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p53 at the crossroads between cancer and neurodegeneration: unveiling molecular circuitries involved in tumorigenesis and neuronal cell death

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p53 at the crossroads between cancer and neurodegeneration: unveiling molecular circuitries involved in tumorigenesis and neuronal cell death

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"I am among those who think
that science has great beauty"

Marie Curie 1867-1934

To my Mother
for teaching me
the importance of
passion and sacrifice

To my Father
for showing me
the beauty of Nature

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2. ABSTRACT

Cancer and neurodegeneration are linked by a relation of inverse comorbidity, cancer patients being at lower risk for neurodegenerative disorders and *vice versa*. Interestingly, many cellular processes and factors contribute to both pathologies, and a central role is played by the transcription factor p53. Best known for its antiproliferative activities following transformation-related *stimuli*, p53 acts to maintain genetic stability and prevent tumour onset by transcriptional and non-transcriptional mechanisms. Recently, a contribution of p53 also in neuronal development and death was unveiled. In the case of Huntington's Disease (HD), p53 mediates cytotoxicity in HD cells and animal models, whereas its inhibition prevents this phenotype.

On these premises, we were prompted to investigate the signalling pathways and protein interactions that modulate p53 activation in both cancer and neurodegeneration with the aim of identifying critical hubs as new targets for therapeutic intervention. We discovered that expression of HD causative agent, i.e. mutant Huntingtin (mHtt) protein, behaves like a genotoxic *stimulus* in inducing phosphorylation of p53 on Ser46, that leads to its modification by phosphorylation-dependent prolyl-isomerase Pin1 and consequent induction of apoptotic target genes. Inhibition of Ser46 phosphorylation by targeting HIPK2, PKCδ, or ATM kinases, as well as inhibition of Pin1, prevented mHtt-dependent apoptosis of neuronal cells. These results provide a rationale for the use of small-molecule inhibitors of stress-responsive kinases and Pin1 as a potential therapeutic strategy for HD treatment.

On the other hand, we investigated the contribution of BRD7, a protein involved in epigenetic regulation, to the p53 pathway. We found that BRD7 is required for the onset of oncogene-induced senescence, a main tumour suppressive p53 activity. In addition, we found that upon oncogene activation BRD7 restrains, independently of p53, the acquisition of malignant phenotypes, such as migration/invasion and stem cell traits. We observed a strong induction of inflammatory genes after depletion of BRD7, whose contribution to the tumorigenic process is currently under investigation. BRD7 takes part into the SWI/SNF and PRC2 chromatin-modifying complexes, appealing targets for cancer therapy for their pleiotropic roles and multi-subunit composition. We will also discuss how this new generated knowledge could be exploited for the treatment of neurodegenerative diseases, in which chromatin alterations are now recognized as drivers of pathogenesis.

3. INTRODUCTION

3.1 Unveiling the link between cancer and neurodegeneration

Medical research tends to adopt a reductionist approach by studying different disorders as isolated phenomena, thus often neglecting the alternative approach of exploring and highlighting those mechanisms that are either common or in inverse relationship to multiple pathological conditions. Back in the 1970s, the clinician, researcher and epidemiologist Alvan Feinstein defined the concept of ***comorbidity*** as "any distinct additional clinical entity that has existed or may occur during the clinical course of a patient who has the index disease under study" (Feinstein, 1970). This definition places one disease in a central position and treats all the other diseases in the same patient as secondary. For example, depression, thyroid disorders, sleep apnoea, osteoporosis and glaucoma are more common in patients with Alzheimer's Disease than in matched individuals without Alzheimer's Disease. If the mechanisms underlying the aetiology of this comorbidity were to be clarified, the potential for developing more specific therapies and improving patient quality of life would be enormous (Barnett et al., 2012). It is important to note that unveiling the mechanisms underlying comorbidity may be the key to comprehend the relationship between the genome and its phenotypic manifestations.

In the recent years, the concept of the association between certain diagnoses and a lower-than-expected probability of developing other diseases, termed ***inverse comorbidity***, has emerged (Ukraintseva et al., 2010). In particular, numerous epidemiological and clinical studies reveal that Down's syndrome, Parkinson's Disease (PD), schizophrenia, Alzheimer's Disease (AD), multiple sclerosis, Huntington's Disease (HD), anorexia nervosa, diabetes and allergy-related diseases seem to protect against many forms of cancer, including solid tumours, smoking related tumours, prostate cancer and Hodgkin's and non- Hodgkin's lymphoma. A recent comprehensive review of the studies performed about the association of cancer risk with neurodegenerative diseases has been done (Catala-Lopez et al., 2014). This review, which comprises more than 40 studies, shows that patients with neurodegenerative disorders have a substantially lower overall risk of developing cancer.

The mechanisms underlying the inverse comorbidity between CNS disorders and cancer are still a controversial matter of debate; indeed there are some interesting theories that

present the problem in an evolutionary perspective. This perspective refers to the concept of ***antagonistic pleiotropy*** (Campisi, 2005; Papazoglu and Mills, 2007) that is the hypothesis that genes or processes that were selected to benefit the health and fitness of young organisms can have unselected deleterious effects that manifest in older organisms and thereby contribute to degenerative processes during ageing (Campisi and d'Adda di Fagagna, 2007). Tumour suppressors, whose loss enhances tumour formation, are the main actors of the surveillance mechanism to avoid aberrant proliferation under normal conditions and represent a very good example of antagonistic pleiotropy, because they promote early-life survival by preventing the development of cancer, but eventually limit longevity due to the depletion of irreplaceable post-mitotic cells in non-renewable tissues or to the elimination of proliferating or stem cell pools in renewable tissues.

In the following paragraphs, we will focus on the inverse comorbidity between CNS disorders and cancers with a particular focus on the possible molecular mechanisms and pathways underlying this phenomenon. A particular relevance will be given to ***p53***, the prototypical tumour suppressor whose pathway is inactivated in most human cancers (Murray-Zmijewski et al., 2008) but whose persistent activation has been shown to play a role in promoting aging and age-related disease such as neurodegeneration. Indeed p53 is activated and integrates the multiple incoming signals that sense different forms of cellular stress, and reacts by orchestrating appropriate cellular fates depending on both the cellular context and the entity and duration of the stress *stimulus*. Cell responses may vary from DNA repair and antioxidant processes to antiproliferative responses including senescence and apoptosis, thus, it is conceivable that small deregulations towards either one or the other side, could favour survival/regeneration or death/senescence of the cells.

Shared cellular pathways and major disease regulators

The cell cycle hypothesis. Trying to define common cellular mechanisms shared by CNS disorders and cancer may be counterintuitive because these two pathologies are often thought as characterized by disease mechanisms lying at opposite ends of a spectrum. Indeed neurons, for their nature of cells that should maintain their integrity throughout the lifespan, are resistant to mitotic signals and, lacking key mitotic proteins, are completely incapable of cell division (Ackman et al., 2007) while cancer cells are defined primarily by

uncontrolled cell growth (Hanahan and Weinberg, 2011). Surprisingly however, inappropriate entry into the cell cycle seems to be a key, early event in the degenerating neurons from Alzheimer's Disease, Parkinson's Disease and other neurodegenerative syndromes (Busser et al., 1998; El-Khodor et al., 2003; Hoglinger et al., 2007; Husseman et al., 2000; McShea et al., 1997; Nagy et al., 1997a; Nagy et al., 1997b; Smith and Lippa, 1995). Importantly, distortion of the cell division plane (which can be parallel or perpendicular to the proliferating epithelium) interferes with progenitor proliferation, which can lead to apoptosis in neurodevelopmental and neurodegenerative disorders such as lissencephaly (Bi et al., 2009) and Huntington's Disease (Godin et al., 2010). HD is particularly interesting under this point of view because alteration of the protein Huntingtin modifies the primary cilia that regulate progenitor cells in the neuroepithelium, disrupting neuroblast migration and differentiation (Keryer et al., 2011). If this effect also occurred in epithelial cells, it would produce a disruption of tumour progression and metastasis leading to cancer regression.

The DNA damage response. A shared hallmark of cancer and neurodegeneration is the presence of alterations in the DNA damage response (DDR) pathway. DDR, a hierarchical process that entails DNA damage *sensors*, *transducers* and *downstream effectors*, acts either by promoting the repair of the lesions or by depleting the cell form the proliferative pool fostering processes such as apoptosis and senescence depending on the length, the intensity and the nature of the damage (Jackson and Bartek, 2009). Being the first line of defence against DNA damage, DDR plays a pivotal role in the maintenance of genome stability and in sustaining cellular homeostasis in a variety of contexts such as development, differentiation and normal cell growth. Defects in the DDR in proliferating cells can lead to genomic instability and ultimately to cancer allowing the bypass of tumour suppressive mechanisms, while DDR defects or aberrant activation in neurons may result in neurodegeneration. Indeed neurons are unique in being terminally differentiated and in sustaining extreme metabolic pressure due to high rates of transcription and translation associated with high mitochondrial activity. This activity, along with high oxygen consumption, creates a stressful environment where reactive oxygen species (ROS) can lead to DNA damage. The inability to respond properly to this damage, therefore, can be detrimental for cell survival and ultimately lead to neuronal cell death, as for example

happens in the case of the deficiency of the apical kinase of the DDR, ATM, whose loss causes ataxia-telangiectasia (AT), characterized by progressive cerebellar degeneration (Barzilai, 2010). In other neurodegenerative pathologies, such as HD, the presence of pathogenic proteins is able to activate, *via* ROS generation, the DNA damage response towards apoptosis, ultimately leading to cell death (Illuzzi et al., 2009).
Conversely, the robust activation of the DNA damage response in normal growing cells following various kind of cellular stress such as telomere attrition and oncogene activation is able to activate oncosuppressive processes among which a central part is played by cellular senescence (d'Adda di Fagagna, 2008).

Cellular senescence: a link between cancer and neurodegeneration. The role of cellular senescence in neurodegeneration and cancer is an emerging topic of interest in ageing and age-related diseases and might provide additional insights into the molecular and genetic basis of the inverse relationship between these two classes of disorders (Campisi et al., 2011). Cellular senescence is an established cellular stress response, defined as an essentially irreversible loss of proliferative ability, that acts both physiologically being activated by telomere attrition and leading to ageing but also to prevent the expansion of cells that experience potentially oncogenic stress. Cellular senescence is a good example of antagonistic pleiotropy. Indeed in this case a tumour suppressive mechanism, which is clearly beneficial early in life for tumour protection, becomes detrimental later in life when neurodegeneration tends to appear. For this aspect in particular there are a lot of evidences involving a cell non-autonomous effect of senescence, the senescence-associated secretory phenotype (SASP), which entails the secretion of numerous cytokines, growth factors and proteases (Coppe et al., 2008; Kuilman et al., 2008) that can have autocrine or paracrine effects. When senescence is activated as a tumour suppressive mechanism, SASP is beneficial because it attracts and activates cells of the immune system favouring the clearance of senescent cells from tissues *in vivo* (Xue et al., 2007) and reinforces the growth arrest (Acosta et al., 2008; Kuilman et al., 2008) thanks to the pro-inflammatory cytokines IL6 and IL8, the protease inhibitor plasminogen activator inhibitor-1 (PAI-1) and the pleiotropic protein insulin-like growth factor binding protein-7 (IGFBP-7). On the other hand SASP can be a detrimental mechanism for neurodegeneration as demonstrated by the fact that expression of a SASP by senescent astrocytes, which has been documented

both in cells that were made senescent in culture as well as cells that were isolated from aged brain tissue, has been proposed to initiate or contribute to neuroinflammation (Bitto et al., 2010; Salminen et al., 2011), a characteristic of many neurodegenerative diseases thought to cause or exacerbate the age-related decline in both cognitive and motor functions.

Cellular pathways altered in cancer and neurodegeneration. In the past years there have been several evidences that important cellular signalling pathways are upregulated in cancer, while downregulated or dysfunctional in neurodegeneration and *vice versa*. To understand the extent of the deregulation in opposite directions of a common set of genes and pathways in cancer and neurodegeneration, Alfonso Valencia and co-workers recently (Ibanez et al., 2014) performed transcriptomic meta analyses of three CNS disorders (Alzheimer's Disease, Parkinson's Disease and schizophrenia) and three Cancer types (Lung, Prostate, Colorectal) previously described with inverse comorbidities according to population studies. Noteworthy, from this study it emerged that 89% of the pathways that were upregulated in cancers were downregulated in CNS disorders and mainly related to Metabolism and Genetic Information processing. By contrast, the pathways downregulated in cancers, found to be upregulated in CNS disorders, were related to the cell's communication with its environment. Interestingly, the analysis also revealed pathways related to protein folding and protein degradation displaying patterns of downregulation in CNS disorders and upregulation in cancers; among these, a particular relevance has to be given to the Ubiquitin/Proteasome system (UPS). The link between the protein degradation system, cancer and neurodegeneration was already known by single-gene analysis. For example, the genes linked to familial PD (the PARK loci) are primarily involved in protein processing and clearance, but many also play a role in development and cell cycle regulation. Parkin (PARK2) and ubiquitin C-terminal hydrolase (UCHL1, or PARK5) form part of the ubiquitin-proteasome system (UPS), the primary cellular pathway by which intracellular proteins are degraded. Dysfunction of the UPS leads to the accumulation of intracellular proteins, the formation of Lewy bodies (the pathological hallmarks of PD) and eventually to apoptosis (Sherman and Goldberg, 2001). Proteasome dysfunction is also linked to sporadic PD (McNaught et al., 2003) and proteasome inhibition can cause PD in animal models (McNaught et al., 2004). In contrast, as already said, upregulation of UPS is

associated with many cancers (Montagut et al., 2006). Given these premises the ubiquitin proteasome system appears to be a common link between cancer and neurodegeneration and an exciting target for new therapies. Beginning with bortezomib for the treatment of multiple myeloma, proteasome inhibitors are already an established anti-cancer therapy (Crawford et al., 2011). Conversely, although largely unexplored, proteasome activation is an attractive area of research for therapeutic treatment of a variety of neurodegenerative diseases (Huang and Chen, 2009).

Single genes. Many proteins when abnormally expressed or aberrantly regulated have been linked to cancer or neurodegeneration. A review of common factors and overlapping pathways identified in the progression of cancer and neurodegeneration is presented in Table 1.

Gene	Function	Role in neurodegeneration	Role in cancer
α-synuclein (PARK1/4)	Unclear	Gain of function leads to PD, main component of Lewy bodies in PD.	α-synuclein is aberrantly expressed and methylated in cancer.
PINK1 (PARK6)	Kinase	Loss of function leads to PD. Loss of PINK1 functions leads to mitochondrial deficits.	Somatic mutations in cancer (COSMIC Web site). Tumour suppressor? Induced by PTEN.
DJ-1 (PARK7)	Unclear	Loss of function leads to PD. DJ-1 might act as a neuroprotective oxidative stress sensor.	Oncogene. Regulates negatively PTEN. Over-expression in several tumous.
LRRK2 (PARK8)	Kinase, GTPase	Gain of function leads to PD. Enzymatic activity thought to play a key role in the disease.	Somatic mutations in cancer (COSMIC Web site). Oncogene?
ATP13A2 (PARK9)	ATPase	Loss of function leads to PD. May alter autophagic lysosomal function.	ALP plays an important role in cancer.
PLA2G6 (PARK14?)	Phospholipase A2	Mutations lead to infantile neuroaxonal dystrophy (INAD), idiopathic neurodegeneration with brain iron accumulation (NBIA) and dystonia-parkinsonism .	PLA2G6 was identified as a risk factor for melanoma.
Tau (MAPT)	Microtubule-associated protein	Mutations in Tau lead to AD and FTDP-17. Tau is the major component of neurofibrillary tangles in AD.	Reduced expression in several tumours.
APP/PS1,2	Unclear	Gain of function leads to AD type. Mutations in APP and the presenilins increase production of Aβ, which is the main component of senile plaques in AD.	APP is overexpressed in acute myeloid leukemia patients with complex karyotypes.
SOD1	Superoxide dismutase	Gain of function leads to ALS. Mutations thought to cause cell death via aggregation and oxidative damage.	Controversial role in breast cancer.
Huntingtin	Unclear	Gain of function leads to HD.	The Huntington disease protein accelerates breast tumour development and metastasis through ErbB2/HER2 signalling.
Parkin (PARK2)	E3 ubiquitin ligase	Loss of function leads to PD. Parkin enzymatic activity is thought to play a key role in the disease. Loss of parkin function leads to mitochondrial deficits.	Tumour suppressor.

ATM	Kinase	Mutations in the *ATM* gene cause ataxia-telangiectasia. ATM inactivation leads to cerebellar neuron loss.	Tumour suppressor. *ATM* mutations carriers at increased risk of developing cancer, especially breast cancer. Role in cell cycle and DNA damage.
CDK5	Kinase	CDK5 can phosphorylate Tau and parkin. Also associated with AD.	Somatic mutations in cancer.
PTEN	Phosphatase	Functional link between PTEN and PINK1, parkin and DJ-1.	Tumour suppressor, mutated in sporadic and inherited tumours.
mTOR	Kinase	May play a role in neurodegeneration through inhibition of autophagy.	Autophagy can be both oncogenic as well as tumour suppressive.
TSC1/TSC2	Vesicular transport	May play a role in neurodegeneration through mTOR-dependent autophagy.	Tumour suppressors.
p53	Transcription factor	Functional link between p53 and parkin, Aβ, APP and Huntingtin.	Tumour suppressor.

Table 1. Genetic determinants at the interface of cancer and neurodegeneration. Common factors and overlapping pathways can be identified in the progression of both cancer and neurodegeneration (Plun-Favreau et al., 2010).

As previously said, among the genetic determinants shared by cancer and neurodegeneration an important role is played by proteins implicated in DNA damage response and the downstream events of cell cycle arrest and apoptosis. In this context, a particular relevance has to be given to p53. Interestingly, although p53 function in repressing carcinogenesis is known since several years (Donehower et al., 1992), its association with neurodegenerative disorders, including HD, AD and PD (Davenport et al., 2010; Dunys et al., 2009; Jacobs et al., 2006), is becoming increasingly evident and will be discussed in next paragraph.

p53 at the crossroads between cancer and neurodegeneration

A common hallmark of cancer is the inactivation of tumour suppressor genes among which a key role is played by p53. As mentioned previously, the p53 protein is activated following transformation-related *stimuli* (genotoxic damage, deregulated oncogenes, hypoxia, etc.), and acts to maintain genetic stability by transcriptional and non-transcriptional mechanisms (Murray-Zmijewski et al., 2008).

