang L, Beniya H, Kinev A, Xue Y, Lane WS, et al. BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. Cell **102,** 257 – 265 (2000).
43. Campisi J. Senescent cells, tumor suppression and organismal aging: good citizens, bad neighbors. Cell **120,** 513 – 522 (2005).
44. Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature **443,** 421 – 426 (2006).
45. Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, et al. Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature **443,** 448 – 452 (2006).
46. Argos M, Kibriya MG, Jasmine F, Olopade OI, Su T, Hibshoosh H, et al. Genomewide scan for loss of heterozygosity and chromosomal amplification in breast carcinoma using single-nucleotide polymorphism arrays. Cancer Genet. Cytogenet. **182,** 69 – 74 (2008).
47. Yu Y, Zhang BC, Xie Y, Cao L, Zhou M, Zhan FH, et al. Analysis and molecular cloning of differentially expressing genes in nasopharyngeal carcinoma [in Chinese]. Acta Biochim. Biophys. Sin. **32,** 327 – 332 (2000).
48. Liu H, Zhang L, Niu Z, Zhou M, Peng C, Li X, et al. Promoter methylation inhibits BRD7 expression in human nasopharyngeal carcinoma cells. BMC Cancer **8,** 253 (2008).
49. Spruck CH, 3rd, Tsai YC, Huang DP, Yang AS, Rideout WM, 3rd, Gonzalez-Zulueta M, et al. Absence of p53 gene mutations in primary nasopharyngeal carcinomas. Cancer Res. **52,** 4787 – 4790 (1992).
50. Ma Z, Gibson SL, Byrne MA, Zhang J, White MF, Shaw LM. Suppression of insulin receptor substrate 1 (IRS-1) promotes mammary tumor metastasis. Mol. Cell. Biol. **26,** 9338 – 9351 (2006).
51. Liu X, Holstege H, van der Gulden H, Treur-Mulder M, Zevenhoven J, Velds A, et al. Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. Proc. Natl. Acad. Sci. USA **104,** 12111 – 12116 (2007).
52. Collavin L, Lunardi A, Del Sal G. p53-family proteins and their regulators: hubs and spokes in tumor suppression. Cell Death Differ., In press. (2010)
53. Flores ER, Sengupta S, Miller JB, Newman JJ, Bronson R, Crowley D, et al. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell **7,** 363 – 373 (2005).
54. Tomasini R, Tsuchihara K, Wilhelm M, Fujitani M, Rufini A, Cheung CC, et al. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. Genes Dev. **22,** 2677 – 2691 (2008).
55. Guo X, Keyes WM, Papazoglu C, Zuber J, Li W, Lowe SW, et al. TAp63 induces senescence and suppresses tumorigenesis in vivo. Nat. Cell Biol. **11,** 1451 – 1457 (2009).
56. Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, et al. A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell **137,** 87 – 98 (2009).
57. Reisman D, Glaros S, Thompson EA. The SWI/SNF complex and cancer. Oncogene **28,** 1653 – 1668 (2009).
58. Reisman DN, Sciarrotta J, Wang W, Funkhouser WK, Weissman BE. Loss of BRG1/BRM in human lung cancer cell lines and primary lung cancers: correlation with poor prognosis. Cancer Res. **63,** 560 – 566 (2003).
59. Fukuoka J, Fujii T, Shih JH, Dracheva T, Meerzaman D, Player A, et al. Chromatin remodeling factors and BRM/BRG1 expression as prognostic indicators in non-small cell lung cancer. Clin. Cancer Res. **10,** 4314 – 4324 (2004).
60. Glaros S, Cirrincione GM, Muchardt C, Kleer CG, Michael CW, Reisman D. The reversible epigenetic silencing of BRM: implications for clinical targeted therapy. Oncogene **26,** 7058 – 7066 (2007).
61. Versteege I, Sevenet N, Lange J, Rousseau-Merck MF, Ambros P, Handgretinger R, et al. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. Nature **394,** 203 – 206 (1998).
62. Sansam CG, Roberts CW. Epigenetics and cancer: altered chromatin remodeling via Snf5 loss leads to aberrant cell cycle regulation. Cell Cycle **5,** 621 – 624 (2006).

ENHANCER-BOUND P53 PRODUCES eRNAs AND AFFECTS LONG DISTANCE TRANSCRIPTION

4

Carlos Melo*, Jarno Drost*, Patrick J. Wijchers, Harmen van de Werken, Elzo de Wit, Ran Elkon, Joachim A. F. Oude Vrielink, Sonia A. Melo, Wouter de Laat and Reuven Agami

* These authors contributed equally to this work

Manuscript in preparation

ABSTRACT

The p53 tumour suppressor protein regulates transcription of genes involved in cell proliferation and survival. In addition to protein-coding genes, several non-coding RNA species were shown to be essential for proper functioning of the p53 pathway. We identified several genomic regions that are bound by p53 with high affinity. These regions carry all the features of enhancer domains, possess high enhancer activity and produce enhancer RNAs (eRNAs) in a p53-dependent manner. SiRNAs targeting the produced transcripts interfere with p53-dependent induction of distantly located genes and a cell cycle arrest, suggesting that eRNAs are functionally relevant. Furthermore, we identified multiple intra-chromosomal interactions between the enhancers and surrounding genes, correlating with p53-dependent transcription of these genes. We propose that p53 can affect target gene transcription by regulating the activity of enhancer domains and eRNA production.

Carlos Melo[1,5], Jarno Drost[1,5], Patrick J. Wijchers[2], Harmen van de Werken[2], Elzo de Wit[2], Ran Elkon[1], Joachim A. F. Oude Vrielink[1], Sonia A. Melo[3], Wouter de Laat[2] and Reuven Agami[1,4]

[1] Division of Gene Regulation, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands; [2] Hubrecht Institute-KNAW and University Medical Centre Utrecht, Uppsalalaan 8, 3584CT Utrecht, The Netherlands; [3] Beth Israel Deaconess Medical Centre, 330 Brookline Avenue, RW 759, 02115 Boston, MA, USA; [4] Centre for Biomedical Genetics, The Netherlands; [5] These authors contributed equally to this work.

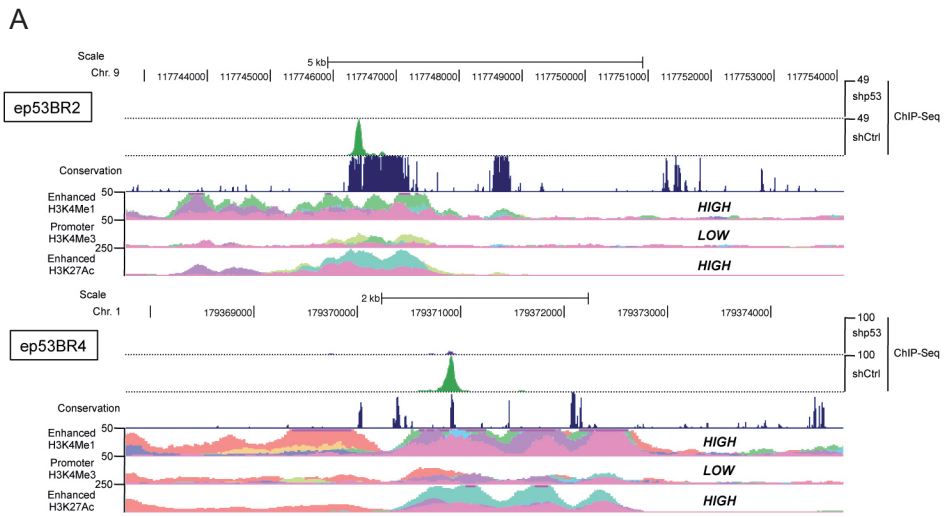
INTRODUCTION

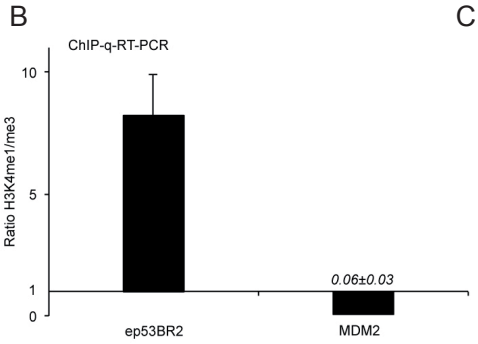
The p53 transcription factor is the most frequently mutated gene in cancer and plays a central role in suppression of tumorigenesis[1]. Chromatin-binding profiles reveal specific interactions of p53 with promoter regions of nearby genes, but also with distant locations that may act as enhancers of transcription. Enhancers are non-protein-coding domains that regulate transcription of genes at long distances that are characterized by specific chromatin signatures of histone methylation and acetylation patterns[2-4]. While p53 binding to promoter regions locally activates gene expression of one gene, enhancers may affect several distant genes in a tissue-dependent manner. Interestingly, RNA polymerase II (RNAPII) was shown to bind to a subset of enhancers and produce transcripts, defined as enhancer RNAs (eRNAs)[5-7]. The transcription of eRNAs was shown to positively correlate with mRNA levels of surrounding protein-coding genes[5]. However, it is unclear if eRNAs have any function in target gene transcription. Here we identified several genomic regions that are bound by p53 with high affinity and harbour all chromatin features of eRNA-producing enhancer domains. We demonstrate that these enhancer domains require p53 for their activity and that p53-dependent production of eRNAs affects transcription of distant genes. In addition, the enhancers appeared to intra-chromosomally interact with multiple surrounding genes, which correlated with p53-dependent transcription of these genes. Thus, our results imply that eRNA production at enhancers is required for long-distance transcription activation by enhancer-bound p53.

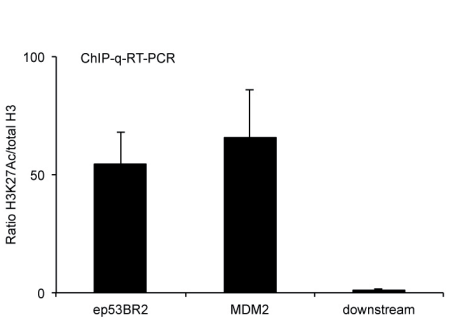
RESULTS

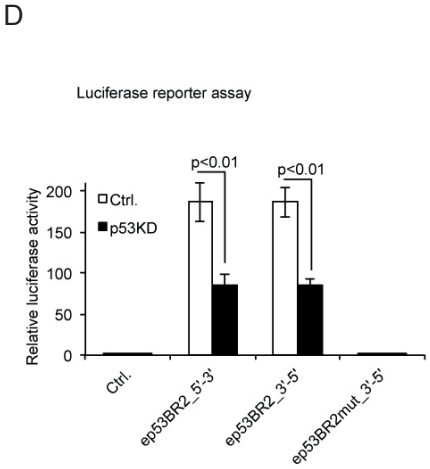
Genome-wide p53 binding analysis identifies p53-dependent enhancer domains

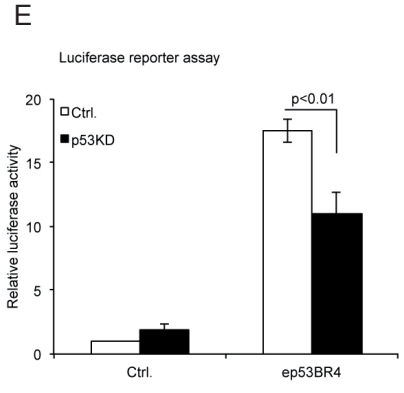
The recent observation that the thus-far known p53 target genes are not required for p53-dependent tumour suppression[8] made us search for novel p53 target genes. Specifically, we searched for putative enhancer regions that are bound by p53 and thus may require p53 for their activity on target gene promoters. To identify p53-bound enhancer domains we made use of publically available genome-wide p53-binding datasets[9,10] and ranked p53-bound regions based on peak height (Supplementary Information, Table S1). In addition to the presence of a significant p53-binding peak, we searched for regions that harbour histone modifications that have been shown to mark active enhancer regions. These include high levels of histone 3 lysine 4 monomethylation (H3K4me1), low levels of H3K4 trimethylation (H3K4me3) and high levels of H3K27 acetylation (H3K27Ac)[3,4,11]. Seven regions contained evident markers of enhancer activity and high affinity p53-binding (Fig. 1a; Supplemental Information, Fig. S1). Compared with p53 binding to the p21 (CDKN1A) promoter, a well-known high affinity p53 target gene, of the seven regions four harboured a p53-binding peak that was significantly higher, while the three others contained a comparable p53 peak (Fig. 1a and Supplemental Information, Fig. S1 and S2). We termed the identified p53-bound enhancers enhancer p53-binding region 1 – 7 (ep53BR1–7). To confirm that the histone


A
Scale
Chr. 9
5 kb
117744000
117745000
117746000
117747000
117748000
117749000
117750000
117751000
117752000
117753000
117754000
ep53BR2
49
shp53
49
shCtrl
ChIP-Seq
Conservation
Enhanced H3K4Me1
Promoter H3K4Me3
Enhanced H3K27Ac
HIGH
LOW
HIGH
Scale
Chr. 1
2 kb
179369000
179370000
179371000
179372000
179373000
179374000
ep53BR4
100
shp53
100
shCtrl
ChIP-Seq
Conservation
Enhanced H3K4Me1
Promoter H3K4Me3
Enhanced H3K27Ac
HIGH
LOW
HIGH


B
ChIP-q-RT-PCR
Ratio H3K4me1/me3
10
5
1
0
0.06±0.03
ep53BR2
MDM2


C
ChIP-q-RT-PCR
Ratio H3K27Ac/total H3
100
50
0
ep53BR2
MDM2
downstream


D
Luciferase reporter assay
Relative luciferase activity
200
150
100
50
0
Ctrl.
p53KD
p<0.01
p<0.01
Ctrl.
ep53BR2_5'-3'
ep53BR2_3'-5'
ep53BR2mut_3'-5'


E
Luciferase reporter assay
Relative luciferase activity
20
15
10
5
0
Ctrl.
p53KD
p<0.01
Ctrl.
ep53BR4

modifications observed in the genome browser are also present in our cell system, we performed chromatin immuno-precipitations (ChIPs) for H3K4me1, H3K4me3 and H3K27Ac on ep53BR2 in primary BJ fibroblasts immortalized by expression of hTERT (BJ/ET). Indeed, H3K4me1 was the predominant modification on ep53BR2 in combination with high levels of H3K27Ac (marking active genomic regions), whereas the promoter of the p53 target gene MDM2 harboured, as expected, predominantly H3K4me3 in combination with high levels of H3K27Ac (Fig. 1b, c). Similar results were obtained in MCF7 cells (data not shown).

Next, we determined whether ep53BR1–7 have p53-dependent enhancing activity using luciferase reporter assays in MCF7 cells. We cloned into luciferase reporter constructs the identified p53-bound regions and some additional flanking sequence (ranging from ~1.8 – ~ 2kb) in front of the SV40 promoter. Transfection of all reporters revealed significant enhancing activity compared to SV40 without enhancer sequence, ranging from about 4-fold to about 190-fold, whereas two control genomic regions (harbouring no enhancer features) did not show any activity (Fig. 1d, e and Supplemental Information, Fig. S3). As expected from an enhancer region, transcription activation was orientation-independent (Fig. 1d). Intriguingly, the enhancer activity was p53-dependent as co-transfection of a p53 knockdown vector ($p53^{KD}$) significantly inhibited enhancer activity, and mutating the p53-binding sites completely abolished the activity of ep53BR2 (Fig. 1d, e and Supplemental Information, Fig. S3). The fact that some enhancing activity was seen in cell transfected with $p53^{KD}$ suggests that residual p53 left in the cells was sufficient to provide some enhancer activity. Altogether, we identified seven p53-bound regions that harbour histone modification marks of enhancer domains and contain p53-dependent enhancer activity.

Ep53BR2 and ep53BR4 produce eRNAs in a p53-dependent manner

Recently, it was shown that RNA polymerase II (RNAPII) binds to a subset of enhancers where it bi-directionally transcribes so-called enhancer RNAs (eRNAs)[5]. In addition to the already mentioned high levels of H3K4me1, low levels of H3K4me3 and high levels of H3K27Ac, eRNA-producing enhancer domains were marked by p300/CBP binding[5]. Remarkably, we found significant RNAPII and

◀**Figure 1.** Identification of p53-dependent enhancer domains. (A) UCSC Genome Browser (hg18 assembly) presentation of the p53-binding pattern[10] and histone modifications at the ep53BR2 (top) and ep53BR4 (bottom) genomic regions. (B) BJ/ET cells were subjected to ChIP for total H3, H3K4me1 and H3K4me3. The abundance of the histone modifications within the indicated genomic regions was calculated as the ratio between the H3K4me1 and H3K4me3, both corrected to total H3. MDM2 was used as promoter control. Graphs show means and s.d. for three independent experiments. (C) Cells from (B) were subjected to ChIP with antibodies against total H3 and H3K27Ac. H3K27Ac abundance within the indicated genomic regions was calculated as the ratio between H3K27Ac and total H3. MDM2 was used as promoter control. Region downstream of ep53BR2 was used as negative control. Graphs show means and s.d. for three independent experiments. (D) MCF7 cells were co-transfected with the indicated reporter construct and either control or p53 knockdown (p53KD) vector. The relative firefly luciferase/renilla activity was determined and compared to the control promoter vector (Ctrl.). Graphs represent mean and s.d. from three independent experiments. *P* values were calculated using *t*-test. Mutant (mut) contains point mutations in the p53-binding motifs of ep53BR2. (E) As (D) but for ep53BR4.

p300 binding at ep53BR2 and ep53BR4 in different cell lines (UCSC genome browser; Fig. 2a) and confirmed this in BJ/ET cells by RNAPII ChIP (Fig. 2b). As RNAPII binds to ep53BR2 and 4, we set out to investigate whether p53 activation induces transcript production from these regions. We treated BJ/ET cells with the MDM2-inhibitor Nutlin-3 or left them untreated and performed quantitative RT-PCR with primers covering the p53-bound regions. This analysis revealed induction of transcripts at both loci upon Nutlin-3 treatment (Fig. 2c, d). Transcript production could also be induced upon ionizing radiation or Nutlin-3 treatment of MCF7 cells (containing wild-type p53), but not by Nutlin-3 treatment of MDA-MB-436 cells, which contain mutant p53 (Supplementary Information, Fig. S4 and Fig. S5), further confirming p53-dependent induction. Last, northern blot analysis verified p53-dependent transcript induction at ep53BR2, and revealed that the size is around 600 nucleotides (Fig. 2e). In conclusion, our observations indicate that ep53BR2 and ep53BR4 produce eRNAs in a p53-dependent manner.