Confirming the importance of p53 for tumour suppression, on average *TP53* is mutated in 31% of all tumours included in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Forbes et al., 2011); mutations, however, occur more frequently in some types of tumours than in others, in particular in ovarian (50% of cases in COSMIC), large intestine (43%) and lung (36%) cancers. In other kind of tumours where p53 mutations occur less

frequently such as breast cancer, hotspot p53 mutations were reported to be associated with more aggressive malignancies and could confer novel phenotypes *in vivo* including an increased metastatic capacity and resistance to chemotherapies (Garritano et al., 2013). In most other cancer cases in which the *TP53* gene is wild-type, genetic lesions in components of the p53 pathway interfere with its activation; a classical example is the increased turnover or sequestration of the p53 protein itself through overexpression of its negative regulator MDM2 (Levine and Oren, 2009). Indeed it has been found a single nucleotide polymorphism (SNP) in the human *MDM2* gene (Bond et al., 2004) that confers higher expression levels of the protein; individuals carrying this polymorphism are predisposed to early-onset cancer, suggesting that subtle differences in basal p53 activity may be enough to affect cancer risk (Whibley et al., 2009).

A further proof of p53 importance as a tumour suppressor is given by the fact that mice engineered to be deficient in *TP53* are developmentally normal, but susceptible to spontaneous tumours (Donehower et al., 1992). In addition, knockout p53 mice ($p53^{-/-}$) have a significantly higher number of proliferating cells (Meletis et al., 2006). Interestingly, these p53-null mice are also mildly resistant to neurotoxicity (Morrison et al., 1996). Indeed, data from *in vitro* and *in vivo* models recently unveiled a possible central role of p53 in neuronal development and death. A strong correlation between p53 expression and excitotoxic neuronal death induced by glutamate, kainic acid and N-methyl-D-aspartate has been established (Cregan et al., 1999; Uberti et al., 1998; Xiang et al., 1998). Moreover, many studies demonstrated that inhibition of p53 prevents cell death in a variety of neurodegenerative models. For example pifithrin-α, a drug that inhibits the transcriptional activity of p53 (Bassi et al., 2002; Komarov et al., 1999), attenuated neuronal death in several different rodent models of stroke (Culmsee et al., 2003; Culmsee et al., 2001; Zhu et al., 2002) and in cultured neurons exposed to DNA-damaging agents, glutamate and Aβ peptide (Culmsee et al., 2001). In the next paragraphs we will provide a brief review on what is known about the involvement of p53 in some of the most common neurodegenerative diseases such as AD, PD, schizophrenia and HD.

Alzheimer's Disease. AD is accompanied by neurodegeneration and neuronal loss in the frontal cortex, leading to a slow but progressive deconstruction of cognition, namely memory, emotion, reasoning and judgement. The most common form of AD is usually characterized by the deposition of amyloid-β (Aβ) protein in the extracellular plaques

within the brain (Baloyannis, 2006; Pereira et al., 2004), which leads to neuronal death, and by intraneuronal neurofibrillary tangles composed of the microtubule-associated protein tau (Seyb et al., 2006; Zheng et al., 2002b). A minor percentage of AD cases is familial (accounting for less than 1% of cases). Most of autosomal dominant familial AD can be attributed to mutations in genes encoding APP (β-amyloid precursor protein) and presenilin-1 and/or presenilin-2, which are parts of the γ-secretase complex involved in the generation of the Aβ peptide from the amyloid precursor protein (APP) (Batelli et al., 2008). The best known genetic risk factor of sporadic AD is the apolipoprotein E (APOE) ε4 isoform. Regardless of how the pathology is triggered, p53 is highly elevated in AD brains (Chung et al., 2000; Kitamura et al., 1997). Interestingly, in human neurons treated with β-amyloid peptides (Paradis et al., 1996) and Alzheimer's brains (Su et al., 1997), it has been reported a decrease of the anti-apoptotic p53 target Bcl-2 with concomitant increase in pro-apoptotic Bax expression. Moreover, p53 appears to mediate apoptosis in primary human neurons expressing Aβ1-42 (Zhang et al., 2002a), and also microglial apoptosis seems to be mediated by p53 in AD (Davenport et al., 2010). Interestingly, a conformational isoform of p53 has been identified to be associated with AD (Lanni et al., 2008), suggesting that p53 is either mutated or misfolded in AD.

Parkinson's Disease. The pathology of PD involves loss of neurons in the *substantia nigra*. It initially starts as a movement disorder that progresses into cognitive and language impairment and eventually dementia. As for AD, elevation in p53 levels is also seen in PD brains (Mogi et al., 2007). p53-mediated neuronal death is observed both in cellular (Lee et al., 2006) and animal (Martin et al., 2006) models. Strikingly, several PD associated genes play a role in inducing p53 expression and/or transcriptional activity and surprisingly, three of these genes are associated with autosomal recessive juvenile PD. For example, loss of parkin function leads to an increase in p53 mRNA levels and transcriptional activity (da Costa et al., 2009). Another trait associated with PD is loss of the function of the protein α-synuclein as mutant α-synuclein expression serves as a murine model of PD. It is interesting to note that Syphilin-1, a binding partner of α-synuclein, inhibits p53 transcriptional activity (Giaime et al., 2006). It has been suggested that α-synuclein may also have a role in inhibiting p53 activation and transcriptional activity (Alves Da Costa et al., 2002), and thus it is conceivable that its loss will foster p53 activity. Considering this, down-modulating neuronal p53 activity may be therapeutic in PD.

Schizophrenia. Schizophrenia is a complex neuropsychiatric syndrome with several symptomatic dimensions, including positive symptoms such as reality distortion (for example hallucinations), negative symptoms (e.g. social withdrawal) and cognitive impairments. It is important to underline that the relationships between schizophrenia and individual types of cancer are more complex than those between the other neurodegenerative pathologies and cancer (Tabares-Seisdedos and Rubenstein, 2013). For example, patients with schizophrenia have a lower risk of prostate cancer and melanoma but a higher risk of breast and lung cancers. Recent evidences suggest that p53 has a role in schizophrenia. More specifically, the genetic evidence of *TP53* as a schizophrenia-susceptibility gene is strong, with five out of six studies reporting a significant association (Tabares-Seisdedos and Rubenstein, 2009). The meaning of this association, however, still needs to be elucidated.

Huntington's Disease. HD is a genetically dominant neurodegenerative disease caused by an expanded, unstable CAG repeat sequence in the *huntingtin* gene leading to abnormal Huntingtin protein product (Htt) (The Huntington's Disease Collaborative Research Group, (1993). Mutant Huntingtin protein (mHtt) contains an elongated polyglutamine (polyQ) trait whose length correlates with an earlier age of disease onset. The disease manifests at a mean age of 35 years and is characterized by progressively worsening chorea, psychiatric impairment and cognitive decline (Ross and Shoulson, 2009). The most striking pathological manifestation of HD is a specific and gradual loss of medium-sized spiny neurons in the caudate and in the putamen, though the *substantia nigra* and the cortex, among the others, are affected by the presence of mHtt. The HD mutation, by virtue of the expanded glutamine tract, is likely to confer toxic properties to the mutant protein. A gain of function mechanism is indeed supported by genetic and experimental data. HD is a dominant disease and in knockout mouse models, complete Huntingtin deficiency does not cause an HD-like phenotype. In most cells, Huntingtin is primarily a cytoplasmic protein associated with various organelles including mitochondria, endoplasmic reticulum and Golgi complex, but a fraction is also found in the nucleus. The pathophysiology of HD has been linked to apoptotic insult, defect in the proteosomal apparatus, glutamate mediated excitotoxicity, and mitochondrial dysfunction (Grunewald and Beal, 1999; Sawa, 2001; Schapira, 1997), but nuclear disturbances are also implicated with HD (Hodgson et al., 1999; Petersen et al., 1999; Schilling et al., 2004; Steffan et al., 2000): recent evidences

suggest that mutant Huntingtin may disrupt normal transcription program in neurons (Figure 1).

In the last decades an involvement of p53 in HD has been clearly demonstrated. First, p53 protein levels are elevated in the brains of Huntington's Disease patients (Bae et al., 2005), while susceptibility of spiny neurons to mHtt-mediated injury was directly correlated to p53 elevation and indirectly correlated to the endogenous levels of the p53-repressed gene Bcl-2 (Liang et al., 2005). mHtt has also been found to interact with p53 in the inclusion body, both biochemically (Steffan et al., 2000) and genetically (Ryan et al., 2006). Moreover, genes regulated by p53 are among a large cohort of genes upregulated in cell cultures transfected with mHtt (Sipione et al., 2002). Confirming these evidences, in 2005 it was demonstrated (Bae et al., 2005) that the DNA damage response engaged by mHtt and mediated by the activation of the ATM/ATR pathways (Illuzzi et al., 2009) is able to activate p53, that in turn mediates mitochondrial dysfunction and cytotoxicity in HD cells and in transgenic animal models whereas its inhibition (either genetic or pharmacologic) prevents these phenotypes.

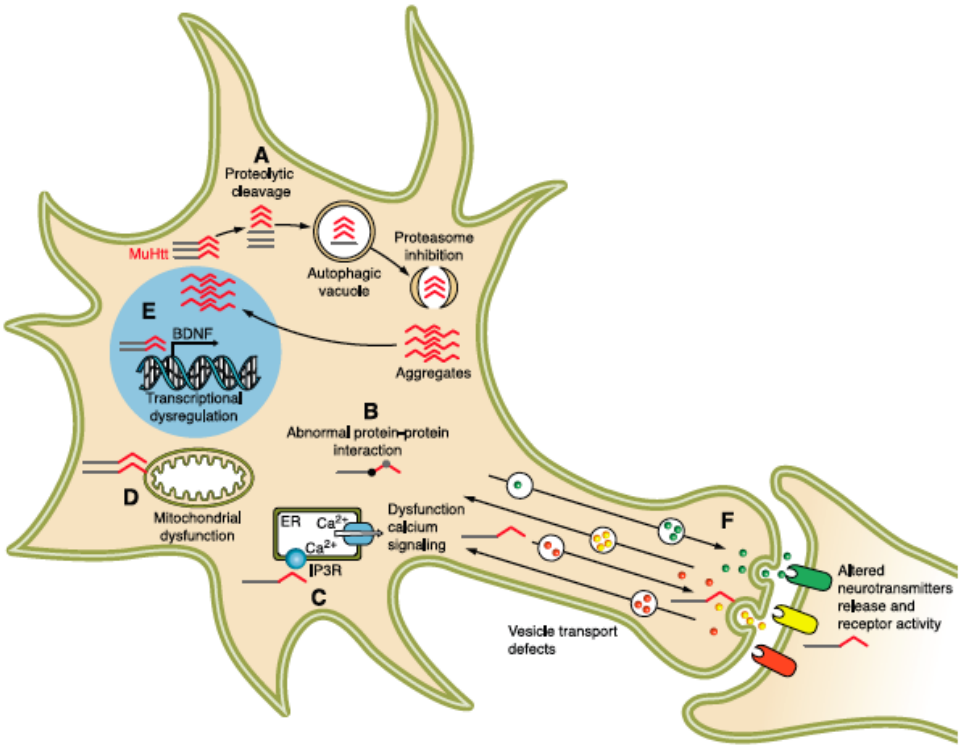


Figure 1. Key pathogenic mechanisms in Huntington's disease (HD). A: the mutation in Huntingtin causes a conformational change of the protein that leads to partial unfolding or abnormal folding of the protein. Full-length mutant Huntingtin is cleaved by proteases in the cytoplasm. In an attempt to eliminate the toxic Huntingtin, fragments are ubiquitylated and targeted to the proteasome for degradation. However, the proteasome becomes less efficient in HD. Induction of the proteasome activity as well as of autophagy

protects against the toxic insults of mHtt proteins by enhancing its clearance. B: NH_2-terminal fragments containing the polyQ stretch accumulate in the cell cytoplasm and interact with several proteins causing impairment of calcium signaling and homeostasis (C) and mitochondrial dysfunction (D). E: NH_2-terminal mHtt fragments translocate to the nucleus where they impair gene transcription or form intranuclear inclusions. F: the mutation in Huntingtin alters vesicular transport and recycling (Zuccato et al., 2010).

In the next session, we will summarize the vast knowledge about the tumour suppressor p53, in particular the cellular events that drive its activation and their different outcomes and the regulation of its activity.

3.2 The tumour suppressor p53

p53 in health and disease

The p53 protein, encoded by the *TP53* gene, is a tumour suppressor able to integrate different physiologic and pathologic *stimuli* and to orchestrate adequate cellular responses in order to maintain genomic stability. Discovered in 1979 (Chang et al., 1979; Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979) as the main interacting partner of the viral SV40 T-antigen, only in the late 1980s its role as tumour suppressor was officially unveiled (Weisz et al., 2007). Since then, p53 has been one of the most intensively studied proteins worldwide, mainly due to the evidence that most of human malignancies bear the abovementioned alterations in its signalling pathway. Indeed, as said, genetic mutations inactivating normal p53 functions can be found in a consistent number of human tumours, with percentages that vary from nearly 50% in ovary cancer to 5,8% in cervical tumours (IARC TP53 Mutation Database).

Since its discovery the p53 protein has best been known and studied as a tumour suppressor for its ability to inhibit cell proliferation, by both blocking transiently or irreversibly cell cycle progression or promoting apoptotic cell death. The importance of these pathways for tumour suppression has been demonstrated *in vivo* by the study of the mechanisms of restoration of p53 in different mouse models (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Restoration of p53 in lymphomas led to their widespread apoptosis, whereas restoration of p53 in sarcomas and hepatocarcinomas led to a senescence-type response. These growth inhibitory functions of p53 are normally held dormant, and the p53 protein, present at low levels in the cells, gets stabilized and

activated in response to different kind of *stimuli* that a cell might encounter during malignant progression such as genotoxic damage, oncogene activation, loss of normal cell contacts and hypoxia (Oren, 2001). In unstressed cells, p53 activity is maintained at low levels through p53 degradation, principally mediated by the MDM2 E3-ubiquitin ligase and by the related protein MDM4 (known as MDMX in humans)(Finch et al., 2002; Lu and Levine, 1995; Momand et al., 1992; Wadgaonkar and Collins, 1999). Stress-induced post-translational modifications of both p53 and MDM2/MDM4 abolishes this interaction leading to p53 accumulation and the unleashing of its transcriptional activity (Toledo and Wahl, 2006); once active, the protein can mediate different biological phenomena depending on the entity and type of damage, among which the best characterized are temporary cell cycle arrest to favour DNA repair, programmed cell death (apoptosis) and permanent cell cycle arrest (senescence).

It is now becoming apparent that the spectrum of p53 activities may be far broader than simply promoting antiproliferative responses to acute stress. Indeed the ability to prevent cancer has been suggested to be an "evolutionarily late" cooption of primordial p53 activities that had initially evolved to protect the germline and monitor development (Aranda-Anzaldo and Dent, 2007; Vousden and Lane, 2007). In fact it is conceivable that protection against tumour formation was probably not the ancestral function of the p53 regulatory network, and support for this idea comes from taking into account the existence of p53 family members in simple, short-lived organisms and protists (Lu et al., 2009). Moreover, the relatively late appearance of *ARF* gene (an MDM2 inhibitor) orthologues in the vertebrate lineage – they are absent from both zebrafish and puffer fish genomes (Gilley and Fried, 2001) - seems consistent with this deduction as the corresponding proteins arguably represent fundamental links between oncogenic stress and p53 (Martins et al., 2006). Also the rapid post-translational regulation of p53 activity by modulating its interactions with MDM2 and MDM4 is a peculiarly vertebrate acquisition: no direct counterparts of MDM2 or MDM4 exist in invertebrates, and invertebrate p53 homologues lack the key residues with which these protein interact (Nordstrom and Abrams, 2000).

It is important to underline that, independently of the specific function, p53 is mainly acting as a transcription factor regulating both positively and negatively the expression of an elevated number of genes, directly binding in tetrameric form to specific target sequences on the DNA (p53-responsive elements, p53RE) (Bourdon et al., 1997; el-Deiry

et al., 1992; Funk et al., 1992; Laptenko and Prives, 2006). However, several transcription-independent activities have been described, mainly involved in potentiating the pro-apoptotic response acting in the cytoplasm and at mitochondria (Chipuk et al., 2005; Moll et al., 2005; Yee and Vousden, 2005).

A snapshot of the stresses activating the p53 pathway and its outcome is reported in Figure 2.

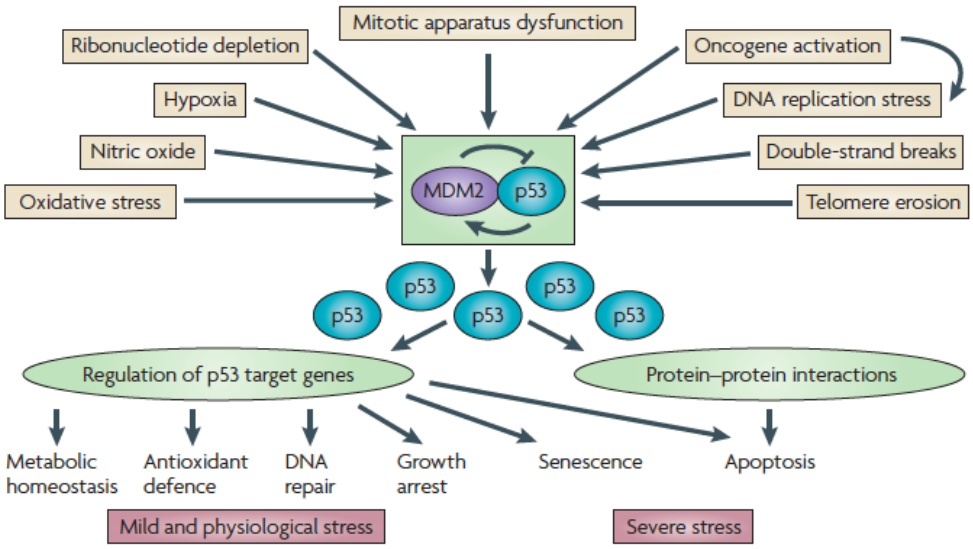


Figure 2. Simplified scheme of the p53 pathway. The p53–MDM2 feedback loop is the 'heart' of the p53 pathway. Under normal conditions, it maintains constantly low steady-state p53 levels and activity. Various stress signals related in many ways to carcinogenesis, impinge on this central loop to release p53 from MDM2-mediated inhibition. The downstream effects of p53 are largely due to its ability to transactivate and repress various subsets of target genes; however, at least in the case of apoptosis, protein–protein interactions in the cytoplasm (primarily with Bcl-2 family members) also have an important role. Recent evidence indicates that p53 also has an important role in enabling the cell to adjust its metabolism in response to mild normal physiological fluctuations, including those in glucose and other nutrient levels, oxygen availability and reactive oxygen species levels (Levine and Oren, 2009).