Inhibition of eRNAs produced at ep53BR2 and ep53BR4 affects transcription of nearby genes

The production of eRNAs at enhancers has been shown to correlate with transcription of neighbouring genes[5]. The genes nearest to ep53BR2 and ep53BR4 are PAPPA (pregnancy-associated plasma protein A; at ~ 210 kb) and IER5 (immediate early response 5; at ~ 50 kb), respectively. Curiously, mRNA levels of both genes decreased (~2 and ~3 fold, respectively) upon knockdown of p53 in BJ/ET cells, as determined by 3'-end mRNA sequencing (Fig. 3a). In addition, Nutlin-3 treatment of BJ/ET cells induced p53-dependent transcription of both PAPPA and IER5 (Fig. 3b-d). These results imply that both genes are transcriptionally regulated by p53. However, no significant p53-binding peak can be detected in the promoters of PAPPA and IER5 by ChIP-seq in BJ/ET cells, arguing that p53 regulates their transcription either from a distant location or via an indirect route.

To determine if the eRNAs produced from ep53BR2 and ep53BR4 are involved in p53-dependent transcriptional regulation of PAPPA and IER5, respectively, we designed siRNAs targeting the eRNA transcripts that are produced form these regions (Supplemental Information, Fig. S6). We transfected cells with a negative control siRNA, two independent siRNAs targeting ep53BR transcripts, or a positive control siRNA targeting p53 and determined the relative induction of PAPPA and IER5 mRNA levels upon Nutlin-3 treatment. As expected, the transcription of PAPPA and IER5 was activated upon Nutlin-3 treatment. This induction significantly decreased when p53 was knocked down, confirming that the induction of transcription of these transcripts is p53-dependent (Fig. 3e, f, left panels). Intriguingly, transfection of either of two independent knockdowns targeting the eRNAs produced from ep53BR2 and ep53BR4 significantly inhibited the induction of PAPPA and IER5, respectively, upon Nutlin-3 treatment (Fig. 3e, f, left panels). In contrast, the induction of p21 following Nutlin-3 treatment was not significantly inhibited by eRNA knockdown, implying that the effect is specific for PAPPA and IER5 (Fig. 3e, f, right panels). These results indicate that eRNA

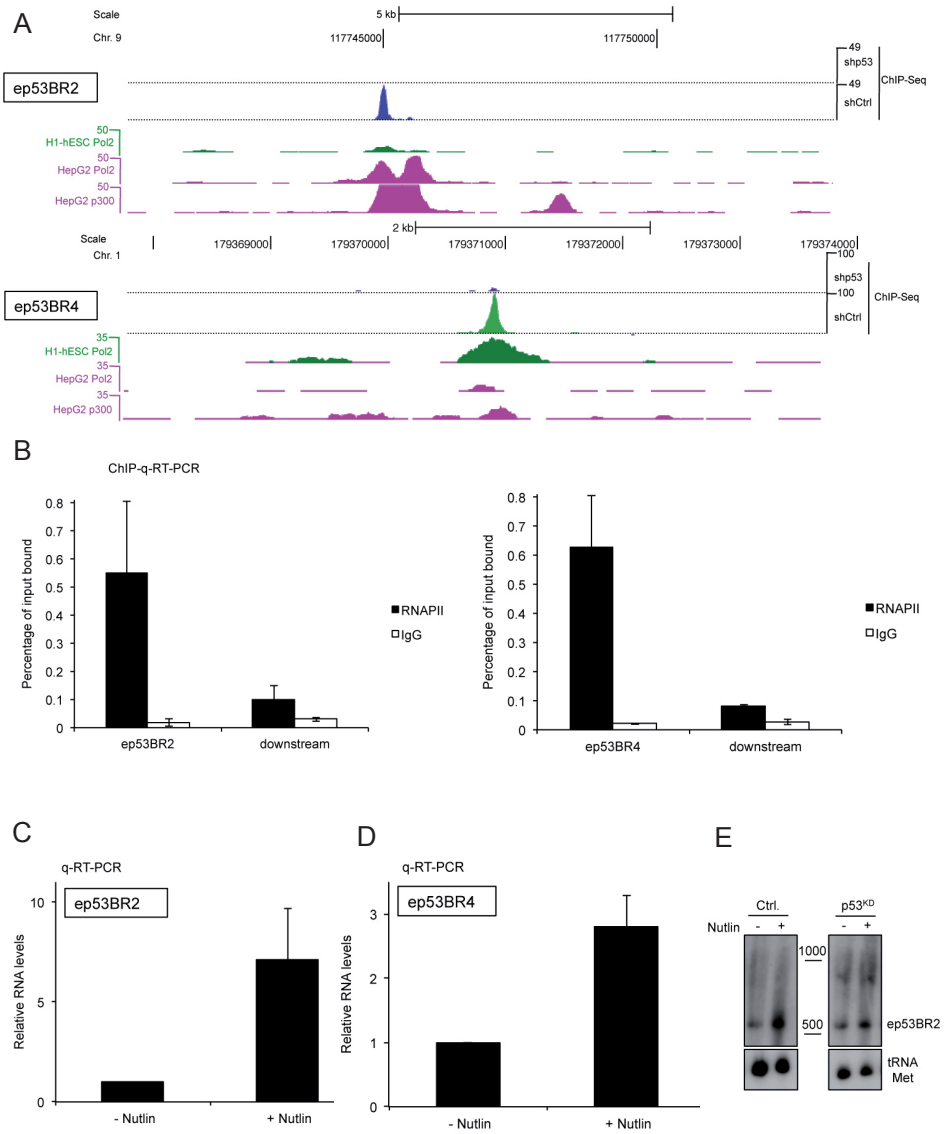
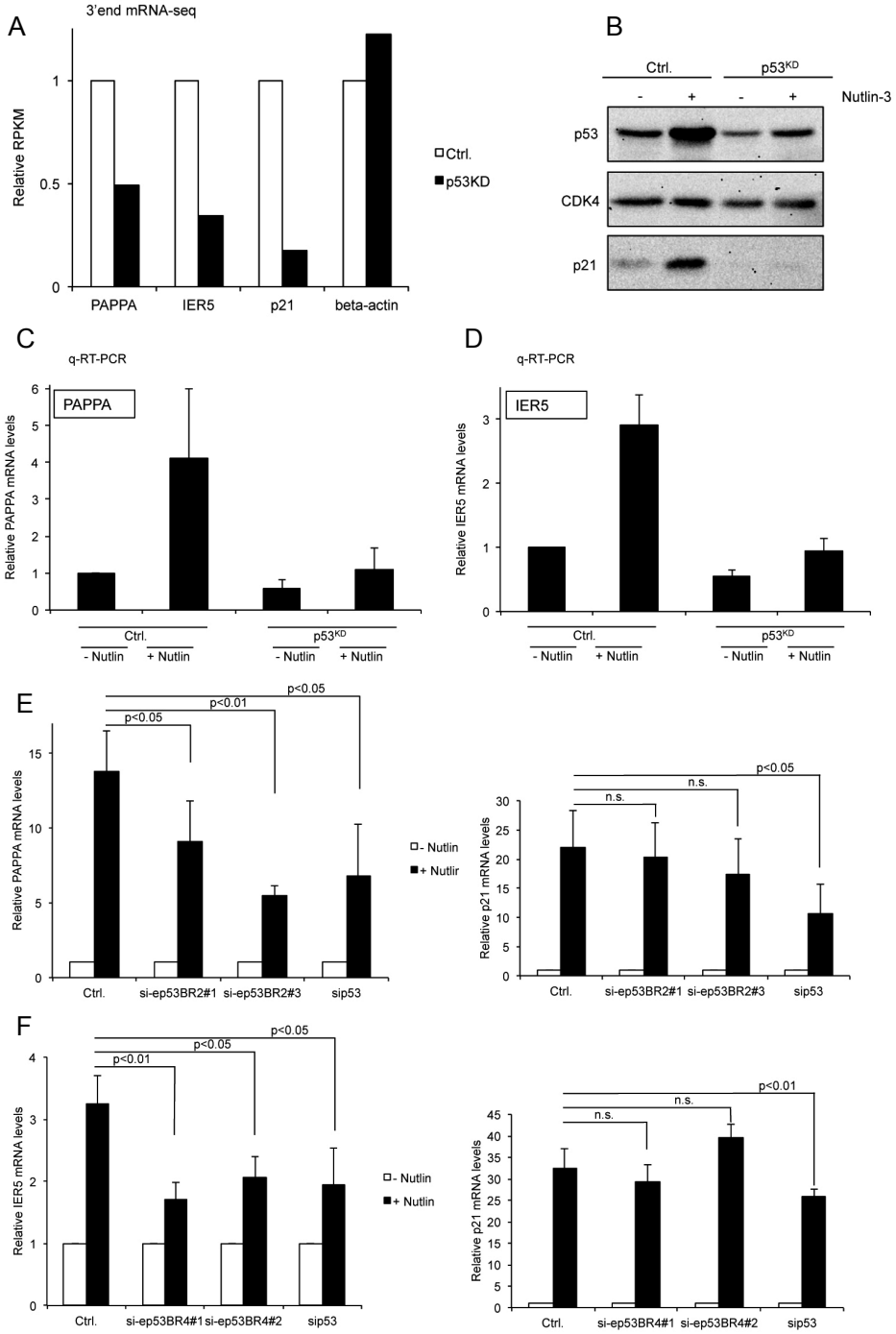


Figure 2. Ep53BR2 and ep53BR4 carry features of eRNA-producing enhancer domains and produce transcripts in a p53-dependent manner. (A) UCSC Genome Browser (hg18 assembly) presentation showing RNAPII and p300 binding at ep53BR2 (top) and ep53BR4 (bottom). (B) BJ/ET cells were cultured in the presence of Nutlin-3 for 24 h and subsequently subjected to ChIP for RNAPII. Rabbit IgG was used as negative control. Protein binding to the indicated genomic regions was quantified by calculating the percentage of input that is chromatin-bound. Graphs show means and s.d. for three independent experiments. (C) + (D) BJ/ET cells were treated with Nutlin-3 for 24 h or left untreated. The relative transcript levels produced at ep53BR2 (C) and ep53BR4 (D) were determined by quantitative RT-PCR in relation to GAPDH. Graphs show mean and s.d. for three independent experiments. (E) MCF7 cells expressing either a control or p53 knockdown vector were treated with Nutlin-3 for 24 h or left untreated. Northern blot analysis was performed to detect transcripts produced at ep53BR2. tRNA-Met was used as loading control.

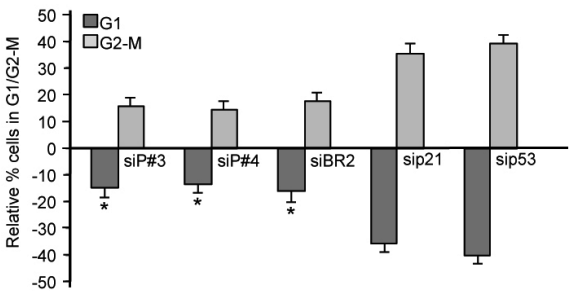

A
3'end mRNA-seq
Relative RPKM
1
0.5
0
PAPPA
IER5
p21
beta-actin
Ctrl.
p53KD
B
Ctrl.
p53KD
- + - +
Nutlin-3
p53
CDK4
p21
C
q-RT-PCR
PAPPA
Relative PAPPA mRNA levels
Ctrl.
p53KD
- Nutlin + Nutlin
D
q-RT-PCR
IER5
Relative IER5 mRNA levels
E
p<0.05
p<0.01
p<0.05
Relative PAPPA mRNA levels
- Nutlin
+ Nutlir
Ctrl.
si-ep53BR2#1
si-ep53BR2#3
sip53
p<0.05
n.s.
n.s.
Relative p21 mRNA levels
F
p<0.05
p<0.05
p<0.01
Relative IER5 mRNA levels
- Nutlin
+ Nutlin
Ctrl.
si-ep53BR4#1
si-ep53BR4#2
sip53
p<0.01
n.s.
n.s.
Relative p21 mRNA levels

production from ep53BR2 and ep53BR4 is required for the induction of PAPPA and IER5 transcription, respectively, following p53 activation.

ERNAs produced at ep53BR2 are involved in the efficient induction of a p53-dependent cell cycle arrest

Next, we set out to investigate if eRNA production is involved in a p53-dependent cell cycle arrest. Therefore, we transfected MCF7 cells with a control siRNA, one of two independent siRNAs targeting the eRNA transcripts, or a p53 siRNA. After treatment with ionizing radiation (IR), cell cycle profiles were analysed by propidium iodide staining. As expected, control transfected MCF7 cells entered a G1 arrest upon IR. This could be alleviated by knockdown of p53 or p21, confirming that the arrest is dependent on a functional p53 pathway. Interestingly, transfection of MCF7 cells with either one of the two siRNAs targeting ep53BR2-produced transcripts resulted in a significant decrease in the amount of cells arrested in G1 (Supplemental Information, Fig. S7a, b). Furthermore, similar to the knockdown of the ep53BR2-produced eRNAs, knocking down PAPPA significantly inhibited the p53-dependent cell cycle arrest as well (Fig. 3g and Supplemental

G



siRNA	G1	G2/M
siPAPPA#3	-14.73	15.47
siPAPPA#4	-13.47	14.23
si-ep53BR2	-16.40	17.33
sip21	-35.6	35.4
sip53	-39.97	39.30

Figure 3. P53-dependent eRNAs are required for transcription of genes lying in proximity of ep53BR2 and ep53BR4. (A) BJ/ET cells expressing either a control or p53 knockdown vector were subjected to 3'-end mRNA sequencing. Depicted are RPKM values as fold difference from control cells. (B) BJ/ET cells expressing either a control or p53 knockdown vector were treated with Nutlin-3 for 24 h or left untreated. Western blot analysis was performed to detect p53, p21 and CDK4 (loading control). (C) + (D) BJ/ET cells expressing either a control or p53 knockdown vector were treated with Nutlin-3 for 24 h or left untreated. Relative PAPPA (C) and IER5 (D) mRNA levels were measured by quantitative RT-PCR and is represented as fold induction from values measured in untreated control cells. Graphs represent mean and s.d. from three independent experiments. (E) BJ/ET (E) and MCF7 (F) cells were transfected with the indicated siRNAs and either treated with Nutlin-3 for 24 h or left untreated. Relative PAPPA (E, left panel), IER5 (F, left panel) and p21 (E, F, right panels) mRNA levels were measured by quantitative RT-PCR and is represented as fold induction from values measured in untreated cells. Graphs represent mean and s.d. from at least three independent experiments. *P* values were calculated using *t*-test. N.s., not significantly downregulated. (G) MCF7 cells were transfected with the indicated siRNAs and 24 h later treated with ionizing radiation (10 Gy). The next day cells were treated with nocodazole for 24 h. Cell cycle profiles were evaluated by propidium iodide staining and compared to cells transfected with control siRNA. Graphs represent mean and s.d. from three independent experiments. *P* values were calculated using *t*-test. * p value < 0.01

Information, Fig. S7c). Altogether, these results indicate that the eRNAs produced from ep53BR2 are required for an efficient p53-dependent cell cycle arrest and that this is likely to be due to inhibition of PAPPA induction by p53.

Ep53BRs interact with multiple surrounding genes that require p53 for their transcription

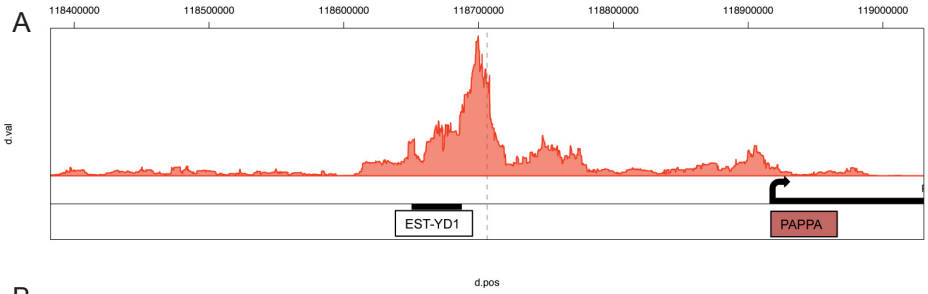
Enhancers control gene expression over a distance and in an orientation independent manner. This happens through so-called DNA looping, which brings the enhancer in close proximity of multiple target promoters. Thus, p53 could potentially regulate transcription of multiple target genes through binding to one enhancer region. To determine whether the identified p53-dependent enhancer domains intra-chromosomally interact with the putative target gene regions, we applied chromosome conformation capture on chip (4C) technology[12,13] for ep53BR2, ep53BR4 and ep53BR5 in BJ/ET cells (Fig. 4a, c, Supplemental Information, Fig. S8). Prominent interaction peaks could be detected nearby PAPPA and IER5 when scanning from ep53BR2 and ep53BR4, respectively (Fig. 4a, c). The interaction was not very local and seemed to spread over several thousands of bases. In addition, all three enhancers appeared to interact with other neighbouring genes. Indeed, 3'-end mRNA sequencing revealed that most of these genes require p53 for efficient expression (Fig. 4b, d, Supplemental Information, Fig. S8). These observations indicate that the identified p53 enhancer regions physically contact multiple distant genomic regions to convey p53-dependent transcription regulation.

DISCUSSION

We show that regulating gene transcription through distant enhancer domains is a mechanism by which p53 induces target gene transcription. We found several enhancer regions that are bound by p53 with high affinity, require p53 for their activity and produce eRNAs in a p53-dependent manner. We present evidence that eRNAs are required for gene transcription. Recently, enhancer-like features were also ascribed to a different class of ncRNAs[14]. Knockdown of these ncRNAs was demonstrated to result in decreased expression of neighbouring genes. In contrast to eRNAs[5], these ncRNAs are poly-adenylated and the regions encoding them harbour the chromatin signature of protein-coding genes (high H3K4me3 at 5´-end and downstream H3K36me3 (ref. 14)). Here we suggest for the first time that eRNAs are involved in target gene transcription as well. The suppression of p53-induced eRNAs resulted in decreased p53-dependent target gene induction. Additional research will be required to resolve how eRNAs induce target gene transcription.

In addition, we show that the identified enhancers physically interact with multiple distant protein-coding genes. Expression of these genes is indeed dependent on p53. As enhancers can interact and regulate multiple target genes (recently reviewed in ref. 15), enhancer-bound p53 would be able to regulate transcription of multiple target genes via one single binding site. We propose that

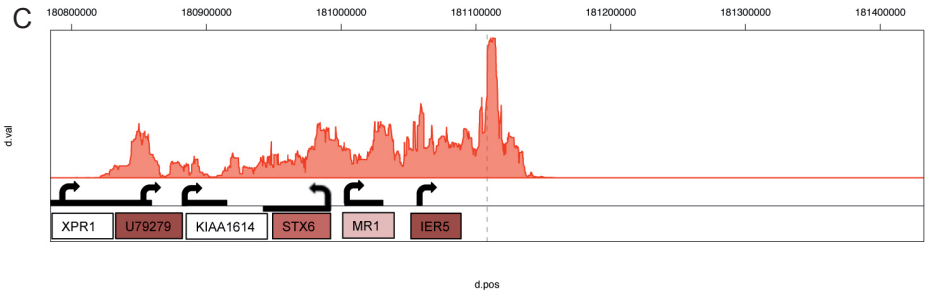
A



B

Chr.9	Ctrl.	p53KD
PAPPA	1	0.49
EST-YD1	n.e.	n.e.