The p53 family

Three members of the p53 family are found in humans: p53, p63 and p73. As shown in Figure 3, all the members have an amino-terminal transactivation domain, a central DNA-binding domain and a carboxy-terminal oligomerization domain, but their primary sequences do not share an elevated homology, being about 30% for the whole proteins while reaching 65% when only the DNA binding domain is considered (IARC TP53 Database).

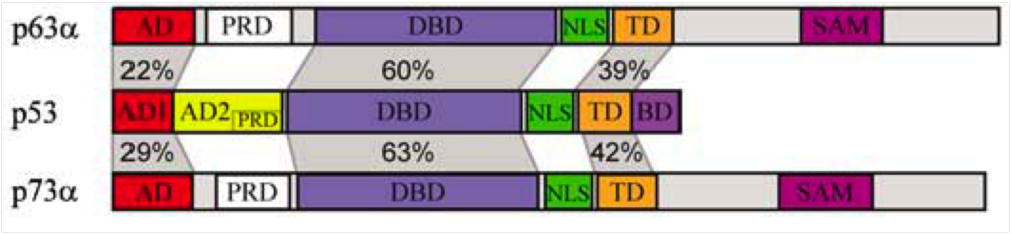


Figure 3. Conserved functional domains of the p53 family. The percentage indicates the degree of identity. AD: transactivation domains. PRD: proline-rich domain. DBD: DNA binding domain. NLS: nuclear localization signal. TD: tetramerization domain. BD: basic domain. SAM: sterile alpha motif(Harms and Chen, 2006).

Notably, p63 and p73 contain a sterile alpha motif (SAM) domain at the extreme C-terminus; this domain probably facilitates protein-protein interactions and, in the context of p73, has been implicated in protein turnover. The SAM domain is absent in p53 and therefore p63 and p73 share a more common ancestor.

p63. The human *TP63* gene is composed of 15 exons, spanning over 270.000 bp on chromosome 3q27. Altogether, the p63 gene expresses at least six mRNA variants, which encode for six different p63 protein isoforms (TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ) (Figure 4). The TAp63 isoforms are able to activate transcription of p53 target genes and induce cell cycle arrest or apoptosis. The ΔNp63 isoforms can bind DNA and can exert dominant-negative effects over p53, p73 and p63 activities by either competing for DNA binding sites or by direct protein interaction (Bourdon, 2007b). ΔNp63 isoforms were also shown to directly activate specific gene targets not induced by TA isoforms. ΔNp63 are abundantly expressed in progenitor cell layers of skin, breast and prostate, while TA p63 isoforms are barely detectable, indicating a switch between isoforms expression during normal cellular differentiation. However, TAp63 isoforms have been found to be expressed also in early embryogenesis to foster the initiation of epithelial stratification and inhibit terminal differentiation in a complex interplay with ΔNp63 isoforms.

p73. The human *TP73* gene is composed of 15 exons spanning on chromosome 1p36.3. The p73 gene expresses at least seven alternatively spliced C-terminal isoforms (α, β, γ, δ, ε, ζ and μ) (Bourdon, 2007b) and at least four alternatively spliced N-terminal isoforms initiated at different ATG (Figure 4). Altogether, the p73 gene expresses at least 35 mRNA variants, which can encode theoretically 29 different p73 protein isoforms. As for what reported for p63, both ΔN and TA isoforms have transactivation capabilities, and ΔNp73 can exert dominant negative functions over the other isoforms.

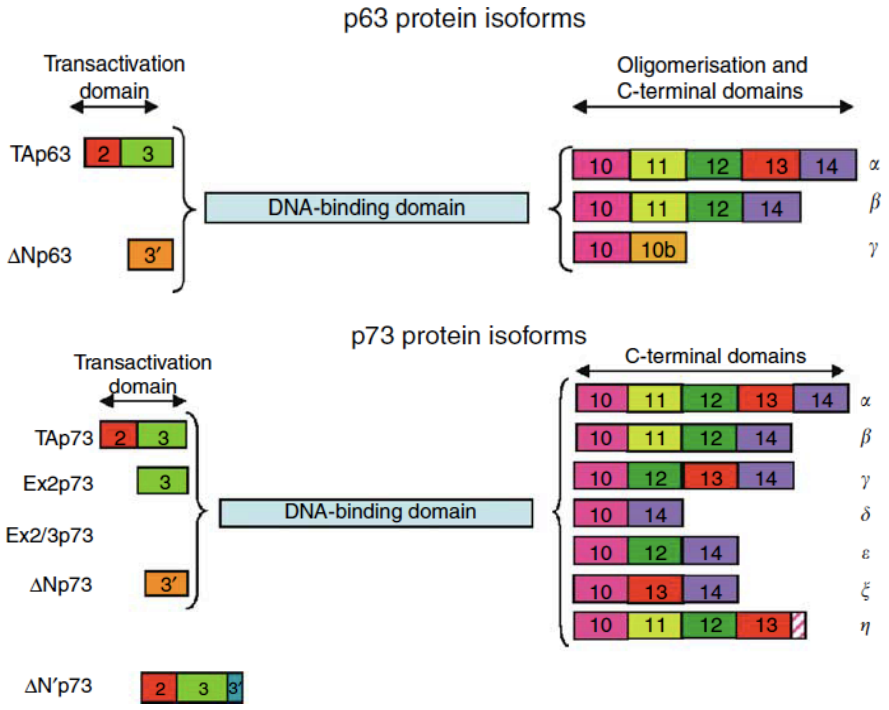


Figure 4. Known isoforms of the p63 and p73 proteins(Bourdon, 2007b).

As a consequence of the partial structural homology, p53 family members have some overlapping functions mediated by the transactivation of common targets (Stiewe, 2007). Indeed, p63 and p73 are able to trigger apoptosis upon DNA damage (Yang et al., 2000) and to induce senescence both *in vitro* and *in vivo* (Guo et al., 2009). Recently, a critical role of p63 in metastasis suppression has emerged (Adorno et al., 2009; Muller et al., 2009). Consistently, the role of p63 and p73 in tumour suppression is highlighted by the findings that compound $p63^{+/-}$ $p73^{+/-}$ mice develop spontaneous tumours and that loss of p63 and p73 can also cooperate with loss of p53 in the development of specific tumour types (Flores et al., 2005).

In addition to their oncosuppressive activities p63 and p73 share with p53 the ability to control also embryonic development and differentiation. Although mice lacking p63 are born alive, they show the most severe developmental phenotype of all the p53 family members (Mills et al., 1999). The limbs are absent or truncated owing to a malfunction of the apical ectoderm ridge. Moreover, the mice fail to develop a stratified epidermis and most epithelial tissues (e.g. hair follicles, prostate and mammary glands) and eventually die from dehydration within hours after birth.

p73 knockout mice are viable, but show a runting phenotype and a high mortality rate within the first two months (Yang et al., 2000). The animals suffer from several neurologic defects (hydrocephaly, hippocampal disgenesis and loss of sympathetic neurons) and have immunological problems characterized by chronic infections and inflammation. Moreover, these mice show abnormal reproductive and social behaviour, which is presumably due to defects in pheromone detection in the vomeronasal organ.

p53 structure and isoforms

p53 isoforms. The mouse p53 gene is composed of 11 exons, spanning over 12.000 bp (GenBank Accession Number: NC_000077) on chromosome 11, while the human p53 gene is composed of 11 exons spanning over 19.200 bp (GenBank Accession Number: NC_000017) on chromosome 17p13.1. The primary transcript can be subjected to alternative splicing; moreover, the presence of alternative promoters allows the production of N-terminal truncated proteins, similarly to what happens for the other members of the family. The canonical form of the human protein, identified as p53α, comprises 393 residues. 8 more isoforms have been described, that are differentially expressed in various tissues (Bourdon et al., 2005)(Figure 5).

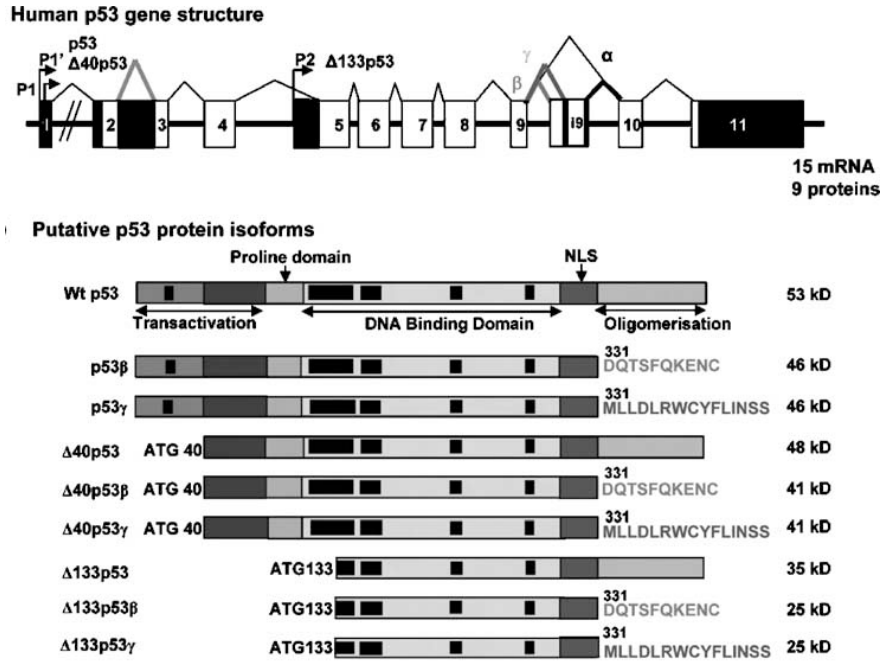


Figure 5. p53 gene and isoforms. Genomic structure of p53: Alternative splicing (α, β, γ) and alternative promoters (P1, P10 and P2) are indicated (upper panel). p53 protein isoforms: p53, p53 β and p53 γ proteins encoded from P1 or P10 promoters contain the conserved N-terminal domain of transactivation (TA). Δ133p53 isoforms encoded from promoter P2 are amino-truncated proteins deleted of the entire TA domain and deleted of part of the DNA-binding domain. Translation is initiated at ATG-133. Δ40p53 protein isoforms encoded from P1 or P10 promoters are amino-truncated proteins due to alternative splicing of exon 2 and/or alternative initiation of translation at ATG-40. Δp53 protein isoform is due to an alternative splicing between the exon 7 and 9 (adapted from (Bourdon, 2007a)).

p53 isoforms can have distinct biochemical activities. p53β binds preferentially the p53-responsive promoters p21 and Bax rather than MDM2, while p53 binds preferentially to MDM2 and p21 rather than Bax promoters. Co-transfection of p53 with p53β increases slightly p53-mediated apoptosis, while co-transfection of p53 with Δ133p53 strongly inhibits p53-mediated apoptosis in a dose dependent manner, consistently with the finding that it is frequently overexpressed in breast tumours. This suggests that a subtle and complex balance between the different p53 isoforms and p53 could regulate cellular fate outcome in response to p53 activation (Bourdon et al., 2005).

Δ40p53 (also named p47 or ΔNp53) is an amino-terminally truncated p53 isoform deleted of the first 40 amino acids. The Δ40p53 protein still contains part of the p53 transactivation domain and can activate gene expression, but can also act in a dominant-negative manner towards full-length p53 inhibiting both p53 transcriptional activity and p53-mediated apoptosis (Mills, 2005; Rohaly et al., 2005). Moreover Δ40p53 can modify p53 cell

localization and inhibits p53 degradation by MDM2.

Functional domains of the p53 protein. As many other transcription factors, p53 has a modular structure composed by evolutionarily conserved functional domains: an N-terminal transactivation domain (aa 1-61), a proline-rich domain (aa 64-93), a central DNA-binding domain (aa 93- 292), an oligomerization domain (aa 325-355) and a C-terminal regulatory domain (Figure 6).

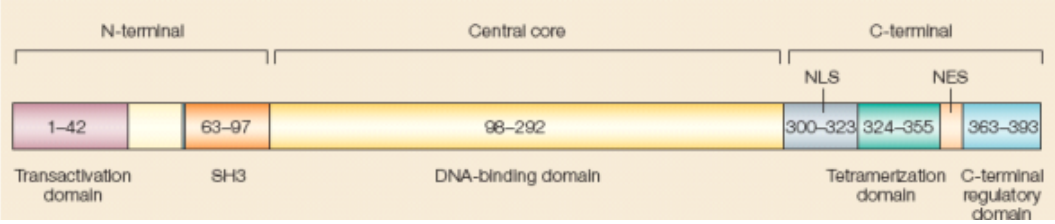


Figure 6. Schematic representation of p53 domains (Bode and Dong, 2004).

The N-terminal **transactivation domain** of p53 contains two acidic domains (TAD1, aa 1-40, and TAD2, aa 40-61) (Bode and Dong, 2004), both required to induce transcription of target genes. These two regions, indeed, interact with components of the transcriptional machinery, such as TBP (TATA box binding protein), TAFs (TBP-associated factors), the p62 subunit of the transcriptional/repair factor TFIIH and the transcriptional coactivators CBP (CREB binding protein) and p300 (Avantaggiati et al., 1997).

The transactivation domain is followed by a **proline-rich domain** (PRD, aa 64-93), which contains five repeats of the amino acid motif PXXP (P= proline; X= any amino acid). The PRD is required for p53 stabilization (Zacchi et al., 2002; Zheng et al., 2002a) and is particularly involved in the ability of p53 to trigger apoptosis, both by transcriptional induction of pro-apoptotic genes (Bergamaschi et al., 2006; Venot et al., 1998) and by direct activity of p53 at mitochondria (Chipuk et al., 2004).

In the PRD is also present the best-characterized p53 polymorphism: in humans, codon 72 can encode either proline or arginine. The distribution of this polymorphism appears to follow a north/south gradient, with the frequency of the Pro72 allele increasing toward the equator, suggesting a possible selection of codon Pro72 variants in areas with high UV light exposure. This polymorphism affects p53 activity: Arg72 variant is more efficient than Pro72 in inducing apoptosis due to both its enhanced localization at mitochondria and

its lower affinity for the iASPP protein, which binds preferentially to the PRD and selectively blocks access of p53 to the promoters of apoptosis-related genes (Bergamaschi et al., 2006). Consistently, expression of p53 Arg72 is associated to a greater sensitivity of tumour cells to anticancer drugs and is predictive of a more favourable clinical response to chemotherapy in head and neck squamous cell carcinomas (Sullivan et al., 2004).

The central core of p53 contains its **DNA binding domain** (DBD, aa 93-292), required to recognize and specifically bind to the cognate sequence 5' – PuPuPuC(A/T)- (T/A) GPyPyPy – 3' (el-Deiry et al., 1992). Besides the interaction with DNA, the DBD mediates the binding between p53 and crucial cofactors in defining p53 functions and activity. Proteins like 53BP1, Hzf, ASPP1 and ASPP2 positively affect p53 activity by interacting with its DBD (Das et al., 2007; Samuels-Lev et al., 2001), while other proteins bind to the DBD and negatively regulate p53. Indeed, MDM2 has been shown to interact also with residues in the DBD and the binding of MDM2 to full-length p53 is 10-fold stronger than binding to the N-terminal domain alone. The frequency of tumour-acquired mutations of TP53 is the highest within this domain, highlighting the critical importance of loss of DNA binding and interaction with pivotal partners for enabling tumour cells to evade stress-induced growth suppression (Brosh and Rotter, 2009).

The **oligomerization domain** (OD, aa 325-355) is required for the formation of a high-affinity DNA binding and transcriptional competent p53 tetramer.

The C-terminal domain of p53, in particular the last 30 amino acids (CT, aa 364-393), has been historically called "**regulatory domain**", since it contains several residues targeted by post-translational modifications that modulate p53 stability and function (Kruse and Gu, 2009). In the C-terminus of p53 is present a cluster of three nuclear localization signals (NLS) that mediate the shuttling of the protein into the cell nucleus. NLSI (aa 316-322) is the most active signal while the other two NLSs, II and III (aa 370-384) appear to be less important for nuclear localization (Shaulsky et al., 1990). p53 contains also two putative nuclear export signals (NES), one in the N-terminus (nNES, aa 11-27) and the other in the OD (cNES, aa 340-351) (Stommel et al., 1999). For both NES, it has been proposed that when p53 needs to be activated, they are masked by the formation of the tetramer, or by direct phosphorylation by DNA-damage activated kinases. More generally, the oligomerization of p53 has been proposed as a mechanism that may regulate its nucleo-cytoplasmic transport by affecting the accessibility of the cNES but also of the NLS to

their respective receptors (Stommel et al., 1999).

Biological activities of p53

In the context of tumour suppression, p53 induction can lead to different biological outcomes, depending on the context. For example, the continued expression of dominant oncogenes *in vivo* can lead to the irreversible withdrawal of cells from the proliferative cycle into a terminal state termed oncogene-induced senescence (Braig and Schmitt, 2006). Such a mechanism has been observed to occur in mouse model of prostate cancer and in human fibroblasts and mammary epithelial cells (Collado and Serrano, 2010). p53 can also suppress tumour development by initiating apoptosis, the major form of programmed cell death, which involves the ordered and rapid destruction of the cell in the absence of an inflammatory response (Benchimol, 2001). For example, p53-mediated apoptosis is thought to protect against the development of lymphoma and, interestingly, mice that express a p53 mutant protein lacking the ability to induce cell cycle arrest but retaining apoptosis-inducing functions are still efficiently protected from spontaneous tumour development (Toledo and Wahl, 2006). Key factors that determine the outcome of p53 induction, at least in cultured cells, are the type and intensity of stress, the cell type and the genetic background. Crosstalk with other pathways, such as survival signalling or the retinoblastoma pathway, can tip the balance between growth arrest and apoptosis. Other mechanisms, such as the prevention of metastasis, are likely to contribute to tumour suppression. However, given the many hundreds of genes that are thought to be regulated by p53 (Vousden and Prives, 2009) and the many varied biological functions to which it is now known to contribute, we do not have a complete picture of how tumour suppression is mediated mechanistically in all instances. The common principle is the protection of the organism either by maintaining the integrity of the cell and its genome or by preventing the proliferation of cancer cells.

Apoptosis. The term “apoptosis” defines the process of programmed cell death that the cell can undergo both physiologically (for example during development) and after pathologic *stimuli* that lead to irreparable damage. The apoptotic cell executes a genetic programme that leads to characteristic modifications such as nuclear condensation, DNA fragmentation

and dilatation of the endoplasmic reticulum; in the final phase of the process the different cellular components are enclosed into the so called "apoptotic bodies" and are phagocytised by macrophages and by nearby cells without causing an inflammatory response.

Apoptosis can start both following the extrinsic route and via intrinsic activation; the first is caused by external *stimuli* as the Fas ligand that, interacting with specific receptors belonging to the TNF-R (*tumour necrosis factor-receptor*) family, activates the cytoplasmic effector proteins called caspases. The intrinsic route is fostered by internal signals as DNA damage or hypoxia (Green, 1998) and, through a direct activity at mitochondria that leads to mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release, ends in caspases activation; indeed in the cytoplasm cytochrome c associates with APAF-1 (*apoptotic protease-activating factor 1*) starting the assembly of the apoptosome that in turn activates the caspases (Li et al., 1997; Zou et al., 1997). Mitochondrial proteins have a fundamental role in the intrinsic pathway, and in particular the Bcl-2 family proteins. The family is divided in anti-apoptotic members, such as Bcl-2, Bcl-xL and Mcl-1, which contain BH1, BH2, BH3 and BH4 domains, and the pro-apoptotic members, which either contain BH1, BH2 and BH3 domains such as the MOMP effectors Bax and Bak, or the BH3-only domain class such as the activators tBid and Bim (Vaseva and Moll, 2009). To effectively induce apoptosis the effector proteins should undergo an activation that involves a conformational change and an homo-oligomerization, which in turn leads to their translocation and insertion into the outer mitochondrial membrane to generate pores that release pro-apoptotic factors from the mitochondrial intermembranous space (e.g. cytochrome c). The BH3-only class of proteins covers the role of favouring such activation, either by stimulating the oligomerization of Bak and Bax (tBid and Bim proteins) or inhibiting the binding between anti-apoptotic proteins and the effectors of apoptosis (Puma, Noxa and Bad proteins).

p53 is able to induce apoptosis acting both on intrinsic and extrinsic pathways, mainly regulating transcriptionally the two routes. Indeed p53 activates the transcription of death receptors localized in the cytoplasmic membrane (among which Fas, DR4 and KILLER/DR5) but also of a number of genes belonging in the Bcl2 family, such as Bax, Bid, Puma, Noxa, p53AIP1 and represses the anti-apoptotic genes Bcl2 and Bcl-xL (p53-target genes involved in apoptosis reviewed in (Vousden and Lu, 2002)). Moreover p53

induces the transcription of effectors of the apoptotic cascade such as Apaf-1 and caspase-6 (MacLachlan and El-Deiry, 2002) (Figure 7).

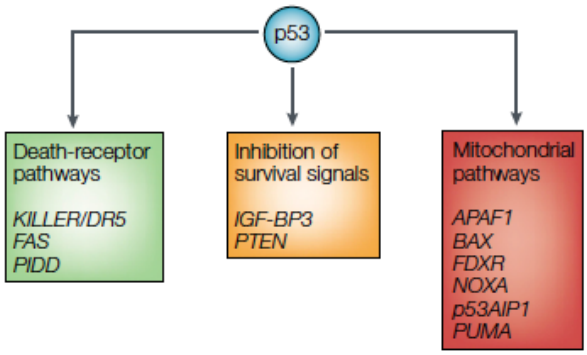


Figure 7. Several apoptotic pathways are activated by p53. p53 can induce the expression of numerous apoptotic genes that can contribute to the activation of both death receptor and mitochondrial apoptotic pathways. p53 can also affect the efficiency of survival signalling (Vousden and Lu, 2002).

In addition to these activities, a p53 transcription-independent route for MOMP has been discovered and starting from this in the last 20 years a lot of effort has been made in elucidating p53 pro-apoptotic activities in the cytoplasm. The hallmark of transcription-independent apoptosis induction by p53 is the stress-dependent accumulation of the protein in the cytoplasm and mitochondria that directly activates the apoptosis effectors Bax and Bak.

Cell cycle arrest. The activation of the p53 pathway can induce cell cycle arrest mostly promoting the induction of three critical target genes: p21, 14-3-3σ and GADD45. The cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ has been the first identified transcriptional target of p53 (el-Deiry et al., 1993). Upon p53 activation, p21 increased levels result in cell cycle arrest in G_1 phase due to inhibition of cyclinA/CDK2, cyclinE/CDK2 and cyclinD/CDK4 (Harper et al., 1993). p21 was demonstrated to participate also in the G_2/M arrest after DNA damage, presumably by blocking PCNA function at replication forks(Ando et al., 2001). However, the p53-induced G_2 arrest is mostly mediated by the activation of other two genes, i.e. GADD45 and 14-3-3σ (Hermeking et al., 1997). Regarding the other proteins, GADD45 is able to destabilize the complexes CDC2/cyclin B, whereas 14-3-3σ sequesters the complexes cyclinB1/CDK1 in the cytoplasm contributing to G_2 arrest. Interestingly the inhibition of 14-3-3σ is sufficient to confer to

primary human epithelial cells the ability to grow indefinitely in culture (Dellambra et al., 2000).

Senescence. Senescence commonly describes a cellular response to different kind of *stimuli* characterized by the irreversible arrest of proliferation. Senescent cells, although remaining vital and metabolically active, lose the ability to divide and to progress in the cell cycle that usually is arrested in the G_1 phase. In 1961 Hayflick and Moorhead (Hayflick and Moorhead, 1961) described for the first time the phenomenon of replicative senescence starting from the observation that human fibroblasts cultured in standard conditions (monolayer, broad space, presence of nutrients and growth factors) at first undergo a rapid cell division that rapidly declines following cellular passages, completely arresting after reaching the intrinsic limit of their replicative potential ("Hayflick limit") given by excessive telomere shortening. In the last years it has emerged the concept that the same phenotype can be originated by various cellular stresses such as oncogene expression (Serrano et al., 1997), DNA damage (Di Leonardo et al., 1994) (Parrinello et al., 2003) or chromatin structure alteration (Munro et al., 2004; Ogryzko et al., 1996). In particular the overexpression of active oncogenes can induce a cellular response phenotypically indistinguishable from replicative senescence, independent from telomere shortening (Jones et al., 2000; Serrano et al., 1997), called oncogene-induced senescence (OIS). Oncogenes have the possibility to induce the transformation of a cell if combined with other mutations. An emblematic role is covered by Ras, a cytoplasmic protein able to transduce extracellular growth signals, which if expressed in the constitutively active form can cooperate with other oncogenes leading to cellular transformation (Malumbres and Barbacid, 2003); when the active form is expressed in an otherwise normal cell, however, it induces an irreversible growth arrest similar to senescence (Serrano et al., 1997). This first observation has been further extended to the various members of the Ras pathway such as the GTPase RAC1 and the kinases RAK1 and MEK whose alteration can induce senescence (Dimri et al., 2000; Michaloglou et al., 2005); it is important to underline that also the loss of PTEN, an inhibitor of the Ras pathway, is able to lead to the same effect (Chen et al., 2005).

Among the mechanisms that induce OIS an important role is played by PML (promyelocitic leukemia). This protein is a key component of nuclear structures called

nuclear bodies (NBs) (Borden, 2002; Salomoni and Pandolfi, 2002; Zhong et al., 2000). It has been demonstrated that the presence of an oncogenic form of Ras induces PML and that this event is necessary for the establishment of OIS in mouse embryo fibroblasts (Pearson et al., 2000). Moreover overexpression of a PML isoform, PML IV, is able to induce senescence in human and mouse fibroblasts and this activity is dependent on p53 acetylation in the nuclear bodies (Pearson et al., 2000).

A common characteristic of OIS is the alteration of DNA replication and the activation of DNA damage response (DDR). It has been demonstrated that the effect due to Ras activation on cell proliferation is biphasic in nature: at first the cells undergo hyper proliferation, rapidly followed by growth arrest and senescence establishment (Di Micco et al., 2006; Sarkisian et al., 2007). This is true also for other oncogenes such as BRAF (Dankort et al., 2007; Michaloglou et al., 2005), E2F (Lazzerini Denchi et al., 2005) and Myc (Dominguez-Sola et al., 2007; Grandori et al., 2003). The activation of DDR coincides with the end of hyper proliferation that is believed to cause replicative fork stalling thus generating DNA damage. Among the first proteins to respond to DSBs is ATM (Ataxia Telangiectasia Mutated). ATM substrates include H2AX, a nucleosomal histone variant, and p53 binding protein-1 (53BP1), which facilitates checkpoint activation and repair. Phosphorylated H2AX (γ-H2AX) and 53BP1 rapidly localize to DSBs, forming characteristic foci. ATM also phosphorylates the DDR kinase CHK2 (checkpoint kinase-2), which promotes growth arrest; NBS1 (Nijmegen breakage syndrome), a member of the MRN (MRE11-RAD51-NBS1) complex that reinforces the DDR and participates in DNA repair; and p53, that orchestrates repair and cell cycle arrest. DSBs that cannot be repaired (e.g., uncapped telomeres) or replicative fork stalling following hyper proliferation driven by active oncogenes cause constitutive DDR signalling, prolonged p53-dependent growth arrest, and eventually an essentially irreversible senescence (d'Adda di Fagagna, 2008). A causal role has been demonstrated through the observation that inactivation of genes involved in DDR is able to avoid senescence and induce transformation following the expression of oncogenes (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007).

Senescence is established and maintained by p53 and pRb pathways (Figure 8).

The INK4a/ARF *locus* is fundamental for the regulation of this process: it encodes for two proteins, $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ in mouse). $p16^{INK4a}$ binds CDK4/CDK6 complex

and induces a conformational change able to disrupt the interaction with cyclin D. In this way pRb is maintained in hypo-phosphorylated form and induces cell cycle arrest in G_1 phase. On the other hand $p14^{ARF}$ binds *mouse double minute 2* (MDM2) displacing its interaction with p53, thereby activating p53 transcriptional activity towards cell cycle arrest genes (Sherr, 1998). The main effector of this response is p21 (Brown et al., 1997). Experimental evidences have demonstrated that reduction of p53, p21 or DNA damage response proteins is able to prevent senescence induced by telomere shortening and DNA damage and in some cases can eventually revert the senescent state (d'Adda di Fagagna et al., 2003; Di Micco et al., 2006; Gire et al., 2004).

Recently some new evidences on the role of other genes transcribed by p53 for senescence induction are emerging, for example the class of microRNAs miR-34 (He et al., 2007). In particular miR34a is induced or more expressed in senescent human fibroblasts and its absence augments their replicative potential. The mechanism of action has been proposed to involve inhibition of the deacetylase SIRT1 (Yamakuchi and Lowenstein, 2009).

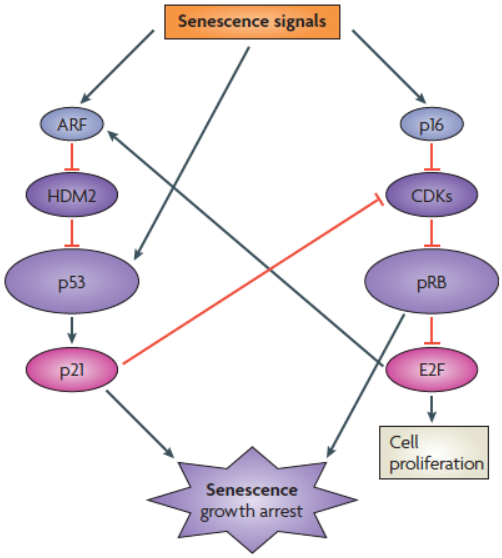


Figure 8. Senescence controlled by the p53 and p16–pRB pathways. Senescence-inducing signals, that usually trigger a DNA damage response (DDR), engage either the p53 or the p16–retinoblastoma protein (pRB) tumour suppressor pathways. Some signals, such as oncogenic Ras, engage both pathways. Active p53 establishes the senescence growth arrest in part by inducing the expression of p21, a cyclin-dependent kinase (CDK) inhibitor that, among other activities, suppresses the phosphorylation and, hence, the inactivation of pRB. Senescence signals that engage the p16–pRB pathway generally do so by inducing the expression of p16, another CDK inhibitor that prevents pRB phosphorylation and inactivation. pRB halts cell proliferation by suppressing the activity of E2F, a transcription factor that stimulates the expression of genes that are required for cell-cycle progression. E2F can also foster proliferation by inducing ARF expression, which engages the p53 pathway. So, there is reciprocal regulation between the p53 and p16–pRB pathways (Campisi and d'Adda di Fagagna, 2007).

Other p53 functions. In the recent years it has emerged the concept that p53 can regulate several aspects of cell metabolism (Shen et al., 2012; Vousden and Ryan, 2009) spanning from direct regulation of glucose metabolism and oxidative phosphorylation to its activity in controlling oxidative stress. Intriguingly, these p53 actions result from a coordination of transcriptional and cytoplasmic activities, whose net balance is not always easy to understand. Regarding the mitochondrial energetic regulation, p53 is able to promote both TCA cycle and oxidative phosphorylation (OXPHOS). Moreover, p53 also regulates glutaminolysis by activating the expression of mitochondrial glutaminase (GLS2), which promotes glutamine to glutamate conversion leading to the formation of α-ketoglutarate, a TCA cycle intermediate (Jiang et al., 2013). As for mitochondrial respiratory chain, p53 is able to foster the pathway by transcriptionally activating "synthesis of cytochrome c oxidase 2" (SCO2) expression, a regulator of complex IV, cytochrome c oxidase (COX) I subunit, and AIF, essential for mitochondrial respiratory complex I function. To counteract the reactive oxygen species produced by the enhancement of OXYPHOS, p53 upregulates antioxidant activities through metabolic processes, for example via the transcriptional induction of "TP53-induced glycolysis and apoptosis regulator" TIGAR (Bensaad et al., 2006) that limits the activity of PFK1 so lowering the rate of glycolysis while promoting pentose phosphate pathway (PPP).

The PPP is a central metabolic pathway that produces NADPH required for regeneration of reduced glutathione (GSH). Interestingly, it has been recently demonstrated that a core of cytoplasmic p53 can inhibit glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme of PPP, through transient interactions. The net effect of nuclear and cytoplasmic activity is not clear and may be cell context and stress-dependent. It is conceivable that inactivation of p53 by different means in cancer cells accelerates glucose consumption and directs glucose for rapid production of macromolecules by an increase in PPP flux thus enhancing biosynthesis. In turn p53 loss could also contribute to the Warburg effect by fostering glycolysis.

In the recent years it has been demonstrated that p53 can also control the process of autophagy, although in an ambiguous fashion (Maiuri et al., 2010). When exposed to stress, nuclear p53 can induce autophagy by regulating several genes involved in the autophagic cascade; first of all p53 can inhibit the negative regulator of autophagy mTOR through inducing transcription of its negative regulators such as TSC2 (tuberous sclerosis

2), the beta 1 and beta 2 subunits of AMP-activated protein kinase (AMPK), an evolutionarily conserved sensor of cellular energy levels which phosphorylates and thus activates TSC1 and TSC2, and sestrins 1 and 2, two main activators of AMPK. Another transcriptional target of p53 involved in autophagy induction is DRAM (damage-regulated autophagy modulator), a lysosomal protein that induces macroautophagy and is also able to regulate cell death. In contrast to the autophagy-promoting functions of p53 in the nucleus, the cytoplasmic pool of p53 suppresses autophagy in multiple experimental settings (Tasdemir et al., 2008), as demonstrated by the fact that pharmacological inhibition of p53 can trigger autophagy in cytoplasts. In human, mouse and nematode cells depletion or inactivation of p53 by different means induced autophagy that relied on mTOR inhibition.
Recently, p53 has also entered the stem cell arena. Indeed a role for p53 in differentiation and development had already been observed in *Xenopus laevis*, where p53 engages in complex interactions with the Smad transcriptional regulators to direct embryonic germ layer specification (Piccolo, 2008). Moreover when p53 is activated in mESCs, it binds to the promoter of Nanog, a master regulator of pluripotency, and represses its expression, leading to the differentiation of the cells and their elimination from the stem cells pool (Brandner, 2010). In addition to the Nanog promoter, p53 was also shown to bind the promoter of Oct4 and repress its expression. On the same line it was recently demonstrated that p53 regulates the polarity of self renewing divisions in mammary stem cells (Cicalese et al., 2009), pushing towards asymmetric division.

Regulation of p53 levels and activities

Given its potent growth suppressive activity, improper activation of p53 must be avoided in growing cells and this is obtained by precise mechanisms that control its stability and activity. When cells undergo stress, p53 stability, promoter recruitment and transcriptional activity are induced by means of multiple post-translational modifications and by interaction with activators and inhibitors (Levine and Oren, 2009). These include proteins that enhance the translation of p53 mRNA, that modify p53 for both stabilization and transcriptional activation, that reverse these modifications or that alter its sub-cellular localization. Rather than simply leading to its rapid activation, all these events finely modulate p53 functions, allowing it to adapt and to coordinate appropriate responses to

specific stimuli.

Regulation of p53 transcription and translation. For a long time, the regulation of p53 abundance has been associated only to its stabilization; nevertheless, relatively recently the modulation of p53 mRNA transcription and translation have also been shown to play a role in determining p53 expression levels. Indeed, despite p53 mRNA levels were originally reported to be almost unaffected by several stresses, p53 transcription was demonstrated to be regulated by BCL6, HOXA5 and CTCF in particular cellular contexts. p53 translation can be regulated by factors able to bind the 5'UTR and 3'UTR secondary structures of the p53 mRNA. Acting on the 5'UTR, p53 translation is modulated in response to DNA damage positively by the ribosomal protein L26(Ofir-Rosenfeld et al., 2008) and negatively by nucleolin.