C



D

Chr.1	Ctrl.	p53KD
IER5	1	0.35
MR1	1	0.89
STX6	1	0.69
KIAA1614	n.e	n.e.
XPR1	1	1
U79279	1	0.34

E

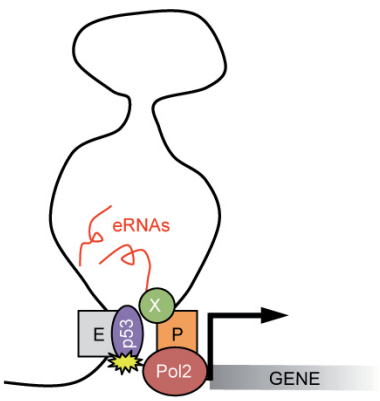


Figure 4. Ep53BRs intra-chromosomally interact with multiple distantly located genes. (A) Intra-chromosomal interactions of ep53BR2 with surrounding regions as determined by 4C analysed by trimmed mean analysis of deep sequencing data. (B) BJ/ET cells expressing either a control or p53 knockdown vector were subjected to 3'-end mRNA sequencing. Colour code depicts degree of p53-dependent regulation. Depicted are RPKM values as fold difference from control cells. N.e., not expressed. (C) Intra-chromosomal interactions of ep53BR4 with surrounding regions as determined by 4C analysed by trimmed mean analysis of deep sequencing data. (D) Same as (B). (E) Schematic model depicting how enhancer-bound p53 may regulate target gene transcription. X represents cofactors that might be required for activation of transcription and/or intra-chromosomal interactions. The involvement of eRNAs produced from the enhancers is under current investigation. Possibly, eRNAs interact with proteins that facilitate enhancer/promoter looping. E, enhancer; P, promoter.

enhancer-bound p53 can activate transcription of distant target genes by getting in close proximity of target gene promoters via intra-chromosomal interactions. Transcription of target genes is induced upon p53 activation, which requires p53-dependent eRNA production (Fig. 4e).

The genomic region including ep53BR2 is located at 9q33.1. This region is associated with loss of heterozygosity (LOH) in human cancer[16,17]. Based on the data we present in this report, it would be interesting to determine whether loss of ep53BRs contributes to human cancer.

METHODS

Cell culture, transfection and retroviral transduction

Cells were cultured in DMEM medium with 10% FCS and antibiotics. Transfection of MCF7 cells for reporter assays was performed with polyethylenimine. SiRNA transfections of BJ/ET and MCF7 cells were performed using Dharmafect 1 and 3 (Dharmacon), respectively. SiRNA sequences were as follows: ep53BR2#1 GCACAGATTCCGTGTAAAT; ep53BR2#3 GCTGGACACTGGGTAAATC; ep53BR4#1 TTAGGGGAAGCTGCTATGT; ep53BR4#2 CAGCCTTGTGGTTTCACAG; sip21 GACCATGTGGACCTGTCAC; sip53 GACTCCAGTGGTAATCTAC. SiRNAs targeting PAPPA were purchased from Dharmacon. Retroviruses were made by calcium phosphate transfection of Ecopack 2 cells (Clontech) and harvesting 40 and 64 h later.

Constructs and antibodies

pRetrosuper (pRS), pRS-blast, pRS-hygro, pBabe-puro-Ras^{V12}ER^{TAM}, pMSCV-blast-Ras^{V12}ER^{TAM} and pBabe-H2B-GFP-hTERT were described previously[18-21]. pRS-p53 was previously described[18]. To monitor enhancer activity the sequences of interest were cloned in pGL3-promoter luciferase reporter vectors (Promega). Primer sequences are shown in Supplementary Information, Table S2. Mutations in the p53-binding sequences of ep53BR2 were made in two steps using Quickchange Site-Directed mutagenesis kit (Agilent) according to manufacturer's instructions (primer 1, 5'-CTTCTGAGAAACTCATGGAGATTTCTGTGCATGCCTGAAC-3'; primer 2, 5'-ATGTCTGTGCATGCCTGAAATTCTCTGACAAAGCCAAGCA-3').

For ChIP antibodies against the following proteins were used: H3 (Cell Signaling Technology), H3K4me1 (Abcam, ab8895), H3K4me3 (Upstate, clone MC315), H3K27Ac (Abcam, ab4729) and RNAPII (Upstate, clone CTD4H8).

For Western blotting antibodies against p53 (DO1), p21 (F5) and CDK4 (C22) were purchased from Santa Cruz Biotechnology. Anti-beta Actin antibody was from Abcam (ab8227).

Quantitative RT–PCR, 3'-end mRNA sequencing and Northern blot

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was transcribed with Superscript III (Invitrogen) using random hexamers in accordance with the manufacturer's protocol. To determine mRNA expression levels, primers were designed with Primer Express v. 3.0 Software.

Real-time PCR was performed with SYBR Green PCR master mix (Applied Biosystems) and Chromo 4 Real Time PCR Detector (Bio-Rad Laboratories). Primer sequences are shown in Supplementary Information, Table S3.

3'-mRNA sequencing was performed according to the manufacturer's instructions (Illumina mRNA-seq). To sequence from the 3'-end, first strand cDNA synthesis was performed using oligo(dT) instead of random primers.

For Northern blot, 30 µg of total RNA (DNase treated) was run on a gel composed of 15 % polyacrylamide (19:1), 48% urea and 1x TBE. RNA was transferred to BrightStar-Plus Positively Charged Nylon Membranes (Ambion) at 200 mA for 1 h in 0.5x TBE. Subsequently, RNA was cross-linked to the membrane (1200 J for 1 min). Pre-hybridization was performed in ULTRAhyb®-Oligo solution (Ambion) for 1 h at 42°C. Hybridization was performed in ULTRAhyb®-Oligo solution (Ambion) containing 150 ng of biotin-tagged oligo (BioTeg; Sigma) by overnight incubation at 42°C. Next, membranes were washed in 2x SSPE/0.5% SDS (2x 15 min), 0.2x SSPE/0.5% SDS (2x 30 min) and 2x SSPE (1x 5 min). Signal detection was performed using BrightStar BioDetect Kit according to the manufacturer's instructions (Ambion). Probe sequence: 5'-CCCCACTTTCCACTGGGTCC-3'.

Luciferase reporter assays

MCF7 cells were co-transfected with 400 ng of pRS or pRS-p53^{KD} in combination with 100 ng luciferase reporter (pGL3-promoter; Promega) and 5 ng renilla plasmid. Three days after transfection, luciferase assays were performed in accordance with the manufacturer's instructions (Dual Luciferase system; Promega).

Chromatin immunoprecipitation assay

ChIP assays were performed as described[22] with some minor modifications. In brief, cells were cross-linked for 8 min with 1% formaldehyde and neutralized with 125 mM glycine. After centrifugation, the pellet was resuspended in lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) and sonicated to achieve an average fragment size of 300 – 800 basepairs. Chromatin was diluted in RIPA buffer (10 mM Tris pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1 % SDS, 0.1 % sodium deoxycholate) and incubated overnight with the indicated antibody. A negative control for immunoprecipitation was performed in the presence of normal rabbit IgG (Santa Cruz). The next day beads were added and incubated for 2 – 3 h. After washing, DNA was eluted overnight in elution buffer (20 mM Tris pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% SDS, 50 µg/ml proteinase K) followed by DNA purification using PCR purification kit (Roche). Real-time PCR was performed with SYBR Green PCR master mix (Applied Biosystems) and Chromo 4 Real Time PCR Detector (Bio-Rad Laboratories). Primers sequences are shown in Supplementary Information, Table S4.

Chromosome conformation capture on chip (4C)

4C templates were prepared as described previously[13]. In brief, at least 10^7 BJ/ET or MCF7 cells were harvested using trypsin-EDTA, and trypsin was quenched with FCS-containing medium. Cells were cross-linked with 2% formaldehyde for

10 min at room temperature, followed by quenching with glycine (125 mM final) and centrifugation for 8 min at 600 g (4°C). Cells were lysed in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1.0% Triton X-100, and nuclei were pelleted for 8 min at 600 x g (4°C). Nuclei were digested overnight with 400 U DpnII (NEB), followed by proximity ligation in 7 ml using 100 U T4 DNA ligase (Roche) overnight at 16°C. DNA circles were phenol-chloroform extracted and ethanol precipitated using glycogen as a carrier (20 μg/ml). DNA circles were further digested with 50 U Csp6I (Fermentas) overnight at 37°C, followed by heat-inactivation and subsequently ligated in 14 ml using 200 U T4 DNA ligase. Trimmed circles, the 4C template, were ethanol precipitated using glycogen as a carrier (20 μg/ml). 16 identical 50 μl PCR reactions were performed per viewpoint using the Expand Long Template PCR system (Roche) with 200 ng 4C template per PCR reaction. PCR conditions were as follows: 95°C for 2 min; 36 cycles of 95°C for 15 s, 55°C for 1 min and 68°C for 3 min; followed by a final step of 68°C for 7 min. All 16 PCR reactions were pooled and purified for next-generation sequencing using the Roche High Pure PCR Product Purification Kit.

4C primers carried additional 5' overhangs composed of adapter sequences for Illumina single read sequencing, and were sequenced on a GA-II or Hi-seq 2000 machine (Illumina).

REFERENCES

1. Vousden, K. H. and Lu, X. Live or let die: the cell's response to p53. Nature Rev. Cancer **2**, 594 – 604 (2002).
2. ENCODE Project Consortium, Birney, E., Stamatoyannopoulos, J.A., Dutta, A., Guigó, R., Gingeras, T.R., Margulies, E.H., Weng, Z., Snyder, M., et al., Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature **447**, 799 – 816 (2007).
3. Heintzman, N.D., Stuart, R.K., Hon, G., Fu, Y., Ching, C.W., Hawkins, R.D., Barrera, L.O., Van Calcar, S., Qu, C., Ching, K.A., Wang, W., Weng, Z., Green, R.D., Crawford, G.E. and Ren, B. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat. Genet. **39**, 311 – 318 (2007).
4. Heintzman, N.D., Hon, G.C., Hawkins, R.D., Kheradpour, P., Stark, A., Harp, L.F., Ye, Z., Lee, L.K., Stuart, R.K., Ching, C.W., Ching, K.A., Antosiewicz-Bourget, J.E., Liu, H., Zhang, X., Green, R.D., Lobanenkov, V.V., Stewart, R., Thomson, J.A., Crawford, G.E., Kellis, M. and Ren, B. Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature **459**, 108 – 112 (2009).
5. Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S., Markenscoff-Papadimitriou, E., Kuhl, D., Bito, H., Worley, P.F., Kreiman, G. and Greenberg, M.E. Widespread transcription at neuronal activity-regulated enhancers. Nature **465**, 182 – 187 (2010).
6. Wang, D., Garcia-Bassets, I., Benner, C., Li, W., Su, X., Zhou, Y., Qiu, J., Liu, W., Kaikkonen, M.U., Ohgi, K.A., Glass, C.K., Rosenfeld, M.G. and Fu, X.D. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. Nature **474**, 390 – 394 (2011).
7. De Santa, F., Barozzi, I., Mietton, F., Ghisletti, S., Polletti, S., Tusi, B.K., Muller, H., Ragoussis, J., Wei, C.L. and Natoli, G. A large fraction of extragenic RNA pol II transcription sites overlap enhancers. PLoS Biol. **8**, e1000384 (2010).
8. Brady, C.A., Jiang, D., Mello, S.S., Johnson, T.M., Jarvis, L.A., Kozak, M.M., Kenzelmann Broz, D., Basak, S., Park, E.J., McLaughlin, M.E., Karnezis, A.N. and Attardi, L.D. Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell **145**, 571 – 583 (2011).
9. 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. A global map of p53 transcription-factor binding sites in the human genome. Cell **124**, 207 – 219 (2006).
10. Drost, J., Mantovani, F., Tocco, F., Elkon, R., Comel, A., Holstege, H., Kerkhoven,

R., Jonkers, J., Voorhoeve, P.M., Agami, R. and Del Sal, G. BRD7 is a candidate tumour suppressor gene required for p53 function. Nat. Cell Biol. **12,** 380 – 389 (2010).

11. Creyghton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J., Hanna, J., Lodato, M.A., Frampton, G.M., Sharp, P.A., Boyer, L.A., Young, R.A. and Jaenisch, R. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc. Natl. Acad. Sci. USA **107,** 21931 – 21936 (2010).
12. Dekker, J., Rippe, K., Dekker, M. and Kleckner, N. Capturing chromosome conformation. Science **295,** 1306 – 1311 (2002).
13. Simonis, M., Klous, P., Splinter, E., Moshkin, Y., Willemsen, R., de Wit, E., van Steensel, B. and de Laat, W. Nuclear organization of active and inactive chromatin domains uncovered by chromosome conformation capture-on-chip (4C). Nat. Genet. **38,** 1348 – 1354 (2006).
14. Ørom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Notredame, C., Huang, Q., Guigo, R. and Shiekhattar, R. Long noncoding RNAs with enhancer-like function in human cells. Cell **143,** 46 – 58 (2010).
15. Ong, C.T. and Corces, V.G. Enhancer function: new insights into the regulation of tissue-specific gene expression. Nat. Rev. Genet. **12,** 283 – 293 (2011).
16. Schultz, D.C., Vanderveer, L., Buetow, K.H., Boente, M.P., Ozols, R.F., Hamilton, T.C. and Godwin, A.K. Characterization of chromosome 9 in human ovarian neoplasia identifies frequent genetic imbalance on 9q and rare alterations involving 9p, including CDKN2. Cancer Res. **55,** 2150 – 2157 (1995).
17. Callahan, G., Denison, S.R., Phillips, L.A., Shridhar, V. and Smith, D.I. Characterization of the common fragile site FRA9E and its potential role in ovarian cancer. Oncogene **22,** 590 – 601 (2003).
18. Voorhoeve, P. M. and Agami, R. The tumor-suppressive functions of the human INK4A locus. Cancer Cell **4,** 311 – 319 (2003).
19. Brummelkamp, T. R., Bernards, R. and Agami, R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell **2,** 243 – 247 (2002).
20. Kolfschoten, I.G., van Leeuwen, B., Berns, K., Mullenders, J., Beijersbergen, R.L., Bernards, R., Voorhoeve, P.M. and Agami, R. A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. Cell **121,** 849 – 858 (2005).
21. De Vita, G., Bauer, L., da Costa, V.M., De Felice, M., Baratta, M.G., De Menna, M. and Di Lauro, R. Dose-dependent inhibition of thyroid differentiation by RAS oncogenes. Mol. Endocrinol. **19,** 76 – 89 (2005).
22. Dahl, J.A. and Collas P. A rapid micro chromatin immunoprecipitation assay (micro-ChIP). Nat. Protoc. **3,** 1032 – 1045 (2008).

SUPPLEMENTARY TABLES AND FIGURES

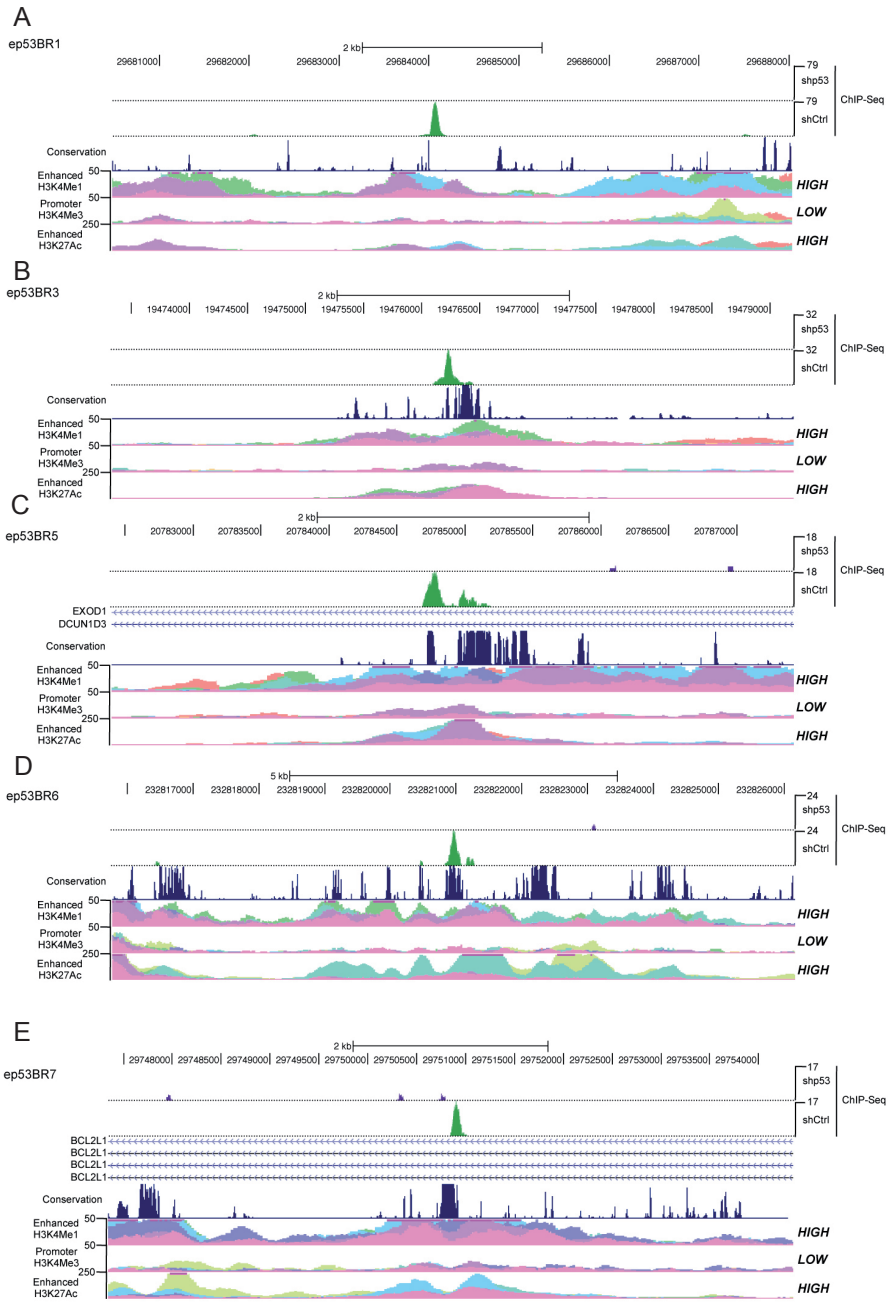


Figure S1. Identification of p53-dependent enhancer domains. (A) – (E) UCSC Genome Browser (hg18 assembly) presentation of the p53-binding pattern[10] and histone modifications around the indicated ep53BRs.