Regulation of p53 stability. Regulation of p53 abundance occurs mainly at the protein level: in physiological conditions, p53 levels are maintained low thanks to efficient degradation mediated by the ubiquitin-proteasome system and in particular this is induced by the product of the p53 target gene MDM2, whose E3 ubiquitin ligase activity establishes a negative feedback loop with p53, leading to attenuation of p53-mediated response under conditions of transient DNA damage. The relevance of MDM2 regulation on p53 function is underscored by the phenotype of MDM2 knockout mouse, whose embryonic lethality is rescued by simultaneous absence of p53 (Jones et al., 1995). Depending on its relative abundance with respect to p53, MDM2 can catalyze either mono- or poly-ubiquitylation of p53, leading to nuclear export and translocation to mitochondria or intranuclear proteasomal degradation, respectively (Li et al., 2003; Marchenko et al., 2007). Connected to MDM2 activity, the tumour suppressor p14 (ARF) is a pivotal positive regulator of p53 stability being able to block MDM2 catalytic activity *in vitro* and the poly-ubiquitylation of p53 *in vivo*.

During the last decade, several other p53's E3-ubiquitin ligases have been described. Among them, Pirh2 and COP1 are, like MDM2, p53-inducible genes that participate in a comparable auto regulatory negative feedback loop (Dornan et al., 2004; Leng et al., 2003).

p53 ubitiquitylation can be reversed by the deubiquitylating enzyme HAUSP (herpes associated ubiquitin-specific protease, also known as USP7), which can stabilize and activate p53 by removing ubiquitin moieties from its C-terminus, thus counteracting

MDM2 function. Moreover, HAUSP is involved in rapid deubiquitylation of p53 at mitochondria, thus generating the apoptotically active non-ubiquitylated form of mitochondrial p53 (Marchenko et al., 2007).

Post-translational modifications. p53 undergoes a great variety of post-translational modifications that influence its stability and its transcriptional activity. At the moment, it is known that more than 36 different amino acids within p53 are modified: the actual pattern of post-translational modifications is complicated since there could be competition for the same residue, which can indeed be modified in different ways by different enzymes (Figure 9).

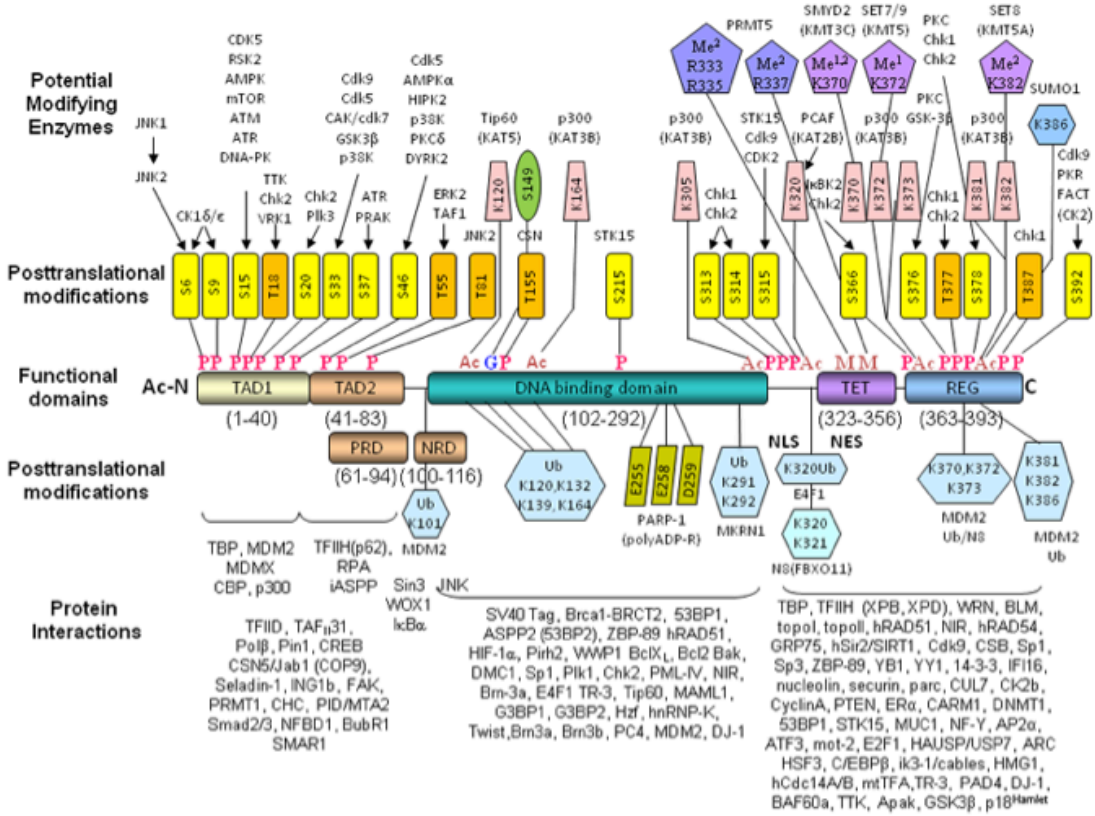


Figure 9. Schematic representation of p53 phosphorylation sites and of the enzyme involved in p53 phosphorylation upon different stimuli. Post-translational modification sites (P, phosphorylation; Ac, acetylation; G, glycosylation; Me, mono(1) or di-(2) methylation; N8, neddylation; Ub, ubiquitylation; polyADP-R, poly-ADP-ribosylation) are indicated together with enzymes that can accomplish the modifications *in vitro*. The C-terminal six lysines (K370, K372, K373, K381, K382, and K386) are the primary site of ubiquitylation by MDM2; whereas K291 and K292 can be ubiqitylated by MKRN1 and K320 is ubiqitylated by E4F1. K373, K372, and K373 are likely sites of attachment for the ubiquitin-like protein NEDD8, and Lys386 may be modified by conjugation with SUMO1, a ubiquitin-like peptide. Adapted from (Meek and Anderson, 2009).

Phosphorylation is the most extensively studied p53 post-translational modification. Many kinases, including ATM, ATR, Chk1, Chk2, CK1, CK2, JNK, Erk, p38, Aurora Kinase A, GSK3β, HIPK2 and DYRK2, have been shown to phosphorylate p53 after DNA damage (Vousden and Prives, 2009). The redundancies observed in p53 phosphorylation can be explained as a failsafe mechanism and also by the fact that distinctive combination of phosphorylated residues could be required for further modifications, leading to maximal activation. Indeed, only a few p53 sites are phosphorylated by one specific protein kinase, as, for example, Ser6, Ser9 and Thr18 by CK1 and Thr18 by JNK (Banin et al., 1998; Waterman et al., 1998). The best-characterized p53 phosphorylations are those at the N-terminus of the protein, in particular modifications that affect Ser15, Thr18, Ser20 and Ser46. Ser15 is phosphorylated in an ATM-dependent manner in response to γ-irradiation (Banin et al., 1998; Waterman et al., 1998) and by ATR and p38 in response to UV light (Bulavin et al., 1999). After Ser15 phosphorylation, p53 is more transcriptionally active, also because of the increased interaction with the acetyltransferase CBP (Lambert et al., 1998), and induces growth arrest and apoptosis (Shieh et al., 1997). Phosphorylation of Ser46 can be performed by several kinases, as HIPK2 (D'Orazi et al., 2006; Hofmann et al., 2002), DYRK2 (Taira et al., 2007), PKCδ (Yoshida et al., 2006) AMPK and p38. This phosphorylation determines selectivity of p53 recruitment to its target promoters, specifically promoting the induction of pro-apoptotic target genes (D'Orazi et al., 2006; Oda et al., 2000). It is likely that an ordered pattern and an interdependence of stress-induced modifications of p53 exist.

p53 can be acetylated at several lysines by different histone acetyltransferases (HATs) as p300/CBP and PCAF (p300/CBP-associated factor), which were shown to acetylate p53 in response to DNA damaging agents, such as UV- and γ-irradiation (Carter and Vousden, 2009). In detail, CBP and p300 acetylate p53 at lysines within the C-terminal domain (Lys 370, 372, 373, 381 and 382)(Avantaggiati et al., 1997). Acetylation at Lys 120 catalyzed by the MYST family acetyltransferases hMOF and TIP60 has also reported to confer promoter selectivity for apoptotic target genes/DNA binding specificity and has also been found involved in transcription-independent apoptotic activity of p53 (Carter and Vousden, 2009).

In the last years several works described the identification of novel p53 modifications mostly on lysines, such as methylation, ubiquitylation, neddylation and sumoylation.

Methylation of p53 by methyltransferases can occur at least at two different sites, reported to lead to opposing effects on p53 function. Indeed, while methylation at Lys372 by the methyltransferase Set9 increases the stability of p53, restricting it to the nucleus and enhancing p53-dependent transcription (Chuikov et al., 2004), the methylation of Lys370, catalyzed by the methyltransferase Smyd2, leads to repression of its transcriptional activity (Huang et al., 2006).

Three lysines that are targeted for ubiquitylation (Lys 370, 372 and 373) are also subjected to NEDDylation. Sumoylation consists in the addition of the small ubiquitin-like protein SUMO1 to the ε-amino group of lysine. In the case of p53, several reports have described the sumoylation of Lys386: however it is still controversial whether this modification modulates p53 transcriptional activity in a positive fashion (Gostissa et al., 1999).

Drugging the p53 pathway: novel insights into therapeutic approaches

As previously said, p53 is inactivated by mutations in over 30% of all cancers (Vogelstein et al., 2000). A high percentage of those mutations is of the missense type, and disrupts its direct binding to DNA or leads to structural perturbations that prevent the correct folding or oligomerization of the tumour suppressor. At other times, loss of p53 function is due to overexpression of p53-regulatory proteins that suppress p53 activity, such as MDM2 and MDMX. Numerous strategies have been devised to correct a dysfunctional p53-regulatory pathway, although it is not a simple task, because p53 is neither a cell surface protein nor a typical enzyme and thus antibodies and low-molecular-mass enzyme inhibitors, which have served as the basis for almost all of the recently developed targeted anticancer therapies, are not pertinent options. In tumours that retain wild-type p53 but have defects in p53-regulatory pathways, such as overexpression or amplification of MDM2 or MDMX or epigenetic silencing of INK4A-ARF locus, the major approach for the restoration of p53 activity has been to inhibit the function of negative regulators of the p53 response(Roxburgh et al., 2012). Although a number of potential therapeutic opportunities have been identified, such as blocking E6-induced proteasomal degradation of p53 in human papillomavirus (HPV)-driven cancers using bortezomib, or inhibiting the enzymatic E3 ubiquitin-ligase activity of MDM2 (reviewed in (Hoe et al., 2014)) the vast majority of the current lines of research and clinical trials are focused on finding small-molecule drugs

that block the binding of p53 with MDM2 or MDMX. A breakthrough in the field was the development of nutlin, the first small molecule inhibitor of the p53-MDM2 interaction (Vassilev et al., 2004). A complementary approach led to the identification of the small molecule inhibitor RITA (Reactivation of p53 and Induction of Tumour cell Apoptosis); whereas nutlins bind to MDM2, RITA binds to p53 and prevents it from being attached by MDM2(Issaeva et al., 2004). RITA is particularly promising because it has a strong apoptotic effect on many tumour cell lines (Grinkevich et al., 2009; Sorrentino et al., 2013).

In cases where the p53 protein is mutated, attempts have been made to find molecules that may act as chaperones by binding to mutant p53 protein and stabilizing its conformation. Indeed, genetic studies that demonstrate reversion of the effects of common p53 mutations by second-site mutations have supported such an approach (Nikolova et al., 2000) by showing that alterations in different regions of p53 can correct the defect induced by the primary mutation. Moreover, about 8% of p53 mutations result in early termination of translation and "read-through drugs" such as the aminoglycoside antibiotic G418 (Hoe et al., 2014) can bypass the effect of stop codons introduced by mutations and thus restore the expression of p53.

Attempts have also been made to manipulate the p53 pathway using gene therapy or immunological approaches (Brown et al., 2009). In particular, adenoviral vector-based delivery of *TP53* was approved in China in 2004 for the treatment of head and neck cancer. An alternative gene therapy strategy was developed at Onyx, California, USA. This is constituted by an oncolytic Adenovirus that is deficient for E1B 55kDa, which binds and inactivates p53. Consequently, this virus can replicate in tumour cells that lack functional p53 and kill them. Although clinical trials yielded promising results, approval for clinical use still needs to be evaluated. A related oncolytic virus, operating on a similar principle, has been approved for cancer therapy in China (Hoe et al., 2014).

As described above, a consistent therapeutic approach targeting p53 has been challenging to design, especially considering that the most desirable effect should be the induction of apoptotic death of cancer cells rather than senescence. Indeed it is still a matter of debate how p53 is instructed to choose between its growth arrest and pro-apoptotic functions and a detailed knowledge of the underlying pathways may help to design more specific and effective therapeutic approaches. Indeed deciphering the mechanisms driving the

multiplicity of p53 responses is of paramount importance in the clinical arena, where the apoptotic potential of p53 could be exploited for selective elimination of cancer cells. On the other hand, activation of p53-dependent apoptosis in healthy tissues as for example happens in neurodegenerative diseases or after chemotherapy is detrimental and should be avoided. Thus, a better comprehension of the mechanisms of p53 activation could provide also possible new targets to escape inappropriate p53 activation.

A great interest comes from the evidence that genes tend to function as networks rather than individually, and this is particularly important for those that are master regulators of many pathways, like p53. Recent studies in model organisms have provided an updated view of gene networks based on flexibility, degeneracy, and redundancy. This is particularly obvious in disorders such as cancer and neurodegeneration. Indeed, a large number of p53 cofactors are emerging as relevant for both pathologies, and it is likely that future therapeutic approaches for these important diseases will rely on combinatorial strategies targeting gene networks. These advanced therapies, however, will require a deep understanding of gene networks relevant to cancer and neurodegeneration.

In the next session we will discuss the importance of understanding the pathways that reinforce and modulate p53 activity; this will allow the development of anticancer therapies to target tumour-supportive cellular machineries but may also be exploited to block unwanted effects of p53 activation.

3.3 The importance of cofactors in the regulation of p53 functions

p53 activities are regulated by its interaction with a high number of cofactors whose different tissue distribution is likely to concur in generating the heterogeneity of p53 responses. Some of these proteins bind the p53 DNA binding domain; an example is given by the members of the ASPP family: ASPP1, ASPP2 and iASPP. This evolutionarily conserved family of transcriptional regulators is able to regulate the proapoptotic function of the p53 family proteins. While ASPP1 and ASPP2 associate with p53 DBD and foster its binding to pro apoptotic promoters such as Bax and PIG3 (Samuels-Lev et al., 2001), iASPP binds also the proline rich domain of p53 and has the opposite effect of inhibiting apoptosis induced by p53 (Bergamaschi et al., 2003).

Some p53 cofactors belong to large cellular machineries such as chromatin modification

complexes or signal transduction cascades whose relationships with both cancer and neurodegeneration are becoming increasingly evident. In this thesis we will analyse bromodomain-containing protein 7 (BRD7), a component of epigenetic regulatory complexes, and the prolyl-isomerase Pin1, an amplifier of phosphorylation-directed signalling pathways as paradigms of cofactors able to differently instruct p53 activity, thereby providing putative new targets for cancer and neurodegenerative disease therapies.

Chromatin modifiers

Alterations in the chromatin remodelling machinery are common events in cancer. Following physiologic or pathologic *stimuli*, chromatin can be modified either by histone modifications (including acetylation, methylation, ubiquitylation, sumoylation and phosphorylation), by exchange in histone variants or by ATP-dependent sliding of nucleosomes. Interestingly, proteins regulating all these processes have been found altered in human malignancies: for example the histone methyltransferase genes MLL2 and MLL3 are the two most commonly mutated genes in medulloblastoma (Parsons et al., 2011), the histone variant macroH2A suppresses melanoma progression (Kapoor et al., 2010) and 43 % of tumours bear mutations in DAXX or ATRX, that encode subunits for a chromatin remodelling complex (Jiao et al., 2011). This widespread pattern of alterations points towards a key role of chromatin remodelling in regulating transformation, as also demonstrated by the clinical success of drugs targeting chromatin modifiers. One of the best examples are histone deacetylase (HDAC) inhibitors (vorinostat and romidespin)(Dobbelstein and Moll, 2014), which have been approved by the FDA for the treatment of cutaneous T cell lymphoma (CTCL). Besides HDACs, also bromodomain-containing proteins have recently received attention as targets for modulating chromatin dynamics. Bromodomains comprise an extensive family of evolutionarily conserved protein modules of 110aa that have the ability to bind to acetylated lysines with high specificity (Haynes et al., 1992; Zeng and Zhou, 2002). The bromodomain is found in many chromatin-associated proteins and derives its name from the *Drosophila* protein Brahma, where it was first identified (Elfring et al., 1998; Haynes et al., 1992). The finding that this domain has specific acetyl-lysine binding properties (Dhalluin et al., 1999; Hudson et al., 2000; Jacobson et al., 2000; Owen et al., 2000) highlighted how some

protein-protein interaction can be modulated by lysine acetylation, with broad implications in a wide variety of cellular processes including chromatin remodelling and transcriptional activation (Dyson et al., 2001; Winston and Allis, 1999).