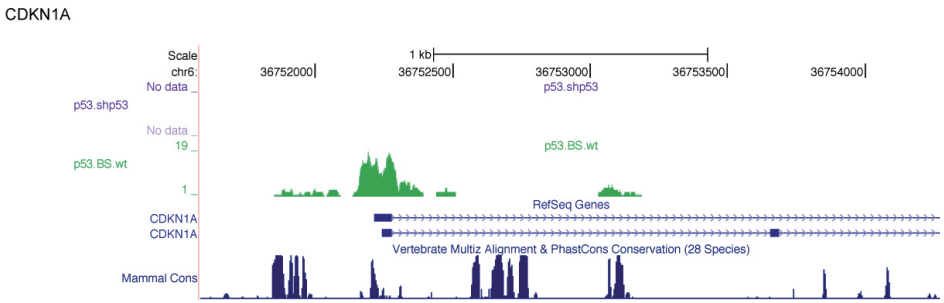


Figure S2. P53 has a high affinity for ep53BRs. UCSC Genome Browser (hg18 assembly) presentation of the p53-binding pattern at the high affinity p53 target gene p21 (CDKN1A).

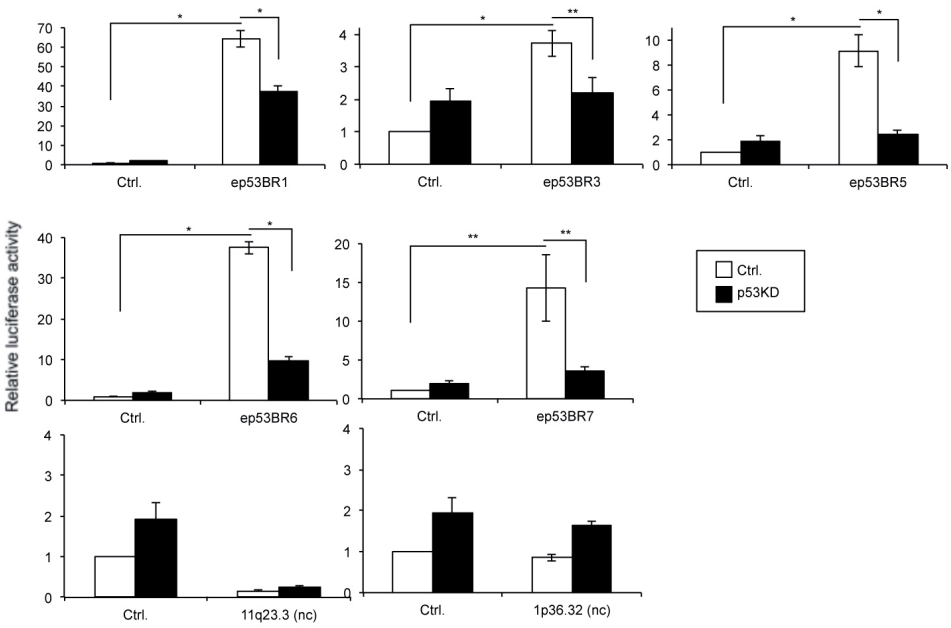


Figure S3. Identified ep53BRs have p53-dependent enhancer activity. MCF7 cells were co-transfected with the indicated reporter construct and either control or p53 knockdown (p53KD) vector. The relative firefly luciferase/renilla activity was determined and compared to the control promoter vector (Ctrl.). Graphs represent mean and s.d. from three independent experiments. *P* values were calculated using *t*-test. Graphs show means and s.d. for three independent experiments. * p value < 0.01, ** p value < 0.05.

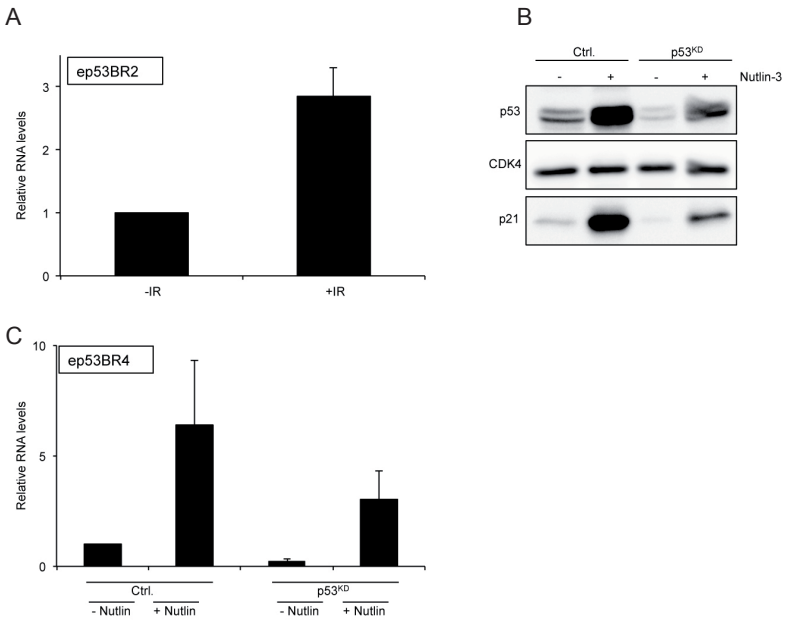


Figure S4. Ep53BR2 and ep53BR4 produce transcripts in a p53-dependent manner. (A) MCF7 cells were treated with ionizing radiation (10 Gy) or left untreated. After 8 h, the relative ep53BR2 transcript levels were determined by quantitative RT-PCR. Graphs show mean and s.d. for three independent experiments. (B) MCF7 cells expressing either a control or p53 knockdown vector were treated with Nutlin-3 for 24 h or left untreated and harvested. Western blot analysis was performed to detect p53, p21 and CDK4 (loading control). (C) Cells form (B) were treated with Nutlin-3 for 24 h or left untreated. The relative ep53BR4 transcript levels were determined by quantitative RT-PCR. Graphs show mean and s.d. for three independent experiments.

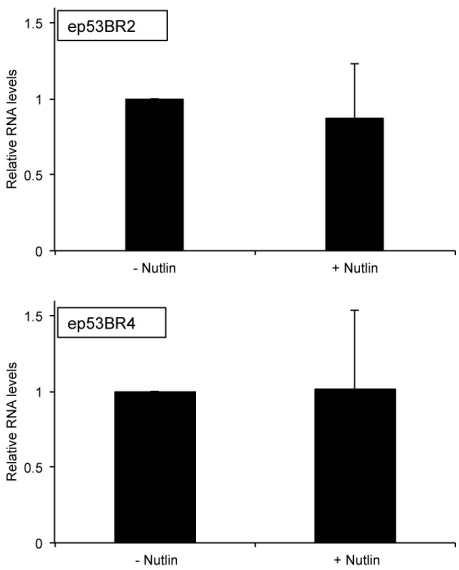


Figure S5. Ep53BR2 and ep53BR4 produce transcripts in a p53-dependent manner. MDA-MB-436 (mutant p53) were treated with Nutlin-3 for 24 h or left untreated. The relative ep53BR2 (top) and ep53BR4 (bottom) transcript levels were determined by quantitative RT-PCR. Graphs show mean and s.d. for three independent experiments.

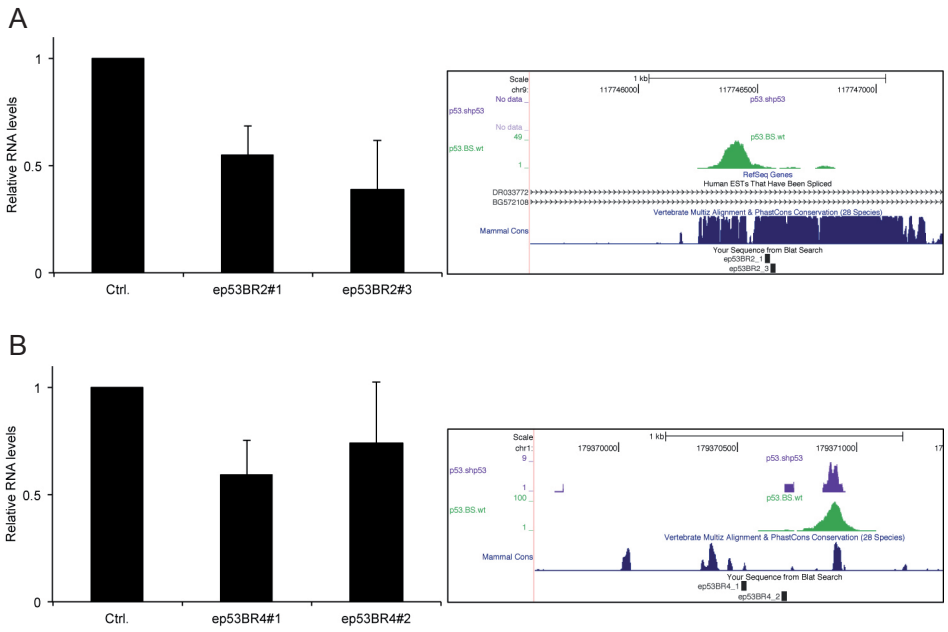
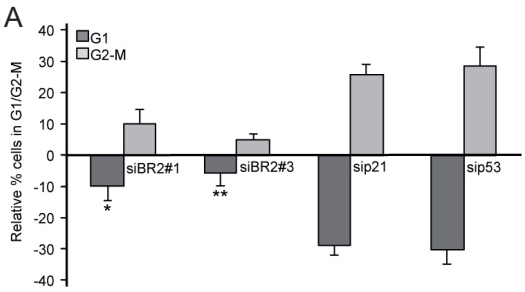


Figure S6. Ep53BR2 and ep53BR4 siRNA locations and efficiencies. Schematic representation of the targeting locations and siRNA efficiencies for ep53BR2 (A) and ep53BR4 (B).



siRNA	G1	G2/M
si-ep53BR2#1	-10.15	9.8
si-ep53BR2#3	-5.98	4.7
sip21	-28.8	25.8
sip53	-30.2	28.3

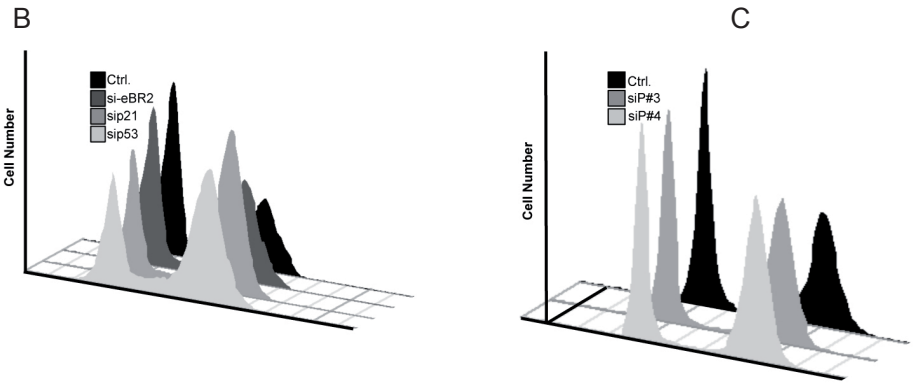
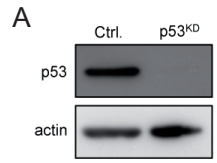
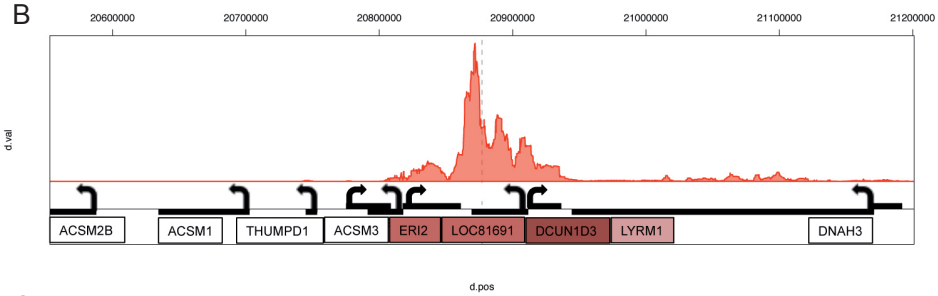


Figure S7. ERNAs produced from ep53BR2 are required for p53-dependent cell cycle arrest. MCF7 cells were transfected with the indicated siRNAs and 24 h later treated with ionizing radiation (10 Gy). The next day cells were treated with nocodazole for 24 h. Cell cycle profiles were evaluated by propidium iodide staining and compared to cells transfected with control siRNA. (A) Graphs represent mean and s.d. from three independent experiments. *P* values were calculated using *t*-test. * p value < 0.01, ** p value < 0.05. (B) FACS plots of the experiment described in Fig. S7a. (C) FACS plots of the PAPPA siRNAs used in the experiment described in Fig. 3g.





C

Chr.16	Ctrl.	p53KD
ERI2	1	0.64
ACSM3	n.e.	n.e.
DCUN1D3	1	0.24
LYRM1	1	0.74
DNAH3	n.e.	n.e.
THUMPD1	1	1.09
LOC81691	1	0.67
ACSM1	n.e.	n.e.
ACSM2B	n.e.	n.e.

Figure S8. Ep53BRs intra-chromosomally interact with multiple distantly located genes. (A) BJ/ET cells expressing either a control or p53 knockdown vector were subjected to Western blot analysis to determine p53 expression levels. Actin was used as loading control. (B) Intra-chromosomal interactions of ep53BR5 with surrounding regions as determined by 4C analysed by trimmed mean analysis of deep sequencing data. (C) BJ/ET cells expressing either a control or p53 knockdown vector were subjected to 3'-end mRNA sequencing. Colour code depicts degree of p53-dependent regulation. Depicted are RPKM values as fold difference from control cells. N.e., not expressed.

Table S1. Top 20 p53-binding peaks identified using p53 ChIP-seq[10]

Rank #	chromosome	start	end	Neighbouring gene	ep53BR
1	chr8	29683912	29684200	DUSP4	1
2	chr9	117746224	117746551	PAPPA	2
3	chr4	77922127	77922401		
4	chr4	76030785	76031048		
5	chr3	154815119	154815393		
6	chr5	52822299	52822583		
7	chr12	19476074	19476349	PLEKHA5	3
8	chr1	179370714	179371060	IER5	4
9	chr3	196389658	196389937		
10	chr6	36752063	36752408	**CDKN1A**	
11	chr16	20784627	20784882	DCUN1D3	5
12	chr4	123938821	123939097		
13	chr8	126270765	126270982		
14	chr17	52149897	52150142		
15	chr1	232820805	232821097	IRF2BP2	6
16	chr10	89592873	89593111		
17	chr13	113573373	113573719		
18	chr4	157912082	157912296		
19	chr20	29750782	29750998	BCL2L1	7
20	chr1	142722044	142722314		

Table S2. Sequences of cloning primers.

Region	Forward (5′-3′)	Reverse (5′-3′)
ep53BR1	GTCTAAAAAGTACCTTTACAGC	GATTAATCCATTGATGAGGGAAGAG
ep53BR2	CTAGTATATGAACTGTTATTGTCC	TTTTTTAGGTAGAATTTTCCAGC
ep53BR3	GAAAGGAGGGCTGGTTGCTTTATTTT-GGCTGAAGGCC	CCTTCTGGGAGTCCCAGAAG-GGCAAGGGTCTCTCAC
ep53BR4	GCTCACGCCTGTAATCTCAGCACTTTG	CTTGTGGCCAACTATGACACTCCAG
ep53BR5	CCAAAAATAAATAAATAAAAATGC	TTGCTGATTGATCATCTGTTG
ep53BR6	CGTGTCTGCTTGGTTTGGGCTC	CTCTGAGCAATTTCTCAATAAAAC
ep53BR7	CGAGTAGCTGGGAATACAGGT-GCGAAAC	GAACTCTTACTTTAATGGGAGAGACAG

Table S3. Sequences of primers used for quantitative RT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
ep53BR2	CCAGTGGAAAGTGGGGGAGT	CCTGAAATCTCTGCTTGGCTTTG
ep53BR4	TGGCACTGGGCTTAGGTCTTTT	CCCACAAGGGCTCTCAAGTTC
p21	TACCCTTGTGCCTCGCTCAG	GAGAAGATCAGCCGGCGTTT
PAPPA	TGCCGAGAGAATAAGCACAAGG	GGTGGAGGTGGGTCACAGG
IER5	CGGGGAGGGAGGGACTTTAC	AGGTGGGGAGGGACAGAAGG

Table S4. Sequences of primers used for ChIP.

Region	Forward (5′-3′)	Reverse (5′-3′)
ep53BR2	GTTACAAATACGGGAGCTGGAC	AAAGGCCAGGGAATGTAGACTG
Downstream ep53BR2	CTAGAACTTTGTCTGATGTGGTCAA	GGACAGCTCTTTATTCCATTATGTC
ep53BR4	ACTCTTAAACAGCCTTGTGGTTTC	CAGAAACACCCCACTACAGTCTC
Downstream ep53BR4	AAGTAGAGTCCTGGGAGAAACAGA	GGCCCTAGACAAACATCTAAAGAAC
MDM2	GGGCTATTTAAACCATGCATTTTC	GTCCGTGCCCACAGGTCTA

5

THE MIR-17-92 CLUSTER IS INVOLVED IN NEOPLASTIC TRANSFORMATION OF HUMAN PRIMARY CELLS

Jarno Drost, P. Mathijs Voorhoeve and Reuven Agami

ABSTRACT

Malignant transformation of non-tumorigenic cells involves multiple genetic and epigenetic alterations. Cells have to bypass several intrinsic defence mechanisms to become a full-blown tumour cell. MicroRNAs are a class of small endogenous non-coding RNAs that have been implicated in tumorigenesis. Here we describe the involvement of the oncogenic miR-17-92 cluster in transformation of human primary cells. We show that expression of this microRNA cluster hampers the retinoblastoma (pRB) tumour suppressor pathway. Furthermore, cluster expression can replace loss of $p16^{INK4A}$, an important component of the pRB pathway, in cellular transformation. Thus, the miR-17-92 microRNA cluster is involved in the malignant transformation of human primary cells.

Jarno Drost[1], P. Mathijs Voorhoeve[1] and Reuven Agami[1,2]

[1] Division of Gene Regulation, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands; [2] Centre for Biomedical Genetics, The Netherlands.

INTRODUCTION

MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs that regulate gene expression. Regulation mainly occurs through association of the so-called mature miRNA with the 3'-untranslated region (3'UTR) of protein-coding genes and subsequent inhibition of translation and/or transcript destabilization[1]. MiRNAs have been implicated in a large variety of cellular processes. Proper regulation of their expression is therefore essential and deregulation has been linked to tumorigenesis. MiRNAs can function both as oncogenes and tumour suppressor genes. Oncogenic properties have, for example, been ascribed to the polycistronic miR-17-92 cluster. The locus encoding miR-17-92 is frequently amplified in lymphomas and lung cancers[2-4]. Furthermore, it was shown that overexpression of this cluster of miRNAs in the Eμ-myc transgenic mouse model of B cell lymphoma dramatically accelerated the onset and progression of the disease[5]. Since then, many reports confirmed the oncogenic potential of the miR-17-92 cluster (reviewed in ref. 6).

The miR-17-92 cluster encodes six miRNAs, namely miR-17, miR-18a, miR-20a, miR-19a, miR-19b and miR-92a. Based on their seed sequence the miR-17/miR-20a and miR-19a/miR-19b families can be distinguished. The exact role of the individual miRNAs in the oncogenicity of the complete cluster has not yet been satisfactorily elucidated (reviewed in ref. 7). All miRNAs in the cluster have been shown to target at least one tumour suppressor gene. In addition, it has been shown that all cluster miRNAs participate in repression of the TGFβ pathway, implying that cooperation between miRNAs may occur[8].

Tumorigenesis is a multistep process involving the inactivation of tumour suppressor genes and activation of oncogenes. *In vitro*, expression of the telomerase reverse transcriptase subunit (hTERT), the Ras^{V12} oncogene and SV40 small t in combination with inhibition of the p53 and pRB tumour suppressor pathways is sufficient to transform human primary fibroblasts into tumour cells[9,10]. This model was used before in our lab to study the oncogenic role of miR-372&3 in oncogene-induced senescence (OIS)[11].

In this study, we used a miRNA expression library that was designed in our lab to screen for miRNAs that are involved in OIS. We identified a role for miR-17, part of the miR-17-92 cluster, in oncogene-induced transformation. We show that expression of 5 miRNAs of the miR-17-92 cluster (miR-17-19) results in an agonistic effect, implying that these miRNAs cooperate to reach their full oncogenic potential. Finally, the miRNAs in this cluster hamper the pRB pathway and their expression allows the transformation of human primary cells.

5

RESULTS

A genetic screen reveals miR-17&18 involvement in the oncogene-induced stress response

To identify miRNAs that are involved in the oncogene-induced stress response, we screened a miRNA-expression library (containing 320 miRNAs) in primary human BJ fibroblasts expressing hTERT and 4-OH-Tamoxifen (4-OHT)-inducible oncogenic H-Ras^{V12} (BJ/ET/Ras^{V12}ER). To reduce the amount of false-positive hits we transduced BJ/ET/Ras^{V12}ER cells with single miRNA-expressing constructs instead of the pooled library. After selection, cells were pooled and cultured for three weeks in the absence or presence of 4-OHT. MiRNA abundance in the Ras^{V12}-expressing population was compared to the untreated population by hybridisation to a miRNA-microarray[11]. Next to the previously identified miR-372&373 (ref. 11) and their relative miR-302, other miRNA-expressing constructs were reproducibly enriched in two independent experiments. Using growth competition assays, the effect could be validated for two of these. Remarkably, both vectors contained and expressed the same miRNAs: miR-17&18 (Fig. 1a and 1b). Expression of the miRNAs was determined using RNase protection assay (RPA; Fig. 1c). These results point to a role for miR-17&18 in the cellular stress response elicited by oncogene activation.

MiR-17-19 – induced growth advantage is independent of the p53 pathway

Proper activation of the p53 tumour suppressor pathway is essential to prevent oncogene-induced transformation of primary BJ fibroblasts[10,12]. To determine if miR-17&18 expression interferes with this pathway, we performed growth competition assays in BJ/ET cells expressing both Ras^{V12} and p53^{KD}. Indeed, additional knockdown of p53 in these cells does not result in a growth advantage, confirming that they lack functional p53 (Fig. 2a). However, expression of miR-17&18 conferred a similar growth advantage to p53^{KD} cells as to control cells (Fig. 1b and 2a). Additionally, this growth advantage was only apparent in BJ/ET cells expressing Ras^{V12} (data not shown). This implies that these miRNAs give a p53-independent proliferation advantage in the presence of Ras^{V12}.

MiR-17&18 are part of the miR-17-92 cluster that, in addition to miR-17&18, contains the miR-17 family member miR-20a, but also miR-19a, -19b and -92 (Fig. 2b). Multiple oncogenic activities have been ascribed to this cluster (reviewed in ref. 6). To determine the effect of miR-17-92 expression on cell proliferation we performed growth competition assays in BJ/ET cells expressing p53^{KD}, Ras^{V12} and a vector expressing all the miRNAs in the cluster (Fig. 2c). Remarkably, miR-17-92 expression gave a significantly increased growth advantage compared to expression of miR-17&18 (Fig. 2a). This effect was similar to knockdown of p16^{INK4A}, which also gives a p53-independent growth advantage[10]. Sequence analysis of the miR-17-92 expression vector revealed a mutation in the miR-92 seed sequence, indicating that miR-92 is probably not involved in the observed effect. To identify the functional miRNA(s) in the cluster, we designed vectors expressing the separate miRNAs or several combinations of them. However,

A

miRNA	Ratio array 1	Ratio array 2	Average
miR-371	3.38	2.99	3.19
miR-302	1.46	2.53	2.00
miR-372	1.93	1.89	1.91
miR-17&18 (c2)	1.87	1.67	1.77
miR-17&18 (c1)	1.88	1.64	1.76

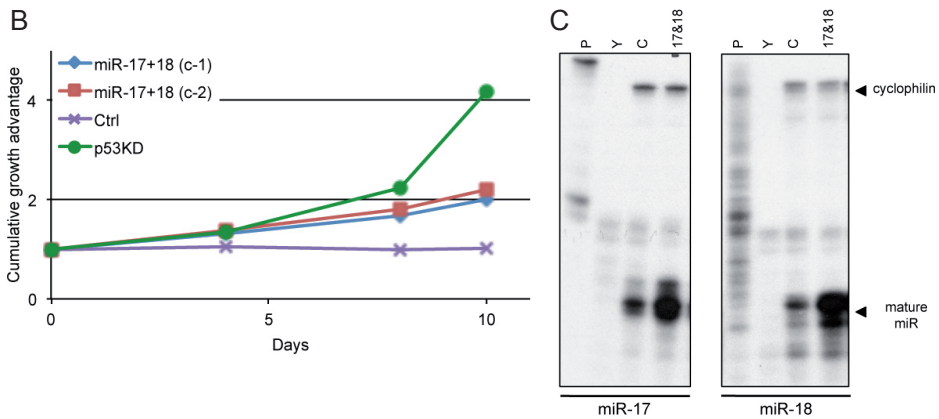


Figure 1. MiR-17&18 are involved in oncogene-induced senescence. (A) Table showing the enriched miRNAs in the Ras^{V12}-expressing population in two independent experiments. In black the previously identified miRNAs[11]. Depicted in red are the newly identified miRNAs. (B) Growth competition assays of BJ/ET/Ras^{V12}ER cells transduced with the indicated vectors and cultured in the presence of 4-OHT (+Ras^{V12}). (C) To check proper miRNA expression from the constructs identified in (A), RPA was performed on RNA from HEK293T cells transfected with either miRVec-Ctrl. (C) or miRVec-17&18. Cyclophilin was used as loading control. P = 10% input probe; Y = yeast control RNA.

expression of none of the combinations conferred a growth advantage to cells similar to expression of the complete cluster (Supplemental Information, Fig. S1), indicating that simultaneous expression of all miRNAs is required for the most efficient effect.

Potential role for miR-17-19 in the pRB pathway

The retinoblastoma (pRB) pathway is another tumour suppressor pathway that prevents the neoplastic transformation of primary cells. Oncogene activation induces the cyclin-dependent kinase inhibitor $p16^{INK4A}$, which inhibits the cyclin D/CDK4 complex from phosphorylating pRB. Hypo-phosphorylated pRB binds E2F and represses transcription of its target genes, which are required for cell cycle progression (ref. 13; Fig. 4). Since expression of miR-17-19 renders Ras^{V12}-expressing cells with a p53-independent growth advantage (Fig. 2a), we set out to investigate if cluster expression affects the pRB pathway. Therefore, we analysed the phosphorylation status of pRB and the activation of the E2F target

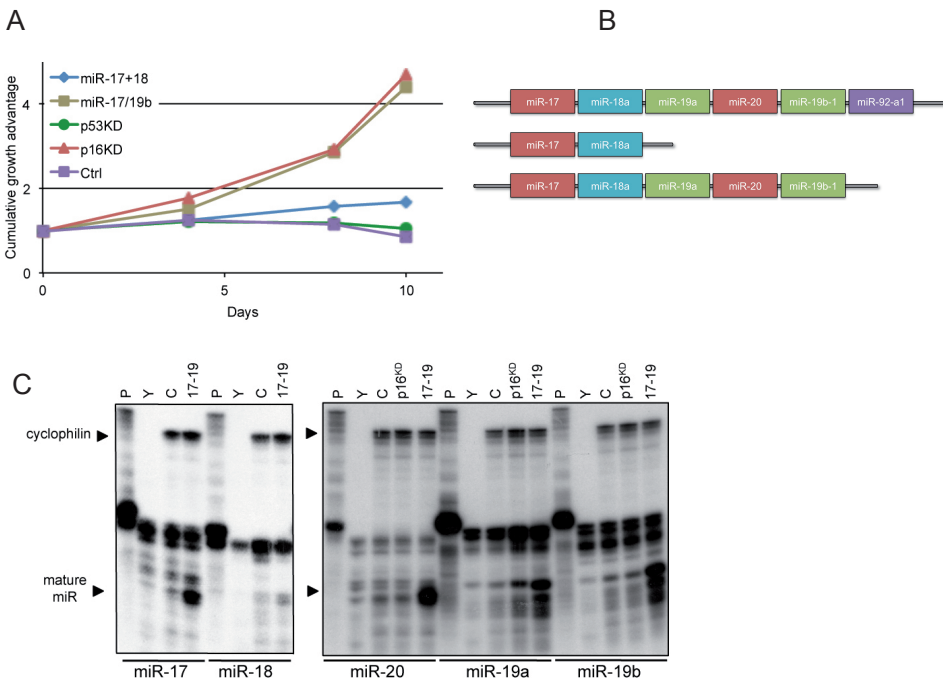


Figure 2. Agonistic p53-independent growth advantage by miR-17-19 expression. (A) Growth competition assays of BJ/ET/Ras^{V12}ER/p53^{KD} cells transduced with the indicated vectors and cultured in the presence of 4-OHT (+Ras^{V12}). (B) Schematic representation of the miR-17-92 cluster and the main constructs used in this study. Family members are depicted in the same colors. Top, miR-17-92; middle, miR-17&18; bottom, miR-17-19. (C) RPA was performed on RNA from HEK293T cells transfected with the indicated vectors. Cyclophilin was used as loading control. P = 10% input probe; Y = yeast control RNA; C = control.

gene cyclin A in normally proliferating and Ras^{V12}-expressing BJ/ET cells. Indeed, Ras^{V12} expression reduced the amount of phosphorylated pRB and cyclin A levels in control cells (Fig. 3a). Interestingly, primary cells expressing Ras^{V12} and the miR-17-19 cluster retained pRB in a hyper-phosphorylated state (Fig. 3a). In addition, cyclin A levels did not decrease upon Ras^{V12} expression confirming that E2F remains transcriptionally active (Fig. 3a).

Senescence is an irreversible growth arrest that can be induced by several cellular stress responses, like oncogene expression[14]. There are several markers used to identify senescent cells, including the formation of senescence-associated heterochromatin foci (SAHF). The formation of SAHF is dependent on a functional pRB pathway[15]. To further confirm that miR-17-19 expression interferes with the pRB pathway, we investigated the formation of SAHF in BJ/ET cells expressing p53^{KD} and Ras^{V12}. Lacking functional p53, these cells bypass OIS. Typically, although proliferating, SAHF could still be detected in these cells, implying that these foci are not functionally involved in senescence induction. This observation was recently confirmed by Di Micco et al., showing that proliferating oncogene-expressing

cells still display SAHF[16]. Still, a significant reduction in the amount of SAHF-positive cells was observed in cells expressing $p16^{INK4A-KD}$, confirming that the pRB pathway is involved in SAHF formation. Similar to knockdown of $p16^{INK4A}$, miR-17-19 expression also inhibited the formation of SAHF in Ras^{V12}-expressing BJ/ET/$p53^{KD}$ cells (Fig. 3b). Altogether, these results suggest that cluster expression interferes with proper functioning of the pRB pathway in response to oncogene expression.

The miR-17-19 cluster supports the transformation of human primary cells

Full neoplastic transformation of human primary cells can be accomplished by expression of hTERT, small t, Ras^{V12} and interference with the p53 and pRB pathways by, for example, knockdown of $p16^{INK4A}$ and p53 (ref. 10). The transformation of cells can be visualized by their acquired ability to grow anchorage-independently in semi-solid media (soft agar assay). To determine whether miR-17-19 expression sufficiently inactivates the pRB pathway to induce cellular transformation, we performed soft agar assays in BJ/ET cells expressing $p53^{KD}$, small t, Ras^{V12}, and either an empty vector, $p16^{INK4A-KD}$ or miR-17-19. As expected, knockdown of $p16^{INK4A}$ increases the efficiency to form colonies in soft agar, confirming the transformed phenotype of the cells. Expression of miR-17-19 increased colony growth similar

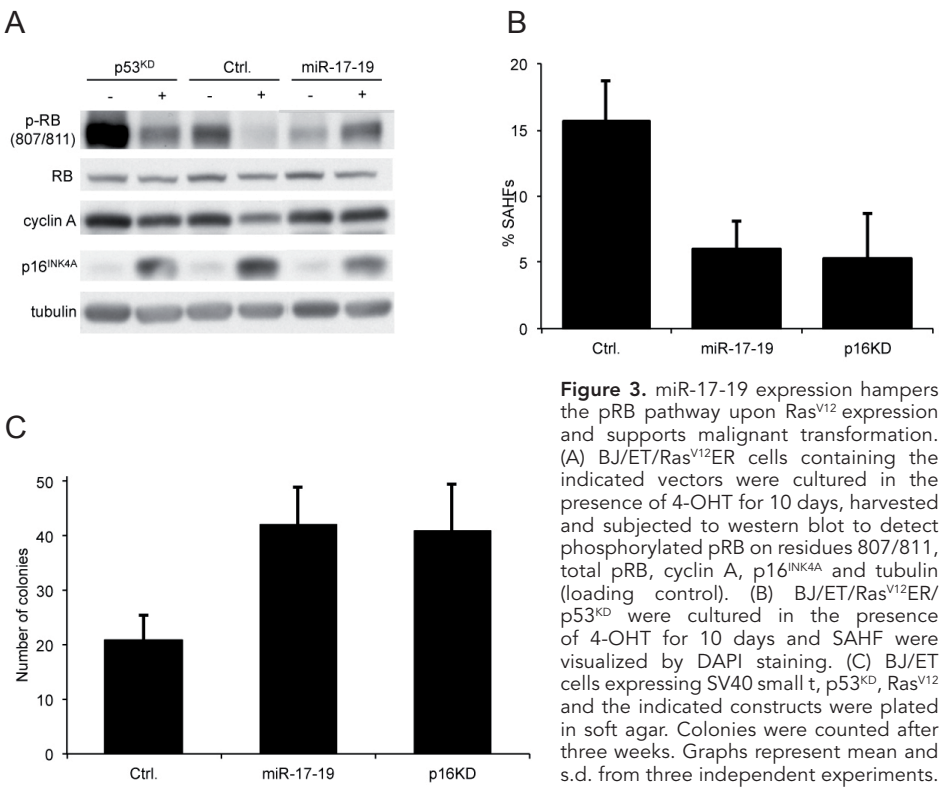


Figure 3. miR-17-19 expression hampers the pRB pathway upon Ras^{V12} expression and supports malignant transformation. (A) BJ/ET/Ras^{V12}ER cells containing the indicated vectors were cultured in the presence of 4-OHT for 10 days, harvested and subjected to western blot to detect phosphorylated pRB on residues 807/811, total pRB, cyclin A, $p16^{INK4A}$ and tubulin (loading control). (B) BJ/ET/Ras^{V12}ER/$p53^{KD}$ were cultured in the presence of 4-OHT for 10 days and SAHF were visualized by DAPI staining. (C) BJ/ET cells expressing SV40 small t, $p53^{KD}$, Ras^{V12} and the indicated constructs were plated in soft agar. Colonies were counted after three weeks. Graphs represent mean and s.d. from three independent experiments.

to p16$^{INK4A-KD}$, indicating that cluster expression can efficiently replace knockdown of p16^{INK4A} in the transformation of human primary cells (Fig. 3c).

In conclusion, our data confirm the oncogenic properties of the miR-17-19 cluster. We show that cluster expression supports the malignant transformation of primary human cells and propose that this is partially achieved via interference with the pRB tumour suppressor pathway (Fig. 4).

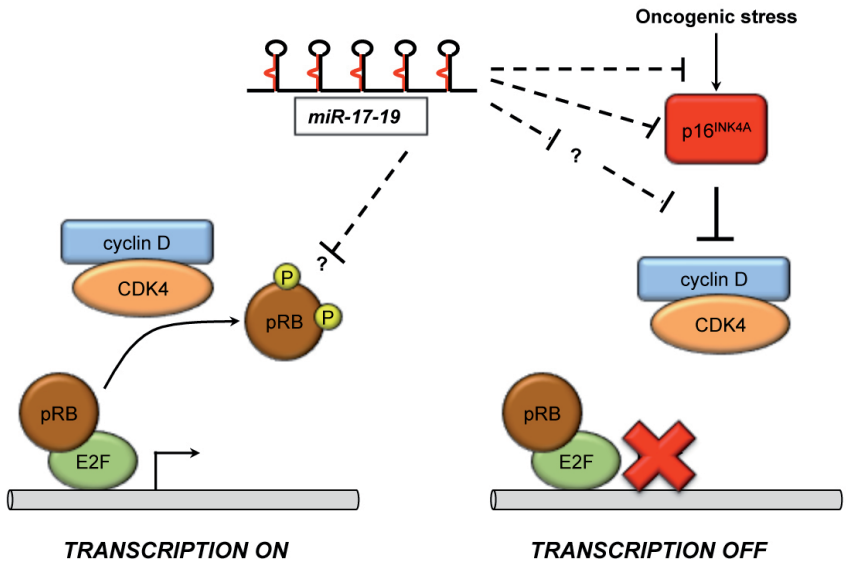


Figure 4. Schematic representation of the pRB pathway and how miR-17-19 expression might interfere.