Some bromodomain proteins present additional domains with specific enzymatic activity, through which they may directly mediate chromatin modifications. Among these p300/CBP (Kraus et al., 1999; Manning et al., 2001), PCAF (Dhalluin et al., 1999), $TAF_{II}250$ (Jacobson et al., 2000) have HAT activity, the transcriptional silencer Ash1L (Gregory et al., 2007) and the tumour suppressor RIZ1 (Steele-Perkins et al., 2001) have histone methyltransferase (HMT) activity and the SNF2α and β subunits of the SWI/SNF complex have ATP-dependent remodelling functions (Smith and Peterson, 2005). Conversely, other bromodomain proteins such as MTA1 (Nicolson et al., 2003), ACF1 (Eberharter et al., 2001) and BRD7, that have no catalytic activity, may mediate communication between chromatin and multiprotein remodelling complexes. These scaffolding functions contribute to epigenetic regulation of transcription through a mechanism called the histone code (Rea et al., 2000; Strahl and Allis, 2000; Turner, 2000), wherein combination of specific histone modifications such as acetylation, mediates the recruitment of different chromatin-remodelling machinery. Interestingly, a bromodomain and extra-terminal (BET) domain protein, BRD4, holds promise as a target in acute myeloid leukaemia (AML)(Zuber et al., 2011). BRD4 was identified in a short hairpin RNA screen from a collection of epigenetic regulators and its downregulation suppressed AML progression in an *in vivo* model, and the same achievement was obtained by a BRD4 inhibitor named JQ1.

In search for novel interactors of p53, in our laboratory it was performed a Yeast Two-Hybrid screening (Van Criekinge and Beyaert, 1999) by using a LexA-p53wt construct lacking the p53 transactivation domain (Δ11-69) as *bait* and a human foetal brain cDNA library cloned into the galactose-inducible expression vector pJG4–5 as *prey* (Gostissa et al., 2004). A new p53 interactor isolated with this technique was the bromodomain-containing protein BRD7. At the same time, our collaborators in the R. Agami's group at NKI (Amsterdam) isolated BRD7 in a loss-of-function screening aimed at identifying genes required for p53-dependent oncogene-induced senescence (OIS). Specifically, primary human BJ fibroblasts expressing the catalytic subunit of human telomerase (hTERT) were used, and transduced with a vector encoding 4-OH-Tamoxifen (4OHT)-

inducible oncogenic $HRas^{V12}$ (BJ/ET/Ras^{V12}ER (Voorhoeve et al., 2006)). In this system induction of constitutively activated mutant Ras^{V12} caused the onset of senescence in a p53-dependent fashion. Importantly, the acceleration of cell proliferation upon inhibition of p53 was mediated through $p14^{ARF}$, while cellular transformation was completely independent of it (Voorhoeve and Agami, 2003). Therefore, to focus on genes acting in the transformation process, the screen was performed in BJ/ET/Ras^{V12}ER cells in which $p14^{ARF}$ was knocked down. Nineteen outliers were selected from which three endowed cells with a growth advantage in the presence of Ras^{V12}, all targeting the same gene, BRD7.

The bromodomain-containing protein BRD7

BRD7 is an evolutionarily conserved and ubiquitously expressed chromatin-binding protein (Staal et al., 2000). Under the name BRD7 fall two isoforms that differ for a single amino acid residue; isoform 1 comprises 652 amino acids while isoform 2 uses an alternate in-frame splice site in the 3' coding region and thus lacks one internal residue compared to isoform 1. The molecular mass of both isoforms is about 75 kDa. The proteins bear a single central bromodomain (Staal et al., 2000), through which they bind specifically to acetylated lysines 9 and 14 on histone H3 and acetylated lysines 8, 12 and 16 on histone H4 (Peng et al., 2006; Sun et al., 2007) (Figure 10).

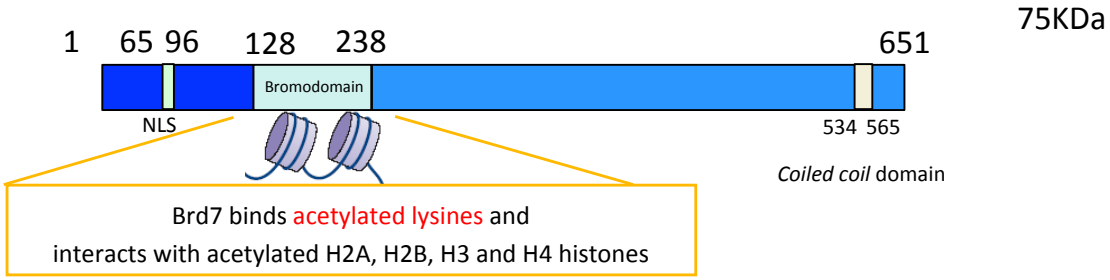


Figure 10. BRD7 structure. Schematic representation of BRD7 protein structure in which is highlighted the bromodomain in position 129-238.

Interestingly, BRD7 is frequently lost in human malignancies. Indeed BRD7 loss is a common hallmark of nasopharyngeal carcinoma (NPC), where its reduced expression has been ascribed to either loss-of-heterozygosity (LOH) on the chromosomal locus hosting

the BRD7 gene (that is one of the most frequent genomic events in NPC) or to aberrant methylation of the BRD7 gene itself (Liu et al., 2008).

Interestingly, loss of BRD7 in other tumour types has been correlated with the acquisition of highly malignant tumoral phenotypes. In high-grade ovarian carcinoma BRD7 mRNA levels have been found decreased compared to normal epithelial tissue or low-grade carcinoma (Bae et al., 2013), whereas in colorectal cancer downregulation of BRD7 protein expression with respect to adjacent normal tissue was found to be significantly correlated with advanced tumour stage and poor pathologic differentiation, as well as with unfavourable prognosis (Wu et al., 2013).

This may be due in part to the fact that BRD7 acts as a negative regulator of cell proliferation. Its ectopic expression in nasopharyngeal carcinoma-derived cell lines lacking endogenous BRD7 is able to inhibit G_1-S progression as a consequence of altering the expression of components of Ras/MEK/ERK and Rb/E2F pathways at the transcriptional level (Zhou et al., 2004). In this experimental system BRD7 has also been shown to negatively regulate cell growth by means of reducing the expression of Cyclin D1, E2F3, MEK (and consequently ERK1/2 phosphorylation) and c-jun and to upregulate expression of α-catenin that retains β-catenin in the cytoplasm thus blocking its translocation into the nucleus (Peng et al., 2007). Furthermore, BRD7 was found to inhibit the growth of prostate cancer cells by decreasing the transcriptional activity of androgen receptor (AR) via inhibition of TRIM proteins (Kikuchi et al., 2009).

In another report BRD7 was shown to interact with the tumour suppressor protein BRCA1, contributing to the transcriptional regulation of a subset of its target genes including oestrogen receptor α (ERα) and the DNA repair gene Rad51. In this set of experiments BRD7 depletion was associated to acquisition of breast cancer cells' resistance to the antiproliferative effects of the antiestrogen drug fulvestrant (Harte et al., 2010).

In addition to repressing cell proliferation, BRD7 appears to be also responsible for other tumour suppressive functions. As mentioned above, it was recently found that overexpression of BRD7 leads to decreased invasiveness of an ovarian cancer-derived cell line in vitro, and this appeared to associate with reduced expression and secretion of MMP2 (Bae et al., 2013).

In contrast to the tumour suppressive activities reported for BRD7, however, an overview of BRD7 gene alterations in human cancers as obtained by analysis of large-scale cancer

genomics data sets reveals a more complex picture, with occurrence of gene deletions, mutations, and amplifications in many different tumour types. This opens the possibility that under particular circumstances, even increased levels of BRD7 may be beneficial for cancer growth and progression.

BRD7 has been shown to participate in both chromatin-modifying complexes, in particular to the histone methyltransferase Polycomb PRC2 complex (Tae et al., 2011), and the chromatin remodeling (SWI/SNF PBAF) complexe (Kaeser et al., 2008), and to interact with several transcription and chromatin-associated factors. In this respect, it has been proposed that BRD7 may provide promoter specificity for the recruitment of epigenetic regulators on its target genes, thereby promoting either activation or repression of a large number of genes in a promoter- and context-specific fashion. Indeed BRD7 participates in SWI/SNF and PRC2 chromatin remodelling complexes, that have been demonstrated to regulate a high number of processes.

For instance, some evidences point towards a role of BRD7 in development; indeed it was demonstrated that BRD7 and the SWI/SNF complex PBAF it belongs to are essential for the neural development of *Xenopus laevis* and drive the formation of multipotent migratory neural crest (NC), a transient cell population that is ectodermal in origin but undergoes a major transcriptional reprogramming event to acquire a remarkably broad differentiation potential and ability to migrate throughout the body, giving rise to craniofacial bones and cartilages, the peripheral nervous system, pigmentation and cardiac structures (Bajpai et al., 2010).

Modulating signal transduction

The reversible phosphorylation of proteins is one of the most important and widespread regulatory mechanism in the cell (Ryo et al., 2003a). Therefore, the ability to define the regulatory components of the phosphorylation/dephosphorylation cascades and their interactions with other cellular networks are critical to our understanding of the molecular mechanisms underlying diverse biological processes and human diseases. Indeed, reversible phosphorylation of certain serine or threonine residues preceding a proline (pSer/Thr-Pro) represents a key switch for controlling the function of many signalling molecules in various cellular processes. Upon Ser/Thr-Pro phosphorylation, proteins

undergo conformational modifications that affect their structure, stability and functions. This depends on the activity of a unique enzyme, the phosphorylation-dependent peptidyl prolyl isomerase (PPIase) Pin1, that catalyses cis/trans isomerization at Ser-Pro or Thr-Pro motifs in which the first amino acid is phosphorylated (pSer/pThr-Pro).

The prolyl-isomerase Pin1

Pin1 structure and functions. The human Pin1 gene maps to chromosome 19p13 and encodes a protein of 163 amino acids with a mass of 18kDa. From a functional point of view, Pin1 is composed of two domains: an amino terminal WW domain (amino acids 1–39) and a carboxy-terminal PPIase domain (amino acids 45–163), which are separated by a short flexible linker region (Figure 11). The N-terminal WW region is characterized by two conserved tryptophan residues and mediates the interaction with the substrates on pSer/Thr-Pro sites, which cannot be bound by any other isomerase (Ranganathan et al., 1997; Yaffe et al., 1997). As a consequence of the interaction, the WW domain targets the Pin1 catalytic domain close to its substrates, so that the PPIase domain can isomerize specific pSer/Thr-Pro motifs and induce conformational changes.

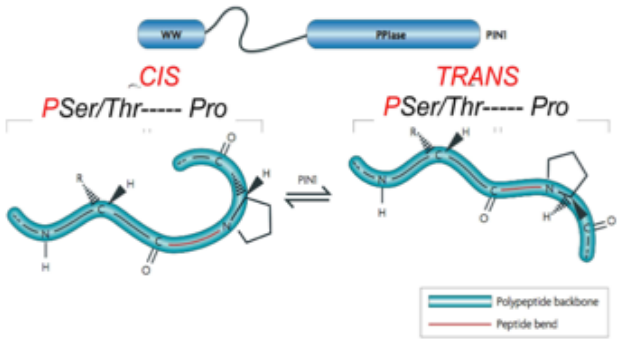


Figure 11. Structure and activity of the prolyl-isomerase Pin1 (Yeh and Means, 2007).

By modification of a plethora of cellular substrates Pin1 governs a variety of cellular processes including cell cycle, transcription and splicing, RNA editing, DNA damage and oxidative stress responses, germ cell development, stem cells self renewal/expansion and neuronal survival (Brenkman et al., 2008; Pinton et al., 2007; Rustighi et al., 2014; Yeh and Means, 2007).

Pin1 mouse models. Pin1 is highly conserved during evolution and is essential for life in *X.Laevis*, *S.Cerevisiae*, *C.Albicans* and *A.Nidulans*, but it is not an essential protein in metazoans such as *D.Melanogaster* or *M.Musculus* (Fujimori et al., 1999; Maleszka et al., 1996). Indeed the absence of *dodo*, the Pin1 homologue in *Drosophila*, results in no obvious phenotypic consequences, but developmental defects in the dorsal-ventral patterning of the egg chamber. Knockout mice for Pin1 gene have been produced in two different backgrounds. The first model, which was obtained in a mixed background (129SvJae/C57BL/6), displays tissue defects that are reminiscent of aging. Along with decreased body weight and testicular atrophy, the most striking alterations involve retina and mammary gland. Indeed, $Pin1^{-/-}$ mice show dramatic impairment in cell survival and proliferation in the retina. Moreover, in pregnant $Pin1^{-/-}$ females, mammary epithelial cells fail to undergo massive proliferation in the development of alveolar structures and ductal side branching (Liou et al., 2002). In a second $Pin1^{-/-}$ model obtained in C57BL/6 background, both male and female Pin1-null mice display a reduced number of germ cells owing to impairment in primordial germ cell (PGC) expansion, thereby causing a profound decrease in fertility (Atchison and Means, 2003).

Pin1 in cancer. In line with its role in sustaining cell proliferation and self-renewal, at least in some cell types, Pin1 expression is induced downstream of oncogenic pathways such as Ras/E2F. Accordingly, Pin1 is found overexpressed in breast, prostate, lung, colon, oesophageal, ovarian and cervical cancers, human oral squamous cell cancer, glioblastoma and melanoma (Atkinson et al., 2009; Bao et al., 2004; Jin et al., 2011; Miyashita et al., 2003). In some cancer subtypes, Pin1 also functions as a prognostic marker (Ayala et al., 2003; Fukuchi et al., 2006; Girardini et al., 2011; Tan et al., 2010; Wulf et al., 2001).
All these evidences underscore a prominent role of Pin1 in up-regulating key cellular pathways that are central during oncogenesis (Lu and Zhou, 2007) (Figure 12).
Pin1 is able to directly raise cyclin D1 levels by fostering its transcription, moreover up-regulation of cyclin D1 expression induced by oncogenic stimuli the Ras, Her2/Neu and Wnt signalling pathways and by NF-κB requires the presence of Pin1 which in turn is indispensable for full activity of these pathways (Liou et al., 2002; Lu and Zhou, 2007; Ryo et al., 2001; Ryo et al., 2003b; Wulf et al., 2001). Pin1 can bind to phosphorylated c-

Jun, β-catenin and NF-κB boosting their protein levels and transcriptional activity, with the consequence of increasing cyclin D1 gene expression. Moreover, Pin1 is able to shield β-catenin and NF-κB from their negative regulators, APC (adenomatous polyposis coli gene product) and IkB, respectively.

One of the key signalling pathways induced upon activation of growth factor receptors is the MAPKs cascade. In response to growth stimuli Ras activates the Raf kinase, which in turn controls the MAPKs. As for other signalling pathways, this kinases cascade must be turned off by a negative feedback mechanism in which MAPKs phosphorylate and inactivate Raf. Notably, Pin1 prevents this negative feedback, by promoting Raf dephosphorylation and consequently maintaining an activated MAPKs cascade (Dougherty et al., 2005).

In the context of triple negative breast cancer it has been demonstrated that Pin1 cooperates with two oncogenes important for this malignancy, namely Notch1 (Rustighi et al., 2009) and mutant-p53 (Girardini et al., 2011). Notch1 is a membrane-bound heterodimeric receptor, normally inactive, that becomes activated upon interaction with its specific ligand (Ranganathan et al., 2011). Pin1 interacts with the Notch1 receptor and increases its stimulus-induced cleavage mediated by γ-secretase, allowing full activation of the pathway and boosting its oncogenic activity both *in vitro* and *in vivo*. Also in this case a positive loop is generated since activated Notch is directly recruited on the Pin1 gene promoter thus inducing Pin1 expression. As a consequence, in human breast cancer samples there is a strong correlation between high levels of activated Notch and Pin1 overexpression (Rustighi et al., 2009). Moreover it was recently demonstrated that Pin1 can modulate Notch levels also post-transcriptionally by regulating the detachment from its negative regulator Fbw7 (Rustighi et al., 2014).

Pin1 and p53. In contrast with its activities in amplifying oncogenic pathways, in normal conditions Pin1 has a central role in transducing genotoxic stimuli and likely also oncogenic stress into full activation of wild-type p53 functions. In particular, Pin1 is indispensable for transducing stress-induced phosphorylation of p53 into conformational changes that affect its stability and function. While almost undetectable in normal conditions, the interaction between p53 and Pin1, mediated by the N-terminal WW domain of the isomerase, can be promoted by several stimuli activating p53, such as γ- and UV

irradiation, treatment with chemotherapeutic drugs and overexpression of activated oncogenes, that result in phosphorylation of different Ser/Thr-Pro motifs in p53, rendering them sites for Pin1 binding. Within the p53 sequence six Ser/Thr-Pro sites are present (Ser33-Pro34, Ser46-Pro47, Thr81-Pro82, Ser127-Pro128, Thr150-Pro151 and Ser315-Pro316). It has been shown that UV irradiation or treatment of cells with doxorubicin recruits Pin1 to Ser33, Thr81 and Ser315 of p53, while γ-irradiation and molecules such as RITA (Sorrentino et al., 2013) stimulate also Pin1 interaction with Ser46. The structural change mediated by Pin1 can trigger various functional outcomes, depending on the specific phosphorylation events and on the cellular context. As a result, many p53 downstream responses, such as transcriptional activation of endogenous target genes as well as induction of apoptosis and growth arrest, are impaired in cells lacking Pin1.

The catalytic activity of Pin1 can modify p53 conformation and this mediates efficient detachment of Mdm2 from p53, resulting in full stabilization of the protein (Zacchi et al., 2002). Accordingly, p53 accumulation following genotoxic stress is reduced in the absence of Pin1 and its half-life is shortened. In addition, it has also been demonstrated that Pin1 is required for efficient binding of p53 to its REs on p21 and Bax promoters upon stress (Mantovani et al., 2007). Moreover, it was shown that Pin1 itself is recruited by p53 on its cognate promoters where it acts enhancing p53 acetylation by p300. Indeed, it was observed that acetylation of chromatin-bound p53 on Lys373 and Lys382 increased upon stress only in the presence of Pin1 (Berger et al., 2005). Another site which may be crucial for Pin1 effect on p53-mediated apoptosis is Ser46-Pro47, as also suggested by the reduced apoptotic potential displayed by the Ser47 allele of the Pro/Ser47 polymorphism (Li et al., 2005).

Pin1 is also an essential regulator of the pro-apoptotic function of the p53-family member p73. Indeed, it has been demonstrated by our group that the binding between Pin1 and p73 is stimulated upon stress conditions by c-Abl and p38 kinases and that this association increases p73 acetylation by p300, thereby stimulating its transcriptional activity towards apoptotic target genes, such as p53AIP1 (Mantovani et al., 2004).