DISCUSSION

We identified miR-17&18 in a screen for miRNAs involved in the cellular stress response elicited upon oncogene expression. Remarkably, expression of the miR-17-92 cluster gives an agonistic effect, suggesting that these miRNAs cooperate to reach the observed effect. This effect is, at least partially, independent of the p53 pathway. The recent observation that miR-17&20 can partially overcome oncogene-induced senescence by suppression of p21 showed that miR-17-92 is also implicated in the p53 pathway[17]. However, our data shows that expression of miR-17&20 in Ras^{V12}-expressing BJ/ET cells gives a weaker effect than expression of the complete cluster, indicating that the other miRNAs are involved as well. On top of that, we observed a miR-17-19-induced proliferation advantage in cells expressing p53^{KD}, implying a p53-independent effect of this cluster.

We propose that miR-17-19 interferes with efficient activation of the pRB pathway. We observed hyper-phosphorylation of pRB and transcriptionally active E2F in miR-17-19 and Ras^{V12} expressing cells. In addition, miR-17-19 expression interfered with p16^{INK4A}/pRB-dependent SAHF formation after oncogene

activation, and could replace loss of $p16^{INK4A}$ in the neoplastic transformation of primary human cells. The by pRB regulated transcription factor E2F1 has been proposed to be a target gene of miR-17&20 (ref. 18). However, besides the fact that inactivation of E2F1 would block rather than support proliferation, we and others have not been able to detect a significant suppression of this protein upon expression of these miRNAs (data not shown; ref. 17).

Obviously, the next step would be to identify the targets responsible for the effects observed upon miR-17-19 expression. Although we approached this issue in several different ways, we were not able to identify the responsible targets. For instance, we combined target prediction programs with micro-array mRNA expression analysis, but none of the targets identified through this strategy could recapitulate the effect given by miR-17-19 expression (data not shown). MiRNAs have the potential to regulate hundreds of target genes and the repression of a combination of these might be responsible for the observed effect. In addition, the miR-17-19 cluster contains five miRNAs, making the search for targets even more complex. It was recently shown that all the miRNAs of the miR-17-92 cluster are involved in repression of the TGFβ pathway in neuroblastoma cells[8]. Mestdagh and colleagues demonstrate that each miRNA contributes to the repression of one or more genes in the TGF-β pathway, thereby efficiently interfering with the pathway.

Several reports claimed to have identified the oncogenic miRNA in the miR-17-92 cluster. Two recent papers propose miR-19 to be the most important miRNA, mainly through regulation of PTEN[19,20]. Other reports describe miR-17&20 as the main oncogenic miRNA by targeting several components of different tumour suppressor pathways. Among these are p21 and E2F1, major downstream effectors of the p53 and pRB tumour suppressor pathways, respectively. Further research will be required to characterize the contribution of each single miRNA and the targets that are involved.

METHODS

Cell culture, transfection and retroviral transduction

BJ primary fibroblasts were cultured in DMEM with 10% FCS and antibiotics. Retroviruses were made by calcium-phosphate transfection of Ecopack 2 cells (Clontech) and harvesting 40 and 64 h later. BJ cells were selected with the proper selection medium 48 h after transduction for at least a week. In the case of Ras^{V12}-encoding retroviruses, cells were kept under selection for the entire duration of the experiment.

Screen of the miRNA expression library

BJ/ET/Ras^{V12}ER cells were plated in 24-well plates and retrovirally transduced with retroviruses containing separate miR-expression constructs[11]. After drug-selection for library–transduced cells, cells were pooled and grown in the presence or absence of 10^{-7} M 4OH-Tamoxifen (4-OHT). Genomic DNA was isolated using

DNeasy tissue kit (Qiagen). MiRNA-encoding sequences were recovered by PCR and subjected to hybridization with a miRNA-microarray, containing all miRNA library inserts[11]. Outliers were picked when enriched in two independent experiments followed by verification using growth competition assays.

Growth competition assays

BJ/ET cells were retrovirally transduced with pMSCV-blast-Ras^{V12}ER, drug selected, and transduced with miRVec-YFP or pRS-GFP constructs expressing the respective miRNA or shRNA, respectively. Efficiency of transduction was determined by flow cytometry (FL1) and cells were plated in medium with and without 10^{-7} M 4-OHT. Cells were propagated, and the percentage of GFP-, or YFP-positive cells was measured two times per week. The relative growth advantage was calculated as described[10].

Transformation assay

BJ/EHT/p53^{KD}/st cells were transduced with miRVec-Ctrl, miRVec-17-19, or pRS-p16, drug selected, and transduced with pBabe-puro-Ras^{V12}. After one week, 5 x 10^4 cells were plated in triplicates in soft agar (30 mm dishes). After three weeks of growth, macroscopically visible colonies were counted.

Constructs and antibodies

The miRNA expression library was described previously[11]. miRVec-blast, miRVec-YFP, pRetrosuper (pRS), pRS-GFP, pBabe-puro-Ras^{V12}, pBabe-puro-Ras^{V12}ER^{TAM}, pMSCV-blast-Ras^{V12}ER^{TAM}, pMSCV-GFP-st, and pBabe-H2B-GFP were described before[10,21,22,23]. pRS-p53 and pRS-p16 shRNA constructs were previously described[10]. miRVec-17-19 was cloned by PvuII digestion of miRVec-17&18, PvuII/NheI digestion of miRVec-18,19a&20 and NheI digestion of miRVec-19b&92. Fragments were ligated and used as template for PCR with 17fw (GCGGATCCGGCATAAATACGTGTCTAAATGGACC) and 92rv (GCGAATTCAATAAATCTAACAACAAAAAATATAG). PCR fragment was subsequently used as template for PCR with 17fw and 19brv (GCGAATTCCACAGCATTGCAACCGATCCCAACCTG) followed by BamHI/EcoRI cloning in miRVec. Antibodies against p16^{INK4A} (C19), cyclin D (M20/H295) and cyclin A (BF683) were purchased from Santa Cruz Biotechnology. pRB (4H1) and phospho-pRB 807/811 were from Cell Signaling.

Staining of senescence-associated heterochromatin foci

Cells seeded on fibronectin-coated coverslips were fixed using 4% paraformaldehyde. Next, they were permeabilized in 0.2% Triton X-100/PBS and stained with DAPI (1:1500 in PBS).

RNase protection assay

RPA was performed using the miRVana probe construction and detection kits (Ambion) according manufacturers instructions. Cyclophilin probe was described previously[11]. Primer sequences are listed in Table S1.

REFERENCES

1. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116,** 281 – 297 (2004).
2. Ota, A., Tagawa, H., Karnan, S., Tsuzuki, S., Karpas, A., Kira, S., Yoshida, Y., and Seto, M. Identification and characterization of a novel gene, C13orf**25,** as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res. **64,** 3087 – 3095 (2004).
3. Rinaldi, A., Poretti, G., Kwee, I., Zucca, E., Catapano, C.V., Tibiletti, M.G., and Bertoni, F. Concomitant MYC and microRNA cluster miR-17-92 (C13orf25) amplification in human mantle cell lymphoma. Leuk. Lymphoma **48,** 410 – 412 (2007).
4. Hayashita, Y., Osada, H., Tatematsu, Y., Yamada, H., Yanagisawa, K., Tomida, S., Yatabe, Y., Kawahara, K., Sekido, Y., and Takahashi, T. A polycistronic microRNA cluster, miR-17-**92,** is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. **65,** 9628 – 9632 (2005).
5. He, L., Thomson, J.M., Hemann, M.T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J., and Hammond, S.M. A microRNA polycistron as a potential human oncogene. Nature **435,** 828 – 833 (2005).
6. Mendell, J.T. miRiad roles for the miR-17-92 cluster in development and disease. Cell **133,** 217 – 222 (2008).
7. Van Haaften, G., and Agami, R. Tumorigenicity of the miR-17-92 cluster distilled. Genes Dev. **24,** 1 – 4 (2010).
8. Mestdagh, P., Boström, A.K., Impens, F., Fredlund, E., Van Peer, G., De Antonellis, P., von Stedingk, K., Ghesquière, B., Schulte, S., Dews, M., Thomas-Tikhonenko, A., Schulte, J.H., Zollo, M., Schramm, A., Gevaert, K., Axelson, H., Speleman, F., and Vandesompele, J. The miR-17-92 microRNA cluster regulates multiple components of the TGF-β pathway in neuroblastoma. Mol. Cell **40,** 762 – 773 (2010).
9. Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. Creation of human tumour cells with defined genetic elements. Nature **400,** 464 – 468 (1999).
10. Voorhoeve, P.M. and Agami R. The tumor-suppressive functions of the human INK4A locus. Cancer Cell **4,** 311 – 319 (2003).
11. Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A.J., Stoop, H., Nagel, R., Liu, Y.P., van Duijse, J., Drost, J., Griekspoor, A., Zlotorynski, E., Yabuta, N., De Vita, G., Nojima, H., Looijenga, L.H., and Agami, R. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumours. Cell **124,** 1169-1181 (2006).
12. Drost, J., Mantovani, F., Tocco, F., Elkon, R., Comel, A., Holstege, H., Kerkhoven, R., Jonkers, J., Voorhoeve, P.M., Agami, R., and Del Sal, G. BRD7 is a candidate tumour suppressor gene required for p53 function. Nat. Cell Biol. **12,** 380 – 389 (2010).
13. Sherr, C.J. and McCormick F. The RB and p53 pathways in cancer. Cancer Cell **2,** 103 – 112 (2002).
14. Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell **88,** 595 – 602 (1997).
15. Narita, M., Nũnez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell **113,** 703 – 716 (2003).
16. Di Micco, R., Sulli, G., Dobreva, M., Liontos, M., Botrugno, O.A., Gargiulo, G., dal Zuffo, R., Matti, V., d'Ario, G., Montani, E., Mercurio, C., Hahn, W.C., Gorgoulis, V., Minucci, S., and d'Adda di Fagagna, F. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat. Cell Biol. **13,** 292 – 302 (2011).
17. Hong, L., Lai, M., Chen, M., Xie, C., Liao, R., Kang, Y.J., Xiao, C., Hu, W.Y., Han, J., and Sun, P. The miR-17-92 cluster of microRNAs confers tumorigenicity by inhibiting oncogene-induced senescence. Cancer Res. **70,** 8547 – 8557 (2010).
18. O'Donnell, K.A., Wentzel, E.A., Zeller, K.I., Dang, C.V., and Mendell, J.T. c-Myc-regulated microRNAs modulate E2F1 expression. Nature **435,** 839 – 843 (2005).
19. Mu, P., Han, Y.C., Betel, D., Yao, E., Squatrito, M., Ogrodowski, P., de Stanchina, E., D'Andrea, A., Sander, C., and Ventura, A. Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas. Genes Dev. **23,** 2806 – 2811 (2009).
20. Olive, V., Bennett, M.J., Walker, J.C., Ma, C., Jiang, I., Cordon-Cardo, C., Li, Q.J., Lowe, S.W., Hannon, G.J., and He, L. miR-19 is a key oncogenic component of mir-17-92. Genes Dev. **23,** 2839 – 2849 (2009).
21. Brummelkamp, T.R., Bernards, R., and Agami, R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell **2,** 243-247 (2002).
22. Kolfschoten, I.G., van Leeuwen, B., Berns, K., Mullenders, J., Beijersbergen, R.L., Bernards, R., Voorhoeve, P.M., and Agami, R. A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. Cell **121,** 849-858 (2005).
23. De Vita, G., Bauer, L., da Costa, V.M., De Felice, M., Baratta, M.G., De Menna, M., and Di Lauro, R. Dose-dependent inhibition of thyroid differentiation by RAS oncogenes. Mol. Endocrinol. **19,** 76-89 (2005).

SUPPLEMENTARY TABLE AND FIGURE

Table S1. Sequences of probes used for RPA.

miR	RPA probe
miR-17	CAAAGTGCTTACAGTGCAGGTAGTCCTGTCTC
miR-18	TAAGGTGCATCTAGTGCAGATACCTGTCTC
miR-20	TAAAGTGCTTATAGTGCAGGTAGCCTGTCTC
miR-19a	TGTGCAAATCTATGCAAAACTGACCTGTCTC
miR-19b	TGTGCAAATCCATGCAAAACTGACCTGTCTC
miR-92	TATTGCACTTGTCCCGGCCTGCCTGTCTC

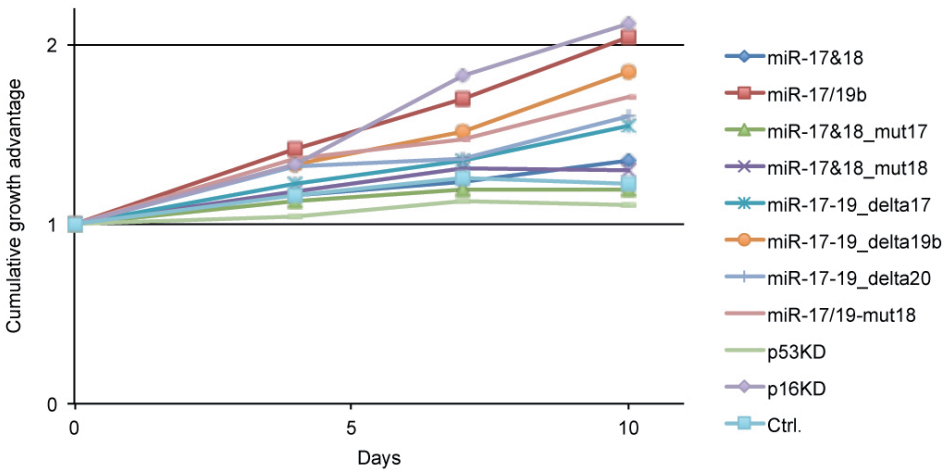


Figure S1. Growth competition assays of BJ/ET/Ras^{V12}ER/p53^{KD} cells transduced with the indicated vectors and cultured in the presence of 4-OHT (+Ras^{V12}).

6

TRANSFORMATION LOCKED IN A LOOP

Jarno Drost[1] and Reuven Agami[1,2,*]

Cell 2009; 139: 654-656

ABSTRACT

During neoplastic transformation, cells can promote their own growth by activating proto-oncogenes. Reporting in *Cell*, Iliopoulos et al. (2009) now show that in certain cell types, a transient oncogenic signal is sufficient to induce neoplastic transformation and to maintain it through a positive feedback loop driven by the inflammatory cytokine interleukin-6.

Jarno Drost[1] and Reuven Agami[1,2,*]

[1]Division of Gene Regulation, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands; [2]Center for Biomedical Genetics, 3584 CG Utrecht, The Netherlands.
*Correspondence: r.agami@nki.nl.

To become fully transformed, primary human cells need to bypass several cellular failsafe mechanisms that normally keep cellular growth in check. In addition, transformed cells can become self-sufficient by producing their own growth signals. Activating mutations in proto-oncogenes are a frequent path to cellular neoplastic transformation, and several reports have described the dependence of cancer cells on the oncogene even after transformation[10]. However, in this issue of *Cell*, Struhl and colleagues[4] report that transient induction of the Src oncogene in nontransformed human mammary epithelial cells results in production of the cytokine interleukin-6 (IL-6), which drives — and maintains — cells in a transformed state. This is mediated by a positive feedback loop involving IL-6, NF-κB, the let-7 micro-RNA (miRNA), and its regulator, LIN28B, resulting in a "snowball" effect.

NF-κB is a transcription factor that can induce the expression of IL-6, a cytokine that plays a crucial role in the immune response and inflammation. LIN28B is a stem cell factor and RNA binding protein that inhibits processing of the let-7 miRNA[8]. Changes in let-7 expression have been associated with tumorigenesis. Although links between inflammation and cancer have been suggested, a link between NF-κB and let-7 has not been reported so far. However, the new work links all of these four players and demonstrates that they cooperate to maintain cellular transformation in response to transient oncogene activation[4].

How transient is the oncogenic signal and how does it work? Iliopoulos et al. expressed a tamoxifen-inducible estrogen receptor-Src (ER-Src) fusion protein in nontransformed human mammary epithelial cells. They show that a 5 min treatment with tamoxifen is sufficient to drive the cells into the transformed state. Next, they provide evidence that this transient treatment induces expression of the inflammatory cytokine IL-6 in an NF-κB-dependent manner. Specifically, they show that Src activates NF-κB, which directly activates the transcription of LIN28B. Indeed, the expression of the mature let-7 miRNA rapidly decreases in response to Src activation, through induction of LIN28B. As a result, IL-6, a direct target of let-7, is derepressed, causing cellular transformation through STAT3, a transcriptional activator that is phosphorylated and activated in response to IL-6 signaling. A positive feedback loop is generated through IL-6, the expression of which is induced by NF-κB, but IL-6 can itself activate NF-κB (Fig. 1a). This loop explains why the transformed state is maintained even after Src expression is shut off. Confirming this model, the authors report that inhibition of any of the components of the feedback loop reverses the transformed phenotype.

The authors provide evidence that their findings may be relevant to some human cancers. In both normal and cancer cells (derived from breast, prostate, and hepatocellular tissues), the authors find a negative correlation between the expression of the let-7 miRNA and that of IL-6. In normal cells, let-7 levels are high and IL-6 levels are low, whereas in cancer cells let-7 levels are low and IL-6 levels are high. These observations suggest that the feedback loop involving IL-6 and let-7 may be active in these cancer cells. Furthermore, interference with any component of the inflammatory feedback loop inhibited the tumorigenicity of

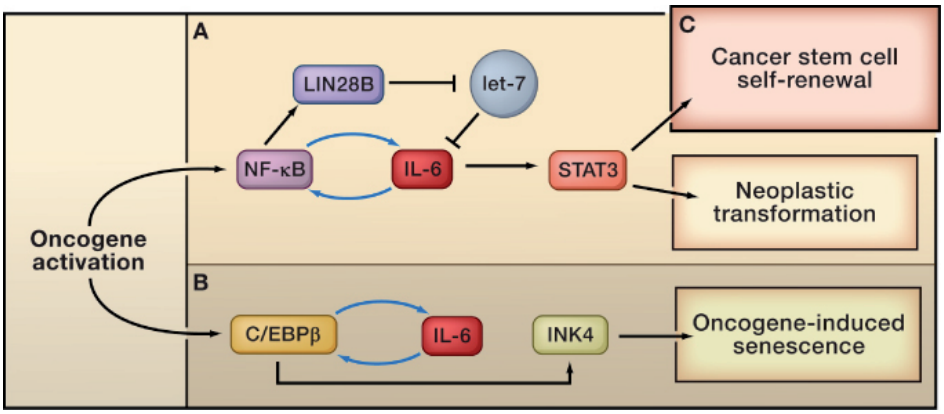


Figure 1. Inflammation and Tumorigenesis. Signaling via oncogenes activates an inflammatory response that drives cells along several different pathways. The response may depend on the cell type or genetic background. (A) An oncogenic trigger activates a positive feedback loop involving IL-6, NF-κB, let-7 miRNA, and LIN28B. IL-6 signaling activates the transcriptional activator STAT3, which drives cells into the transformed state; IL-6 also activates NF-κB and so completes the loop[4]. (B) Oncogene expression can also activate the transcription factor C/EBPβ. C/EBPβ activates transcription of IL-6 and expression of the INK4 locus, resulting in cellular senescence. IL-6 positively regulates C/EBPβ, thereby generating a positive feedback loop. (C) A proportion of cells in which the positive feedback loop (IL-6, NF-κB, let-7 miRNA, LIN28B) is activated acquire characteristics of stem cells, such as self-renewal, and may act as cancer stem cells in tumors.

several human cancer cell lines. However, despite this striking negative correlation in certain human cancers, in vivo tumor models will be needed to prove a causal role for this regulatory circuit in driving tumorigenesis. Intriguingly, mice lacking IL-6 are more resistant to the formation of tumors driven by the Ras oncogene and to developing tumors of the colon associated with inflammatory colitis than normal mice, suggesting causality[1,3]. Future experiments should address whether the IL-6/NF-κB/LIN28B/let-7 loop is involved in the formation of tumors in these animal models.