On the other hand, Pin1 has been linked to mestastasis, aggressiveness and poor prognosis in triple negative breast cancer through cooperation with mutant p53 following oncogenic stress. In particular, it has been described that Pin1 binds to phosphorylated mutant p53 in breast cancer cell lines and tumours and boosts its oncogenic functions, by favouring

mutant p53-dependent inhibition of the antimetastatic factor p63 as well as induction of a mutant p53 transcriptional program to increase aggressiveness. This cooperation correlates with poor prognosis in breast cancer patients and promotes migration and metastasis *in vivo*. Thus, ablation of Pin1 lowers the oncogenic gain of function of mutant p53 with reduced formation of breast cancer metastasis into the lung (Girardini et al., 2011).

Pin1 in neurodegeneration. As opposed to the majority of highly differentiated cells in the body, where Pin1 levels are greatly reduced, Pin1 expression is induced during neuronal differentiation and is observed in neurons at considerable high levels. Recent studies have indicated that Pin1 regulates several neuronal substrates (among them, noteworthy are tau and amyloid precursor protein) (Figure 12), and that it plays an important role in age-dependent neurodegeneration. However, the role of Pin1 in healthy neurons and during development of the nervous system remains largely unknown.

Although Pin1 is expressed in most human neurons, in human AD neurons it has been found to be inhibited or its levels downregulated (Wang et al., 2007), underlying AD associated neurofibrillary tangle and amyloidogenic Aβ formation (Lu et al., 1999; Pastorino et al., 2012). It has been demonstrated that Pin1 activity can directly restore the conformation and function of phosphorylated tau by indirectly promoting its dephosphorylation. Specifically, Pin1 acts on the phosphorylated Thr231-Pro in tau to promote PP2A-mediated dephosphorylation and restore its microtubule binding function. In addition, Pin1 also regulates APP processing and Aβ production. In mouse brain, the absence of Pin1 increases amyloidogenic APP processing and elevates the toxic species of Aβ 42 in an age-dependent manner.

In Parkinson's Disease Pin1 accumulates in Lewy bodies and enhances the formation of α-synuclein inclusions by protecting α-synuclein from degradation (Ryo et al., 2006). It has been discovered that Pin1-mediated prolyl-isomerization modulates α-synuclein aggregation indirectly by acting on its regulatory protein, Synphilin-1. However, the mechanisms underlying the role of Pin1 in PD remain largely unknown (Ghosh et al., 2013).

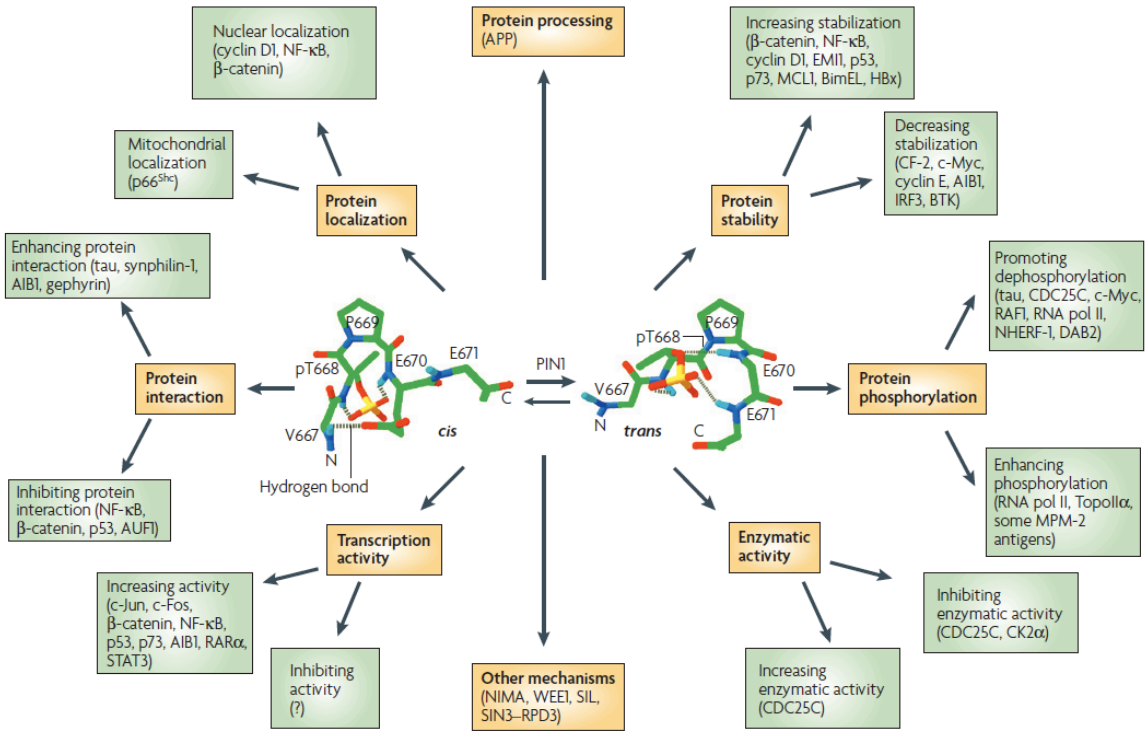


Figure 12. PIN1 regulates many substrates involved in cancer and neurodegeneration (Lu and Zhou, 2007).

4. AIM OF THE THESIS

Neoplastic and neurodegenerative disorders are two fields of research in which, although massive efforts to obtain a clear comprehension of the underlying molecular pathways have recently resulted in several specific therapeutic advances, still raises a profound need for innovative and more powerful approaches. Interestingly, a number of cellular pathways contribute to both pathologies, although with inverse effects, and this suggests the possibility of translating the knowledge obtained in one field to the other.

One of the genes that stand at the crossroads between cancer and neurodegeneration is the tumour suppressor p53 whose key roles in preventing cancer development have been extensively studied in the last 30 years. Intriguingly, p53 is strongly emerging as a regulator of neuronal cell death.

Aim of this thesis is to understand the pathways through which the p53 response is finely orchestrated and regulated during both neoplastic transformation and neurodegenerative disease, with the purpose of highlighting common molecular mechanisms. The first part of this study will focus on the role of the chromatin remodelling machinery, and in particular of the bromodomain-containing protein BRD7, in regulating p53 oncosuppressive functions following oncogenic stress. In the second part we will investigate the role of the prolyl-isomerase Pin1 in regulating activation of p53 apoptotic function in Huntington's Disease.

These studies, conducted with the aim of achieving greater knowledge on the regulation of the p53 pathway in both contexts and of underscoring similarities and differences, might be relevant for designing new targeted treatments for neoplastic and neurodegenerative disorders.

5. RESULTS PART 1

Dissecting the tumour suppressive roles of the bromodomain-containing protein BRD7

As mentioned in the Introduction, the p53 pathway plays a central role in the maintenance of genomic integrity and in tumour suppression. Alterations (polymorphisms, mutations or deletions) in genes of the p53 network, encoding for factors that sense cellular stress and modulate p53 activity, can have a role in tumorigenesis determining fundamental aspects of neoplastic disease such as incidence, age of onset, prognosis and response to therapy of specific tumour types. Indeed the identification of novel p53 cofactors and of their possible alterations in human malignancies is particularly important because it may provide new prognostic markers but also potential therapeutic targets to develop specific anticancer therapies.

By means of Yeast Two-Hybrid screening we identified the bromodomain-containing protein 7, BRD7, as a novel interactor of p53. Moreover at the same time a loss-of-function screening in primary human BJ fibroblasts expressing inducible oncogenic $HRas^{V12}$ (BJ/ET/Ras^{V12}ER cells) suggested a potential role of BRD7 in regulating oncogene-induced senescence.

Based on these evidences, we decided to dissect the relationship between BRD7 and p53 from a functional point of view.

BRD7 interacts physically with p53 and regulates oncogene-induced senescence

By means of co-immunoprecipitation analysis, we first demonstrated that the expression of Ras^{V12} in BJ/ET cells was able to increase the interaction between endogenous BRD7 and p53 (Figure 13A); moreover, by proliferation assays we also confirmed that BRD7 cooperated in the induction of p53-mediated oncogene-induced senescence (Figure 13B).

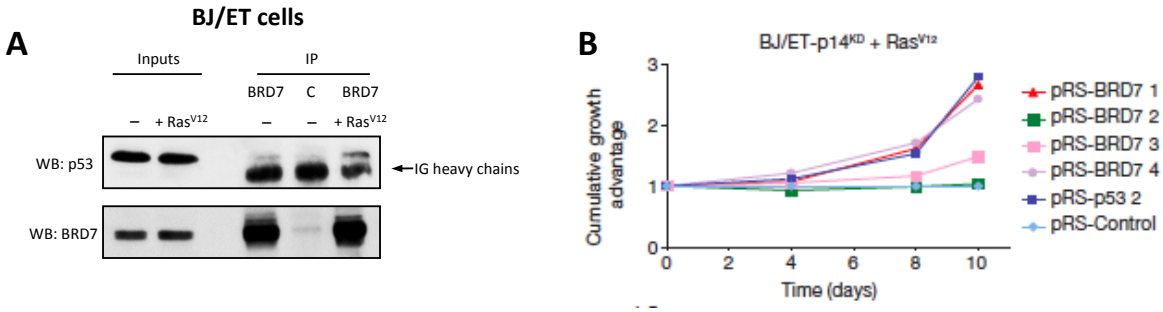


Figure 13. BRD7 interacts physically with p53 and cooperates in the induction of oncogene-induced senescence.

A) Co-immunoprecipitation of endogenous BRD7 and p53 in BJ/ET/Ras^{V12}ER cells cultured in the absence (–) or presence (+Ras^{V12}) of 4OHT. Lysates were immunoprecipitated with anti-BRD7 antibody. Control IP (C) was performed with anti-HA antibody. The migration of immunoglobulin heavy chains is shown.

B) GFP-growth competition assays of BJ/ET/p14$^{ARF\ KD}$/Ras^{V12}ER transduced with the indicated pRetroSuper (pRS) knockdown vectors and cultured in the presence of 4OHT (+Ras^{V12}).

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

As BRD7 is known to bind acetylated lysines in histones through its bromodomain (Peng et al., 2006; Sun et al., 2007) and has also been found to be a component of chromatin-remodelling complexes (Kaeser et al., 2008; Tae et al., 2011), we investigated whether BRD7 was required for the transcriptional activation of p53 target genes involved in cell cycle arrest. We performed mRNA-expression array analysis comparing Ras^{V12}-expressing BJ/ET cells containing either one of two different BRD7^{KD} constructs, a p53^{KD} construct, or a control vector. Gene-ontology (GO-) analysis revealed that BRD7^{KD} significantly affected the expression of several p53 target genes, among those p21 (CDKN1A) and HDM2 (Figure 14A), leaving unvaried pro-apoptotic genes such as Puma, Fas and Bax.

As BRD7 might act as a transcriptional cofactor of p53 for a subset of target genes, we determined whether it was found within transcriptional complexes loaded on the p53 cognate sites in these promoters. Chromatin IP (ChIP) for BRD7 from BJ/ET cells expressing Ras^{V12} showed specific binding of BRD7 at the upstream (-2.3 kb) p53-binding site in the p21 promoter, and within the p53-binding region of the HDM2 gene (Figure 14B), which both require BRD7 for efficient transcriptional activation. In search for a mechanism of BRD7 activity on p53-target promoters, we determined the acetylation status of histone 3 at lysine 9 (H3K9), a marker of active chromatin (Kouzarides, 2007). ChIP analysis revealed that loss of BRD7 expression resulted in a great reduction of H3K9

acetylation around p53-binding sites in the p21 and HDM2 promoters induced by either Ras^{V12} expression or etoposide treatment (Figure 14C).

Activated p53 induces the p21 promoter by recruiting p300, as well as other histone-acetyl transferases (HATs), to modify histone tails (Barlev et al., 2001; Espinosa et al., 2003; Zhao et al., 2006). We demonstrated that endogenous BRD7 and p300 proteins are found in complex in Ras^{V12}-expressing BJ/ET cells. Interestingly, we also demonstrated that loss of BRD7 resulted in a marked reduction of p300 loading on the p21 promoter in Ras^{V12}-expressing BJ/ET cells, event that led to a decrease in histones acetylation and in p53 acetylation on K382 (model in Figure 14D), a key lysine modified by p300/CBP after Ras^{V12} induction (Pearson et al., 2000) and important for p21 transcription (Dornan et al., 2003; Pearson et al., 2000).

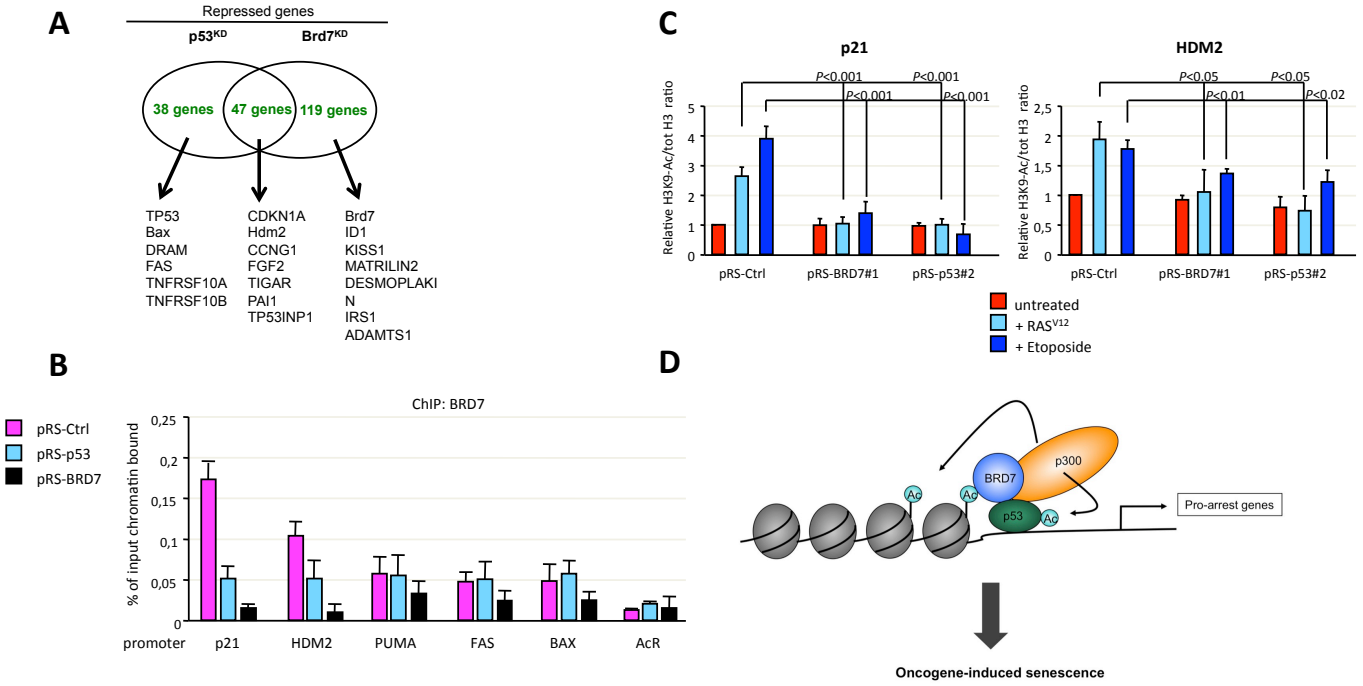


Figure 14. BRD7 is required for efficient transcriptional activation of a specific subset of p53 target genes, binds to selected p53 target gene promoters in a p53-dependent fashion and affects histone acetylation.

A) 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.

B) BJ/ET/Ras^{V12}ER cells containing a control vector, $BRD7^{KD}$ vector (BRD7#4), or $p53^{KD}$ vector were cultured in the presence of 4OHT for 48 hrs and subjected to ChIP for BRD7.

C) BJ/ET/Ras^{V12}ER cells expressing the indicated knockdowns were cultured in the presence or absence of 4OHT or treated with etoposide, and subjected to ChIP with antibodies against acetylated H3K9 or total H3 histone.

D) A schematic model showing how BRD7 may serve as a p53 cofactor to regulate the transcriptional activity of p53 target genes.

Inhibition of BRD7 cooperates in oncogene-induced transformation of epithelial mammary cells

BRD7 is encoded in a locus on chromosome 16q12 that is a well-known hotspot for LOH in breast cancer (Argos et al., 2008), a tumour in which p53 is frequently wild-type. Prompted by this observation, analysing CGH data of a group of 68 sporadic breast tumours in which the p53 status was determined by sequence analysis it emerged that the clearest significant difference was a loss on chromosome arm 16q. Importantly, the genomic position of BRD7 is at 48.9 Mb, at the centre of the first differential loss (Figure 15A). However, our data does not exclude that other genes in this region are involved.

Based on these evidence, we decided to employ a common cellular model of breast carcinogenesis, the normal mammary epithelial cells MCF10A (bearing wild-type p53) transduced with the oncogene Ras^{V12} (Datta et al., 2007), to investigate the role of BRD7 in imposing a barrier to neoplastic transformation in normal breast cells. MCF10A cells are spontaneously immortalized and bear a deletion in the locus 9q21, which contains the oncosuppressors INK4A/p16 and ARF/p14 (Kadota et al., 2010; Soule et al., 1990); moreover MCF10A cells also display *MYC* amplification (Kadota et al., 2010; Soule et al., 1990). In this context we tested whether loss of BRD7 was sufficient to inactivate the p53 pathway and induce cellular transformation, evaluated by the ability to grow anchorage-independently in semi-solid medium (soft agar assay). Notably, knockdown of BRD7 resulted in efficient colony formation (Figure 15B) demonstrating that efficient activation of p53 by oncogenes, as a mechanism against transformation, requires BRD7.