Many reports have shown that IL-6-mediated inflammation and cancer are linked. However, both tumor-suppressive and tumor-promoting activities have been ascribed to IL-6. In accordance with the observations of Iliopoulos et al. suggesting a tumor-promoting role for IL-6, a recent study showed that IL-6 expression, induced by the Ras^{V12} oncogene, is required for tumor growth of human kidney cells in vivo[1]. On the other hand, oncogene-induced IL-6 upregulation in primary human fibroblasts results in senescence[5]. This antiproliferative response is mediated, at least in part, by the induction of the cyclin-dependent kinase inhibitor $p15^{INK4B}$ (Fig. 1b). How can these results be reconciled with the findings of Iliopoulos and colleagues? Remarkably, the spontaneous immortalization of the mammary epithelial cells used by Iliopoulos et al. is associated with a deletion on chromosome some 9p21. This deletion includes part of the INK4/ARF locus, such that these cells lack the genes encoding the cyclin-dependent kinase inhibitors

p15^{INK4B} and p16^{INK4A} (ref. 2). This raises the exciting possibility that the type of IL-6 response that is induced after oncogene activation depends on the status of the INK4/ARF locus. Consistent with this hypothesis, the human kidney cells used in the study of Ancrile et al. in which IL-6 promoted tumor growth, expressed SV40 large T antigen. SV40 large T antigen inactivates the ARF/p53 and INK4/Rb tumor suppressor pathways, thereby neutralizing any IL-6-dependent cell cycle-inhibitory effects via p15^{INK4B} and p16^{INK4A}. It would be interesting to determine whether p15^{INK4B} and p16^{INK4A} are still transcriptionally activated in response to oncogene expression. In contrast to mammary epithelial cells, transient activation of the Ras^{V12} oncogene in a primary fibroblast transformation model[9] was not sufficient to maintain transformation, perhaps indicating that the IL-6 response might be cell-type dependent.

Iliopoulos et al. made another interesting observation. They report that their positive feedback loop is required for the self-renewing capacity of tumor-initiating cells, so-called cancer stem cells (Fig. 1c). A small proportion of the mammary epithelial cells expressing the ER-Src fusion protein that were transiently treated with tamoxifen acquired properties of cancer stem cells, such as self-renewal capacity. The inflammatory loop was more active in this population compared to cells that did not self-renew. When the authors blocked the function of several components in the feedback circuit, they saw a decrease in the self-renewal capacity of the cancer stem cells. This is not the first time a transient signal was found to induce stem cell formation. For example, it is well established that reprogramming of adult fibroblasts back to a stem cell-like state can occur after transient expression of the reprogramming factors OCT4, SOX2, c-MYC, and KLF4 (ref. 7), pointing to the involvement of a positive feedback loop. The results of Iliopoulos et al. suggest that the IL-6/NF-κB/LIN28B/let-7 loop could also be at work in the reprogramming of fibroblasts. Given that LIN28B can also be used as a reprogramming factor, it is tempting to speculate about the involvement of the positive feedback loop identified by Iliopoulos et al. in stem cell maintenance. Intriguingly, the INK4/ARF locus serves as a barrier to efficient reprogramming[6], again implying that this locus plays an important role in the effects observed by Iliopoulos et al.

The Iliopoulos et al. study shows that a transient oncogenic trigger can lead to cellular transformation. This is mediated through an inflammatory signal and subsequent activation of a positive feedback loop containing IL-6, NF-κB, let-7, and LIN28B. From a therapeutic point of view, this study and others raise the possibility that tumors with this overactive positive feedback loop —LIN28B^{HIGH}/Let-7^{LOW}/IL-6^{HIGH}— may be eradicated efficiently by interference with this loop, which also may inhibit the growth of cancer stem cells.

REFERENCES

1. Ancrile, B., Lim, K.H., and Counter, C.M. *Genes Dev.* **21**, 1714–1719 (2007).
2. Cowell, J.K., LaDuca, J., Rossi, M.R., Burkhardt, T., Nowak, N.J., and Matsui, S. *Cancer Genet. Cytogenet.* **163**, 23–29 (2005).
3. Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G.Y., Vallabhapurapu, S., Scheller, J., Rose-John, S., Cheroutre, H., Eckmann, L., et al. *Cancer Cell* **15**, 103–113 (2009).
4. Iliopoulos, D., Hirsch, H.A., and Struhl, K. *Cell*, this issue (2009).
5. Kuilman, T., Michaloglou, C., Vredeveld, L.C.W., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. *Cell* **133**, 1019–1031 (2008).
6. Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Canamero, M., Blasco, M.A., and Serrano, M. *Nature* **460**, 1136–1139 (2009).
7. Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G.W., Cook, E.G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., et al. *Cell* **136**, 964–977 (2009).
8. Viswanathan, S.R., Daley, G.Q., and Gregory, R.I. *Science* **320**, 97–100 (2008).
9. Voorhoeve, P.M., and Agami, R. *Cancer Cell* **4**, 311–319 (2003).
10. Weinstein, I.B., and Joe, A. *Cancer Res.* **68**, 3077–3080 (2008).

7

GENERAL DISCUSSION

The transformation of a normal cell to a tumour cell is caused by genetic and epigenetic changes in genes involved in cell proliferation and survival. Two pivotal players in tumour suppression are the p53 and pRB pathways, of which proper functioning is frequently altered in human cancer. However, in many cases the causative hit remains elusive. The identification of new proteins and non-coding RNAs that act in the p53 and pRB pathways could potentially explain a subset of these cases and give new insights in treatment therapies.

BRD7 is a transcriptional cofactor of p53

In the first study described in this thesis (chapter 2 and 3) we present BRD7 as a novel cofactor of p53. BRD7 is a bromodomain-containing protein and part of the polybromo-associated BRG1-associated factor (PBAF) SWI/SNF chromatin-remodelling complex[1]. We show that BRD7 interacts with p53 and that it is required for transcription of a specific subset of p53 target genes[2]. The genes that require BRD7 for their transcription mainly include the high affinity pro-cell cycle arrest p53 target genes and exclude most low affinity pro-apoptotic targets. These observations were functionally confirmed by showing that BRD7 is required for oncogene-induced senescence (OIS), but dispensable for p53-dependent apoptosis. BRD7 is required for transcription through recruitment of the histone-acetyl transferase p300 to p53 target genes. This results in acetylation and activation of p53, as well as acetylation of surrounding histones, thereby opening the chromatin and facilitating binding of factors required for transcription. Remarkably, although BRD7 is not required for transcription of pro-apoptotic p53 target genes, its binding could be detected on the promoters of these genes. It would be of interest to determine why BRD7 presence not necessarily implies involvement in transcription. It seems likely that other cofactors are involved in the transcriptional regulation of these genes. hCAS/CSE1L, for example, is specifically required for transcription of pro-apoptotic p53 target genes[3].

Another interesting observation we describe in chapter 2 is the increased interaction between BRD7 and p53 upon oncogene expression, whereas no changes in their protein and mRNA levels could be observed. This may imply that posttranslational modifications are involved in the stabilization of the interaction. Although p53 can be acetylated at multiple lysine residues, neither these lysines nor BRD7's bromodomain are involved in the interaction. Another possibility could be that a protein that is regulated by stress stabilizes the interaction. The possible involvement of posttranslational modifications and differential protein binding partners is under current investigation.

The results we present in chapter 2 were recently confirmed by Burrows et al., who describe the identification of BRD7 in a screen for genes that are required for replicative senescence[4]. In addition to BRD7, they show that another component of the PBAF complex (BAF180) is required for p53-dependent transcription and senescence. Also other SWI/SNF complex components are able to interact with p53 and affect p53-dependent transcription, including BRG1 and BAF60a[5-7]. Thus, it seems likely that p53 recruits chromatin-remodelling complexes to the

promoter of target genes through direct interaction with one or more complex components. It would be interesting to determine if the composition of these complexes vary with different types of cellular stresses.

Loss of BRD7 expression in human cancer

A possible role for BRD7 in tumour suppression was first postulated in a search for genes that are differentially expressed in nasopharyngeal carcinoma (NPC). Low expression of BRD7 was found in NPC and it was subsequently shown that promoter methylation is one of the underlying causes[8,9]. Additionally, overexpression of BRD7 suppressed the growth of a NPC cell line[10]. Although p53 mutations are rarely found in NPC, no functional connection was made between the two genes. We discovered decreased BRD7 expression in a subset of breast tumours, specifically in those harbouring wild-type p53 (chapter 2). In addition to promoter methylation, we show that deletion of the region including the BRD7 gene is another cause of its loss of expression.

BRD7's role in tumour suppression was further emphasized by the observation that it interacts with and serves as a transcriptional cofactor of the tumour suppressor BRCA1 (ref. 11). Remarkably, BRCA1 is specifically required for transcription of p53 target genes that are involved in cell cycle arrest and DNA repair[12,13]. Therefore, BRD7 and BRCA1 might also cooperate in the regulation of p53 target genes.

Enhancer RNAs enter the p53 pathway

In chapter 4 we describe the identification of several enhancer domains that produce transcripts in a p53-dependent manner. Several recent reports describe the production of these so-called enhancer RNAs (eRNAs), but no clear function has been assigned to them[14,15]. ERNA-producing enhancer domains are marked by monomethylation of histone 3 at lysine 4 (H3K4me1), a lack of trimethylation at H3K4 (H3K4me3), p300 and RNA polymerase II (RNAPII) binding. RNAPII at these enhancers produces transcripts bi-directionally[14,15].

To identify non-coding p53 target genes we analysed genome-wide p53-binding patterns[2,16]. We identified seven regions that are bound by p53 with high affinity and carry all the features of enhancer domains. We were able to show that these regions have enhancer activity that depends on functional p53 and produce transcripts in a p53-dependent fashion. Knockdown of the transcripts (eRNAs) produced from one of the enhancers interferes with a p53-dependent cell cycle arrest, showing for the first time that eRNAs may have functional relevance. Expression analysis of primary fibroblasts revealed decreased mRNA levels of several genes encoded nearby the enhancer domains upon p53 knockdown. Remarkably, our ChIP-seq did not detect any p53 binding in the promoters of these genes, suggesting that p53 regulates their transcription in an alternative manner. We found that the enhancers interact with multiple surrounding loci, including regions nearby the genes that we found to be regulated by p53. Since enhancer activity is p53-dependent, we suggest that p53 might regulate

transcription through binding to enhancer regions and affecting their activity or even their binding to target genes. We are currently investigating the involvement of p53 in the regulation of enhancer activity.

The miR-17-92 cluster participates in oncogenic transformation at multiple levels

In chapter 5 we describe a role for the miR-17-92 cluster in tumorigenesis. We identified the miR-17&18 component of this oncogenic cluster in a screen for miRNAs whose expression allows bypass of oncogene-induced senescence. Remarkably, expression of the complete cluster gives an agonistic effect. The miRNA cluster consists of 6 miRNAs, which were all previously implicated in cancer (reviewed in ref. 17). For instance, miR-17&20 were shown to interfere with the p53 pathway by targeting p21 (ref. 18,19). However, we show that the cluster also dampens the pRB pathway (chapter 5). This implicates that overexpression of the miR-17-92 cluster neutralizes two important tumour suppressor pathways. Indeed, the miR-17-92 cluster is one of the most potent oncogenic miRNAs. Both genomic amplification and increased expression are found in several types of human cancer[20-22].

Several reports claim the identification of the most important oncogenic component of the miR-17-92 cluster. Some point to miR-17&20 via inhibition of p21 and the E2F transcription factors. Others keep miR-19a&b responsible, via regulation of PTEN. We, and others, show that expression of all the miRNAs is most effective in transforming human primary cells (this thesis; ref. 19). In agreement with this, cooperation between the single miRNA components of miR-17-92 is required for the most potent inhibition of the TGFβ pathway[23]. Unfortunately, we were unable to find the target(s) responsible for the observed effects. It seems likely that, as in the TGFβ pathway, multiple miRNAs (and thus multiple targets) are involved in the ultimate blockage of the pRB pathway. Additional research will be required to find these targets.

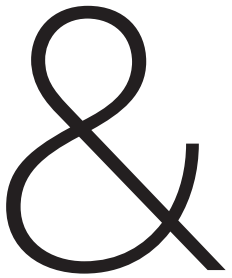
REFERENCES

1. Kaeser, M. D., Aslanian, A., Dong, M. Q., Yates, J. R. III & Emerson, B. M. BRD7, a novel PBAF-specific SWI/SNF subunit, is required for target gene activation and repression in embryonic stem cells. J. Biol. Chem. **283,** 32254 – 32263 (2008).
2. Drost, J., Mantovani, F., Tocco, F., Elkon, R., Comel, A., Holstege, H., Kerkhoven, R., Jonkers, J., Voorhoeve, P.M., Agami, R., and Del Sal, G. BRD7 is a candidate tumour suppressor gene required for p53 function. Nat. Cell Biol. **12,** 380 – 389 (2010).
3. Tanaka, T., Ohkubo, S., Tatsuno, I., and Prives, C. hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. Cell **130,** 638 – 650 (2007).
4. Burrows, A.E., Smogorzewska, A., and Elledge, S.J. Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence. Proc. Natl. Acad. Sci. USA **107,** 14280 – 14285 (2010).
5. Hendricks KB, Shanahan F, Lees E. Role for BRG1 in cell cycle control and tumor suppression. Mol. Cell. Biol. **24,** 362 – 376 (2004).
6. Kang H, Cui K, Zhao K. BRG1 controls the activity of the retinoblastoma protein via regulation of p21CIP1/WAF1/SDI. Mol. Cell. Biol. **24,** 1188 – 1199 (2004).
7. Oh J, Sohn DH, Ko M, Chung H, Jeon SH, Seong RH. BAF60a interacts with p53 to recruit the SWI/SNF complex. J. Biol. Chem. **283,** 11924 – 11934 (2008).
8. Yu Y, Zhang BC, Xie Y, Cao L, Zhou M, Zhan FH, et al. Analysis and molecular

cloning of differentially expressing genes in nasopharyngeal carcinoma [in Chinese]. Acta Biochim. Biophys. Sin. **32,** 327 – 332 (2000).

9. Liu H, Zhang L, Niu Z, Zhou M, Peng C, Li X, et al. Promoter methylation inhibits BRD7 expression in human nasopharyngeal carcinoma cells. BMC Cancer **8,** 253 (2008).
10. Peng C, Liu HY, Zhou M, Zhang LM, Li XL, Shen SR, et al. BRD7 suppresses the growth of Nasopharyngeal Carcinoma cells (HNE1) through negatively regulating beta-catenin and ERK pathways. Mol. Cell. Biochem. **303,** 141 – 149 (2007).
11. Harte MT, O'Brien GJ, Ryan NM, Gorski JJ, Savage KI, Crawford NT, et al. BRD7, a subunit of SWI/SNF complexes, binds directly to BRCA1 and regulates BRCA1-dependent transcription. Cancer Res. **70,** 2538 – 2547 (2010).
12. MacLachlan TK, Takimoto R, El-Deiry WS. BRCA1 directs a selective p53-dependent transcriptional response towards growth arrest and DNA repair targets. Mol. Cell. Biol. **22,** 4280 – 4292 (2002).
13. Ongusaha PP, Ouchi T, Kim KT, Nytko E, Kwak JC, Duda RB, et al. BRCA1 shifts p53-mediated cellular outcomes towards irreversible growth arrest. Oncogene **22,** 3749 – 3758 (2003).
14. Kim, T.K., Hemberg, M., Gray, J.M., Costa, A.M., Bear, D.M., Wu, J., Harmin, D.A., Laptewicz, M., Barbara-Haley, K., Kuersten, S., Markenscoff-Papadimitriou, E., Kuhl, D., Bito, H., Worley, P.F., Kreiman, G., and Greenberg, M.E. Widespread transcription at neuronal activity-regulated enhancers. Nature **465,** 182 – 187 (2010).
15. Wang, D., Garcia-Bassets, I., Benner, C., Li, W., Su, X., Zhou, Y., Qiu, J., Liu, W., Kaikkonen, M.U., Ohgi, K.A., Glass, C.K., Rosenfeld, M.G., and Fu, X.D. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. Nature **474,** 390 – 394 (2011).
16. 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. A global map of p53 transcription-factor binding sites in the human genome. Cell **124,** 207 – 219 (2006).
17. Van Haaften, G., and Agami, R. Tumorigenicity of the miR-17-92 cluster distilled. Genes Dev. **24,** 1 – 4 (2010).
18. Ivanovska, I., Ball, A.S., Diaz, R.L., Magnus, J.F., Kibukawa, M., Schelter, J.M., Kobayashi, S.V., Lim, L., Burchard, J., Jackson, A.L., Linsley, P.S., and Cleary, M.A. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol. Cell. Biol. **28,** 2167 – 2174 (2008).
19. Hong, L., Lai, M., Chen, M., Xie, C., Liao, R., Kang, Y.J., Xiao, C., Hu, W.Y., Han, J., and Sun, P. The miR-17-92 cluster of microRNAs confers tumorigenicity by inhibiting oncogene-induced senescence. Cancer Res. **70,** 8547 – 8557 (2010).
20. Ota, A., Tagawa, H., Karnan, S., Tsuzuki, S., Karpas, A., Kira, S., Yoshida, Y., and Seto, M. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res. **64,** 3087 – 3095 (2004).
21. Rinaldi, A., Poretti, G., Kwee, I., Zucca, E., Catapano, C.V., Tibiletti, M.G., and Bertoni, F. Concomitant MYC and microRNA cluster miR-17-92 (C13orf25) amplification in human mantle cell lymphoma. Leuk. Lymphoma **48,** 410 – 412 (2007).
22. Hayashita, Y., Osada, H., Tatematsu, Y., Yamada, H., Yanagisawa, K., Tomida, S., Yatabe, Y., Kawahara, K., Sekido, Y., and Takahashi, T. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. **65,** 9628 – 9632 (2005).
23. Mestdagh, P., Boström, A.K., Impens, F., Fredlund, E., Van Peer, G., De Antonellis, P., von Stedingk, K., Ghesquière, B., Schulte, S., Dews, M., Thomas-Tikhonenko, A., Schulte, J.H., Zollo, M., Schramm, A., Gevaert, K., Axelson, H., Speleman, F., and Vandesompele, J. The miR-17-92 microRNA cluster regulates multiple components of the TGF-β pathway in neuroblastoma. Mol. Cell **40,** 762 – 773 (2010).

SUMMARY
NEDERLANDSE SAMENVATTING
CURRICULUM VITAE
LIST OF PUBLICATIONS



SUMMARY

Cancer is a DNA disease originating from changes in expression of genes that regulate cell proliferation and survival. Genes serve as blueprints for the production of proteins and non-coding RNAs. Aberrant gene expression can occur upon changing the DNA code (mutations). The human body consists of approximately 100 trillion cells. Sporadically, mutations occur during DNA replication, a phase where cells duplicate their DNA prior to cell division. However, also cells that do not divide can gain errors in their DNA through stresses from the outside, like UV-radiation. Changes in the DNA will be passed on to the daughter cell, meaning that they will accumulate, as cells get older. Eventually, cells might gain the capacity to divide uncontrollably and cancer can arise. Fortunately, cells have mechanisms to protect them from uncontrolled division. These mechanisms are activated upon certain stresses, like DNA damage and excessive mitogenic signalling (hyperactive growth signalling). Genes that support cell growth are called oncogenes, whereas genes that inhibit growth are termed tumour suppressor genes. Activating and inactivating mutations in oncogenes and tumour suppressor genes, respectively, underlie the development of cancer. The pathways in which oncogenes and tumour suppressor genes are functioning are complex and for most of them not all components are known. Clarifying how these pathways are activated by stress and how they function to protect cells from turning malignant might have future implications in the treatment of cancer. In this thesis we describe the identification of novel components of two major tumour suppressor pathways and how they may contribute to the proper functioning of these pathways.

The first chapter gives a short overview of the current knowledge regarding the research described in the other chapters. It also describes the model that we used to screen for tumour suppressor pathway components: the *in vitro* transformation model. With this model we can transform "normal" (primary) cells into tumour cells by simultaneous inhibition and activation of defined tumour suppressor genes and oncogenes, respectively (Chapter 1, Figure 1). Using this model, as well as other approaches, we identified novel components of the p53 and pRB tumour suppressor pathways.

In chapter 2 we describe the identification of bromodomain-containing 7 (BRD7) as a cofactor of the p53 tumour suppressor protein. The gene encoding the p53 protein is the most mutated gene in human cancer. When the p53 gene is wild-type (unaltered) alterations in components of the pathway neutralize its activation. However, in many of the cases the alteration responsible for pathway inactivation is unknown. The main mechanism by which the p53 pathway utilizes its tumour suppressive function is through its central role in senescence induction. Senescence is an irreversible growth arrest that can be induced by several stresses, including the expression of oncogenes. It is an important *in vivo* tumour suppressive mechanism and escape from senescence could ultimately lead to cancer. We show that the ability of p53 to efficiently induce senescence is dependent on BRD7. Mechanistically, BRD7 binds to p53 and is required for p53-dependent transcription of a subset of target genes. Proper activation of p53

upon stress requires acetylation of the protein. We show that BRD7 recruits the p53 acetylator p300 to target genes where it not only acetylates (and activates) p53, but also surrounding histones. This keeps the chromatin at these locations in an open and transcriptionally active state. It was shown earlier that loss of BRD7 is common in nasopharyngeal carcinoma, a type of cancer that rarely harbours mutations in the p53 gene. One of the described causes for loss of BRD7 expression is methylation of the BRD7 gene. We describe in this thesis that BRD7 is also lost in breast cancer, specifically in tumours that harbour wild-type p53. We show that deletion of the genetic locus encoding BRD7 is another cause of BRD7 loss in cancer. Our observation that BRD7 is specifically downregulated in tumours with wild-type p53 confirms that BRD7 plays an important role in the p53 pathway. In conclusion, loss of BRD7 can explain how a subset of tumours can inactivate the p53 pathway without altering the p53 gene itself. The observations described in chapter 2 are further discussed in chapter 3 and the results are linked to previously published literature about related topics.

The p53 protein is a transcription factor that regulates transcription of dozens of target genes. Recently, several non-coding RNAs (transcripts that do not code for proteins) were added to the list of p53 target genes. Non-coding RNAs are implicated in important cellular processes, including cell proliferation and survival. In chapter 4 we use the genome-wide p53-binding pattern (described in chapter 2) to identify novel non-coding p53 target genes. We describe the identification of seven enhancer domains that require p53 for their activity and produce non-coding transcripts in a p53-dependent manner. The regions contain all the in literature described features that mark enhancer RNA (eRNA)-producing enhancer domains. Although eRNAs were described before, we are the first to show that their inhibition has functional implications, namely a less efficient induction of a p53-dependent cell cycle arrest upon stress. Finally, we propose that p53 regulates transcription of distantly located genes by affecting enhancer activity and eRNA production.

In chapter 5 we set out to identify microRNAs (miRNAs) that are involved in the transformation of primary cells into tumour cells. MiRNAs are small endogenous non-coding RNAs that regulate protein expression by binding to the 3' untranslated region (UTR) of messenger RNAs (mRNAs, the products of DNA transcription). When bound to its target mRNAs miRNAs can regulate their expression by inhibition of translation (the conversion of mRNA into protein) or mRNA degradation. We describe the oncogenic role of the miR-17-92 cluster in cellular transformation. This miRNA cluster contains six miRNAs (miR-17, miR-18a, miR-20a, miR-19a, miR-19b and miR-92) and to all of them oncogenic activities have been ascribed. We show that this cluster dampens the pRB tumour suppressor pathway. Cluster expression positively regulates transcription of genes required for progression through the cell cycle. It does so, at least partially, by affecting the phosphorylation status of the pRB protein. Normally, expression of an oncogene results in less (hypo-) phosphorylated pRB, which can then bind to E2F transcription factors and inhibit transcription of E2F target genes. These are mainly genes required for cell cycle progression. Expression of both an oncogene

and the miR-17-19 cluster (without miR-92) keeps pRB in a (hyper-) phosphorylated and E2F unbound state. This means that E2F can activate transcription and cells can continue to proliferate. Although we were unable to find the responsible target(s), we argue that all the miRNAs in the cluster are required for the most efficient inhibition of the pRB pathway. This implies that the miRNAs act at multiple levels in the pRB pathway and that cooperation takes places between them.

In this thesis we investigate how a normal cell can turn into a tumour cell. We present several novel components of the p53 and pRB tumours suppressor pathways and discuss how their identification helps us understand the way tumour cells can bypass intrinsic cellular safety mechanisms.

&

NEDERLANDSE SAMENVATTING

Het menselijk lichaam bestaat uit ongeveer 100 biljoen cellen. Goede communicatie tussen deze cellen is essentieel om een functioneel organisme te kunnen handhaven. De deling van cellen is één van de processen waarbij communicatie tussen cellen een grote rol speelt. Wanneer deze communicatie verstoord raakt en een cel niet meer luistert naar groeiremmingssignalen kan ongecontroleerde celgroei en kanker ontstaan. Miscommunicatie wordt vaak veroorzaakt door veranderingen in genexpressie. Genen bevatten de code voor boodschapper-RNA's (mRNA's) die als blauwdruk fungeren voor het maken van eiwitten. Eiwitten zijn moleculen die betrokken zijn bij het uitvoeren van allerlei processen in een organisme. De ontwikkeling van een tumor wordt veroorzaakt door veranderingen in expressie van genen coderend voor eiwitten die een rol spelen bij het reguleren van celdeling en celdood. Voordat cellen delen moet het genetische materiaal, het DNA, gerepliceerd (verdubbeld) worden. Hierbij kunnen soms fouten (mutaties) in de DNA-sequentie ontstaan. Deze veranderingen worden doorgegeven aan de dochtercellen waardoor mutaties kunnen ophopen naarmate cellen ouder worden. Uiteindelijk kunnen deze veranderingen ongecontroleerde celgroei veroorzaken wat tot kanker en uiteindelijk de dood van het organisme kan leiden. Cellen hebben beveiligingsmechanismen om excessieve groei tegen te gaan. Deze mechanismen kunnen door verschillende stimuli worden geactiveerd, zoals DNA-schade en te veel groeisignalen (hyperactieve groeistimuli). Genen coderend voor moleculen die groei remmen worden tumorsuppressorgenen genoemd en genen coderend voor moleculen die een positieve invloed hebben op groei heten oncogenen. Zowel tumorsuppressorgenen als oncogenen maken deel uit van complexe signaleringsroutes waarvan vaak nog niet alle componenten bekend zijn. Het achterhalen van de precieze werking van deze routes in het beschermen van cellen tegen ongecontroleerde groei kan belangrijke implicaties hebben voor eventuele behandelstrategieën. In dit proefschrift beschrijven we de identificatie van een aantal nieuwe componenten van twee belangrijke tumorsuppressorroutes. Verder laten we zien hoe deze componenten betrokken kunnen zijn bij de werking van deze routes.

Hoofdstuk 1 geeft een beknopt overzicht van de huidige status van het onderzoek naar de onderwerpen die worden bestudeerd in dit proefschrift. Verder wordt in hoofdstuk 1 het model beschreven dat bij een aantal onderzoeken is gebruikt: het *in vitro* transformatiemodel. Dit model beschrijft hoe we in een celkweeksysteem (*in vitro*) een normale (primaire) cel kunnen veranderen in een kankercel. Dit wordt bewerkstelligd door gelijktijdige activatie en remming van, respectievelijk, gedefinieerde oncogenen en tumorsuppressorgenen. Hierdoor kunnen we onderzoeken in welke route een bepaald gen een rol speelt en hoe essentieel deze rol is.

In hoofdstuk 2 beschrijven we de identificatie van een bromodomein bevattend eiwit, BRD7, en de rol die het speelt in tumorgenese als cofactor van het p53 tumor-suppressoreiwit. Het gen coderend voor p53 is het vaakst gemuteerde gen in kanker. Tumoren met een intact p53 gen hebben de route geremd door andere componenten dan p53 te inactiveren. In dit geval kan vaak niet worden

achterhaald wat de inactiverende mutatie is. Dit zou betekenen dat niet alle componenten van de route bekend zijn. Het p53 gen codeert voor een eiwit dat de transcriptie (het maken van mRNA) reguleert van genen die betrokken zijn bij celgroei en geprogrammeerde celdood (apoptose). Het belangrijkste tumorsuppressormechanisme dat wordt gereguleerd door p53 is senescence. Senescence is een onomkeerbaar cel cyclus arrest dat door verschillende soorten cellulaire stress kan worden geïnduceerd, waaronder hyperactieve groeistimuli. In hoofdstuk 2 beschrijven we dat p53 afhankelijk is van BRD7 in het efficiënt induceren van senescence. We laten zien dat BRD7 aan p53 bindt en nodig is voor p53 afhankelijke transcriptie. Dit wordt op twee manieren bewerkstelligd. Ten eerste, de aanwezigheid van BRD7 is nodig voor acetylatie van p53 na stress. Acetylatie van p53 is essentieel voor de activatie van het eiwit. Zonder BRD7 is er minder geacetyleerd (en dus minder actief) p53 waardoor er minder transcriptie is, wat resulteert in minder efficiënte senescence inductie. We laten zien dat BRD7 bindt aan promotoren van p53 afhankelijke genen en p300 rekruteert. P300 is een eiwit dat p53 kan acetyleren. Ten tweede, als BRD7 p300 rekruteert naar de promotoren acetyleert het behalve p53 ook omliggende histonen. Dit houdt het chromatine (het DNA-verpakkingsmateriaal) in een open en transcriptioneel actieve staat.

&

Verlies van BRD7-expressie werd voor de eerste maal geobserveerd in nasopharynx carcinoom, een type kanker waarin p53 mutaties zelden worden aangetroffen. Verlies van BRD7-expressie in deze tumoren kan optreden door methylatie van het gen. Wij laten in hoofdstuk 2 zien dat verlies van BRD7 ook optreedt in borsttumoren, maar specifiek in de tumoren met een intact p53 gen. We hebben gevonden dat verlies van BRD7 ook kan worden veroorzaakt door deletie van de genomische regio waarin BRD7 gecodeerd is. Onze observatie dat verlies van BRD7 vooral optreedt in tumoren met een intact p53 gen bevestigt dat het eiwit een belangrijke rol speelt in de p53 route. Verlies van BRD7 is dus een mogelijke manier waarmee kankercellen de p53 route kunnen inactiveren zonder het p53 gen te muteren. In hoofdstuk 3 bediscussiëren we de resultaten die worden gepresenteerd in hoofdstuk 2 en leggen we verbanden met de al bekende literatuur over gerelateerde onderwerpen.

Naast eiwitten kunnen genen ook coderen voor RNA's waarvan geen eiwit gemaakt wordt, zogenoemde niet-coderende RNA's (ncRNA's). Ook ncRNA's spelen een belangrijke rol bij deling en apoptose van cellen. Als transcriptiefactor kan p53 de transcriptie van tientallen genen reguleren. Recentelijk is aangetoond dat ook ncRNA's door p53 gereguleerd worden. In hoofdstuk 4 beschrijven we de identificatie van zeven regio's die in een p53 afhankelijke manier ncRNA's produceren. We laten zien dat deze ncRNA's nodig zijn voor een efficiënt p53 afhankelijk cel cyclus arrest. De regio's dragen alle beschreven kenmerken van een zogenaamd versterkingsdomein. Een versterkingsdomein versterkt transcriptie van genen die op verre afstand van het domein liggen. We laten zien dat p53 nodig is voor de activiteit van het versterkingsdomein en op die manier transcriptie van genen reguleert.

In hoofdstuk 5 bestuderen we een andere klasse ncRNA's, namelijk microRNA's (miRNA's). MiRNA's zijn kleine, niet-coderende RNA's die mRNA-expressie kunnen

reguleren door te binden aan de 3'UTR van mRNA's. Dit kan leiden tot zowel remming van translatie (het maken van eiwit uit mRNA) of afbraak van mRNA. We beschrijven de studie naar het miR-17-92 miRNA-cluster en de rol die het speelt in de transformatie van normale cel naar tumorcel. Het miR-17-92 cluster bevat zes miRNA's (miR-17, miR-18a, miR-20a, miR-19a, miR-19b, miR-92) en voor alle is een oncogene functie beschreven. Wij laten zien dat expressie van het cluster interfereert met de pRB tumorsuppressorroute. Expressie van een oncogen leidt normaal gesproken tot lage niveaus gemodificeerd pRB eiwit waardoor het aan de E2F transcriptiefactor kan binden en transcriptie remt. Wanneer zowel een oncogen als miR-17-19 (miR-17-92 zonder miR-92) tot expressie wordt gebracht blijft pRB gephosphoryleerd en ongebonden, waardoor E2F transcriptie van genen die nodig zijn voor de celdeling kan blijven activeren. De verantwoordelijke mRNA's waaraan de miRNA's binden hebben we niet kunnen vinden, waarschijnlijk doordat de miRNA's op meerdere niveaus in de route acteren door meerdere mRNA's te reguleren. We stellen voor dat alle miRNA's uit het cluster nodig zijn voor de meest efficiënte remming van de pRB-route en dat er dus samenwerking plaatsvindt tussen de miRNA's.

In dit proefschrift doen we onderzoek naar tumorsuppressorroutes die betrokken zijn bij de transformatie van een normale cel naar een tumorcel. We presenteren een aantal nieuwe componenten van deze routes en laten zien hoe deze componenten functioneren in – en bijdragen aan – het tumorsuppressoreffect van de routes.

CURRICULUM VITAE

Jarno Drost was born on the 21st of March 1982 in Nunspeet (The Netherlands). He received his secondary school HAVO diploma from the Christelijk College Nassau-Veluwe (Harderwijk, The Netherlands) in 1999. In the same year he started his study Hoger Laboratorium Onderwijs (HLO) (Utrecht, The Netherlands) and received his Bachelor's degree in the specialization molecular biology in 2003. During this study he carried out an internship at the department of Immunotherapy at the Wilhelmina Kinderziekenhuis, University Medical Center, Utrecht. Under supervision of dr. L. Steeghs, he investigated the role of Nods in the immune stimulating properties of *Neisseria meningitidis* lipopolysaccharide. Next, he continued his study at the VU University (Amsterdam, The Netherlands) where he received his Master's degree in Biomolecular Sciences (*cum laude*) in August 2005. As part of this study he performed two internships. The first was carried out at The Netherlands Cancer Institute (Amsterdam) in the laboratory of prof.dr. R. Agami at the Division of Tumor Biology. Under supervision of dr. P.M. Voorhoeve he screened for novel regulators of the p53 pathway. He did his second internship at the Hubrecht Institute (Utrecht) in the laboratory of prof.dr. H. Clevers. Under supervision of dr. J.H. van Es he worked on the identification of genes contributing to Goblet cell conversion upon disruption of the Notch signalling pathway. In September 2005 he joined the group of prof.dr. R. Agami at the Division of Gene Regulation at The Netherlands Cancer Institute in Amsterdam and conducted the research described in this thesis.

LIST OF PUBLICATIONS

Elkon R, **Drost J***, Jenal M*, Schrier M, Oude Vrielink JA, van Haaften G, Agami R.
Extensive modulation of alternative polyadenylation associated with cellular proliferation and transformation.
(Manuscript in preparation)

Jenal M, Elkon R, Loayza-Puch F, van Haaften G, Kuehn U, Menzies FM, Oude Vrielink JA, Bos A, **Drost J**, Rooijers K, Rubinsztein DC, Agami R.
The Poly(A)-Binding Protein Nuclear 1 (PABPN1) suppresses alternative cleavage and polyadenylation sites.
(Manuscript in preparation)

Melo C*, **Drost J***, Wijchers PJ, van de Werken H, de Wit E, Elkon R, Oude Vrielink JA, Melo SA, de Laat W and Agami R.
Enhancer-bound p53 produces eRNAs and affects long distance transcription.
(Manuscript in preparation)

Mantovani F*, **Drost J***, Voorhoeve PM, Del Sal G, Agami R.
Gene regulation and tumor suppression by the bromodomain-containing protein BRD7. *Cell Cycle* **9**, 2777-2781 (2010).

Drost J*, Mantovani F*, Tocco F, Elkon R, Comel A, Holstege H, Kerkhoven R, Jonkers J, Voorhoeve PM, Agami R, Del Sal G.
BRD7 is a candidate tumour suppressor gene required for p53 function. *Nat. Cell Biol.* **12**, 380-389 (2010).

Drost J, Agami R.
Transformation locked in a loop. *Cell* **139**, 654-656 (2009).

Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, **Drost J**, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R.
A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* **124**, 1169-1181 (2006).

* These authors contributed equally