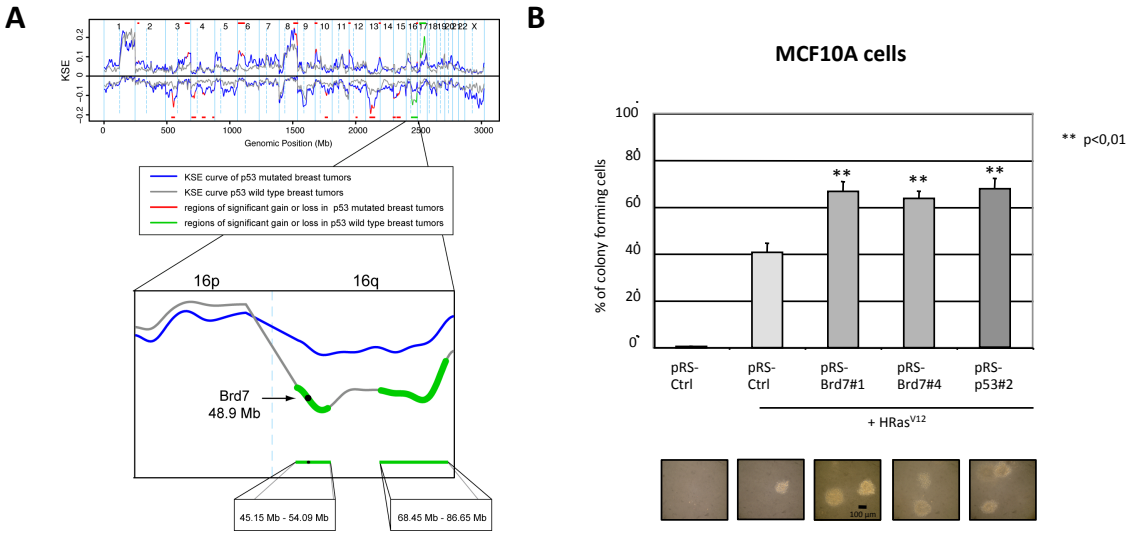


Figure 15. BRD7 expression is lost in a subset of breast tumours containing wild-type p53 and BRD7 knockdown enhances oncogene-induced transformation of MCF10A cells.

A) 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 to the p53 mutant tumours) are depicted with a green bar on top or below the figure, respectively, and with a green line in the grey KSE curves. The significant changes in p53 mutant tumours (compared to p53 wild-type) are shown in red.

B) Normal MCF10A breast epithelial cells stably expressing the indicated knockdowns were transduced with pLPC-puro-HRas^{V12} or with the empty vector. After selection the cells were seeded in soft agar and colonies were counted 2 weeks later. Histograms show the percentage of colony-forming cells and represent mean and s.d. of three independent experiments.

The results reported in this part have been published in the original article:

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

The expression of BRD7 is decreased in high-grade breast cancer relative to normal tissue or low-grade breast cancer

Starting from the abovementioned gene expression analysis that we performed in BJ/ET human fibroblasts expressing Ras^{V12} (Figure 14A) we analysed the functional categories of the genes repressed only by BRD7 knockdown. Interestingly, these genes appear to be involved in the processes of cancer progression (blood vessel morphogenesis and development) and metastasis (cell chemotaxis, migration and motility). This prompted us to investigate the roles of BRD7 in repressing breast cancer aggressive phenotypes as for example the capacity to metastasize and relapse after chemotherapy.

Due to the poor quality of the BRD7 probe employed on microarray platforms, the analysis of BRD7 mRNA levels in publicly available datasets has been highly problematic and unreliable. For this reason we decided to analyse the methylation status of BRD7 promoter on genomic DNA from breast cancer samples. A CpG island was recently identified in the BRD7 gene that is methylated in a large percentage of NPC tumours (Liu et al., 2008), therefore we performed the analysis of the methylation of BRD7 promoter from genomic DNA of a subset of 104 primary breast cancers (Girardini et al., 2011); this analysis pointed out that BRD7 promoter methylation is significantly more frequent in G3 (and thus more aggressive) tumours (Figure 16B).

Due to the intrinsic heterogeneity of breast cancer, BRD7 may be lost only in some cells of the tumour mass (Polyak, 2011); to verify this hypothesis we decided to examine BRD7 expression by immunohistochemistry in different subsets of breast cancer tissues. Although the analysis is still being performed, we observed that in part of breast cancer tissues, BRD7 is lost or localizes in the cytoplasm in some cells (Figure 16C). Interestingly, there are also some tumours that bear enhanced levels of BRD7 expression. Further studies will be performed to complete the statistical analysis and to assess the correlation of BRD7 levels with cancer aggressiveness.

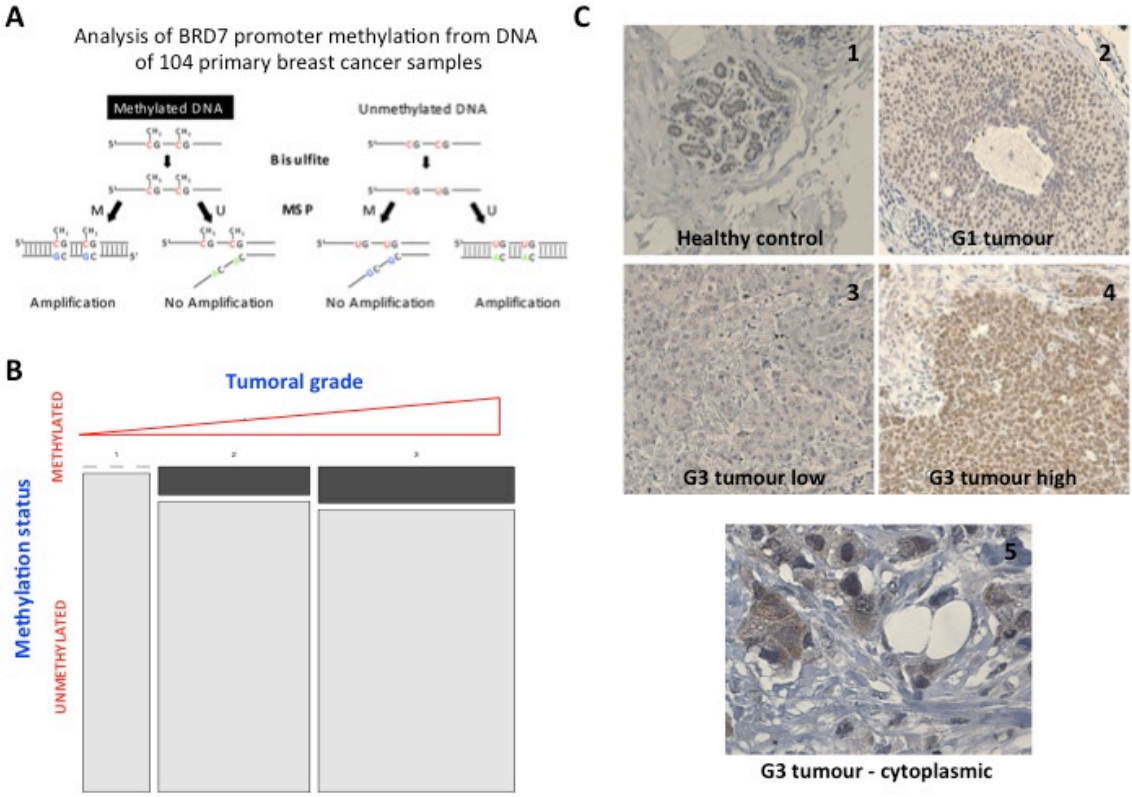


Figure 16. BRD7 loss fosters the acquisition of an aggressive tumour phenotype in breast cancer.
A) Analysis of BRD7 promoter methylation was performed on genomic DNA from a cohort of 104 primary breast cancer samples. B) The analysis unveils BRD7 loss in G3 tumours in respect to G1 tumours.
C) Staining of BRD7 by IHC shows clear nuclear localization of the protein in healthy control tissue and low-grade tumours, whereas in high-grade tumours BRD7 is either lost (3) or overexpressed (4). In some cases of triple negative breast cancer, BRD7 also displays a strong cytoplasmic localization (5). All the pictures were acquired with a 10X objective except picture (5) that was acquired with a 40X objective to better show the cytoplasmic localization.

Downregulation of BRD7 fosters the acquisition of a transformed phenotype in normal breast epithelial cells subjected to oncogenic stress

To dissect the role of BRD7 in repressing breast cancer aggressive phenotypes we took advantage of the already used normal mammary epithelial cells (MCF10A) in which we overexpressed the oncogene $HRas^{V12}$, a well-known model of mammary transformation (Datta et al., 2007): as shown in Figure 17B, we observed that the cells depleted of BRD7 upon $HRas^{V12}$ overexpression acquired an enhanced fibroblast-like, mesenchymal phenotype compared to the control cells, and that this process can be assimilated to that of epithelial-to-mesenchymal transition (EMT) (Kalluri and Weinberg, 2009). This was

confirmed by analysis of EMT markers (reduction of the epithelial marker E-cadherin and induction of mesenchymal markers, in particular Vimentin) by WB, qRT-PCR and IF (Figure 17C-E).

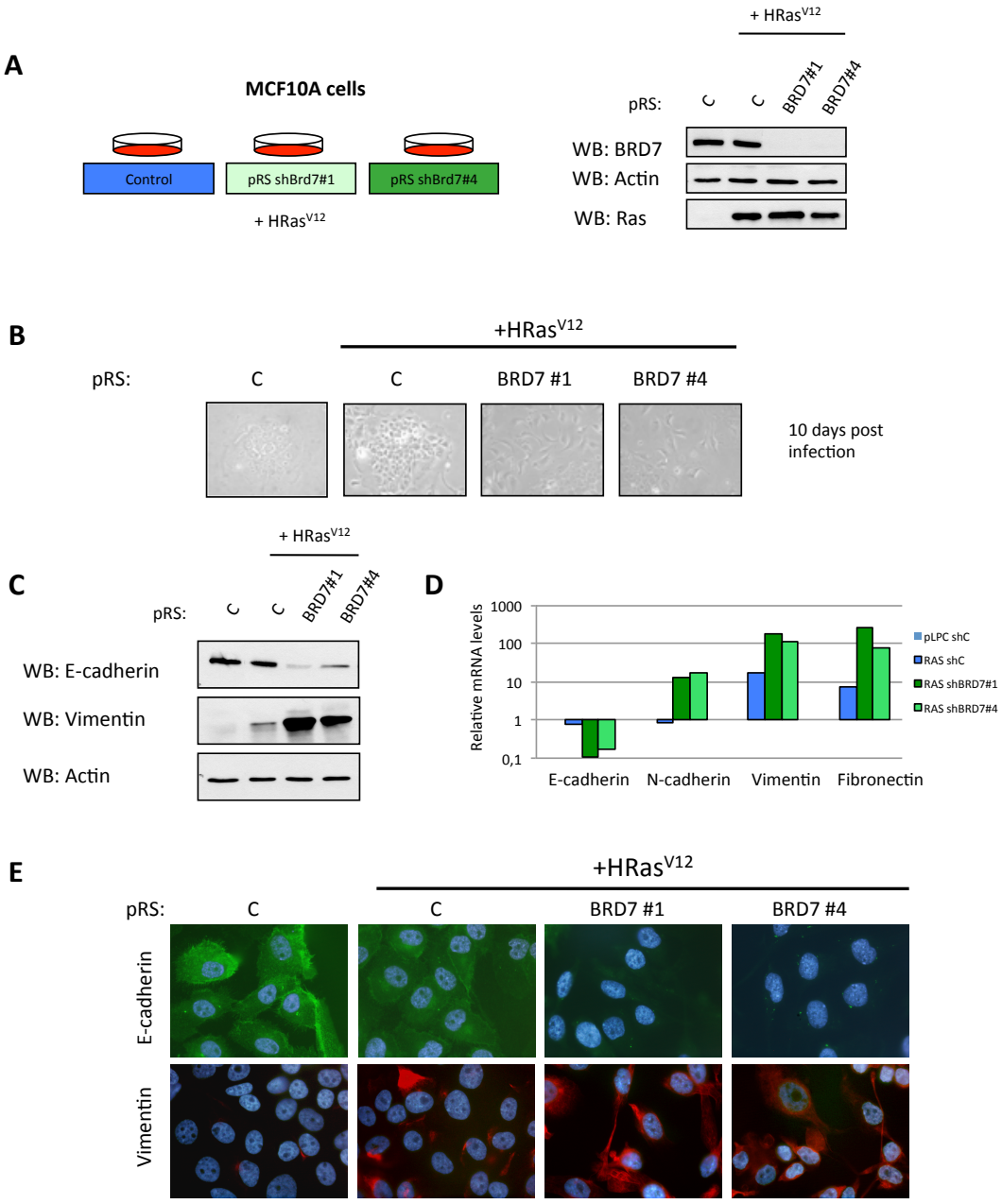


Figure 17. BRD7 loss fosters the acquisition of fibroblast-like, mesenchymal phenotype in MCF10A-HRas^{V12} cells.

MCF10A cells stably silenced for BRD7 expression were transduced with HRAS^{V12}; Western blot analysis was performed to detect BRD7, Ras and actin (loading control) (panel A). After 10 days phase-contrast images of the cells were acquired (panel B) and the status of EMT markers (E-cadherin: epithelial marker; vimentin, fibronectin and N-cadherin: mesenchymal markers) was evaluated by WB (panel C), qRT-PCR (panel D) and immunofluorescence (panel E).

As the process of EMT has been associated with increased cell motility and invasion, we analysed the role of BRD7 in suppressing oncogene-induced migration and invasion of breast epithelial cells. Indeed, BRD7 downregulation resulted in an increase of at least 3-fold in the ability of MCF10A HRas^{V12} cells to migrate as compared to the effect induced by HRas^{V12} expression alone (Figure 18A). Similarly, knockdown of BRD7 was able to foster MCF10A HRas^{V12} invasion across a transwell membrane (Figure 18B).

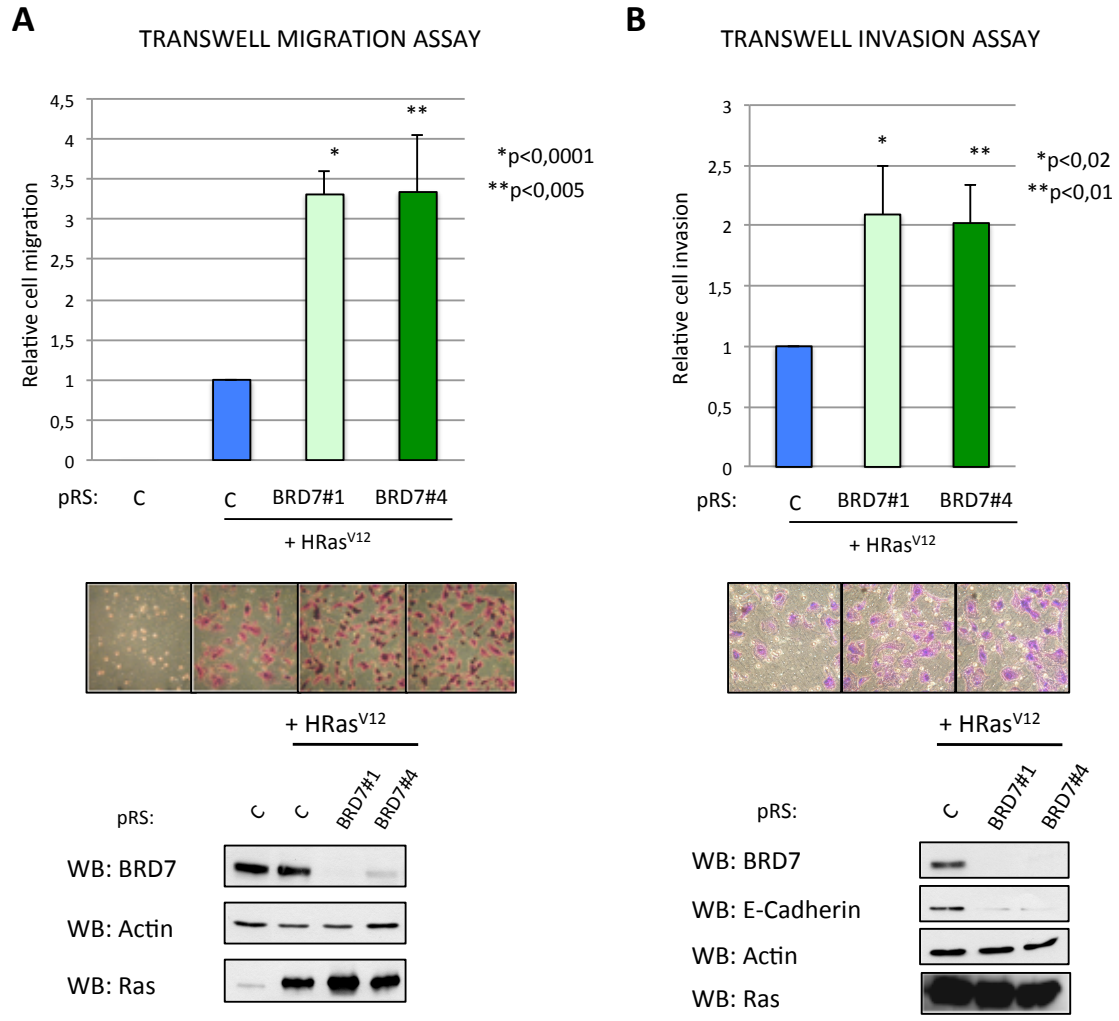


Figure 18. BRD7 inhibits migration and invasion of MCF10A HRas^{V12} cells.
A) MCF10A cells stably silenced for BRD7 expression were transduced with HRAS^{V12}. After 10 days the cells were seeded in 8-µm pore transwell filters and allowed to migrate towards their bottom side for 16 hours.
B) The same cells of the experiment in panel A) were subjected to invasion assay with transwell filters coated with matrigel.

Downregulation of BRD7 impacts on cancer stem cell content and affects acinar structure of MCF10A HRas^{V12} cells

Referring to the recent findings that EMT can promote the acquisition of stem cell traits (Mani et al., 2008) and that the progression to more malignant stages is accompanied by an increased proportion of cancer stem cells within tumours (Pece et al., 2010), we investigated whether BRD7 could regulate the formation and propagation of cancer stem cells. At first we observed that stable silencing of BRD7 in both normal MCF10A and MCF10A HRas^{V12} cells fosters the appearance of a large subpopulation characterized by stem-cell epithelial surface markers (high levels of CD44 and low levels of CD24) (data not shown). Prompted by this indication, we assessed the self-renewing potential of these cells *in vitro* by performing a mammosphere formation assay (Dontu et al., 2003). As can be seen in Figure 19, depletion of BRD7 can foster Ras-induced formation of both primary and secondary mammospheres in a manner comparable to the silencing of p53, an essential regulator of breast cancer stem cell propagation (Cicalese et al., 2009).

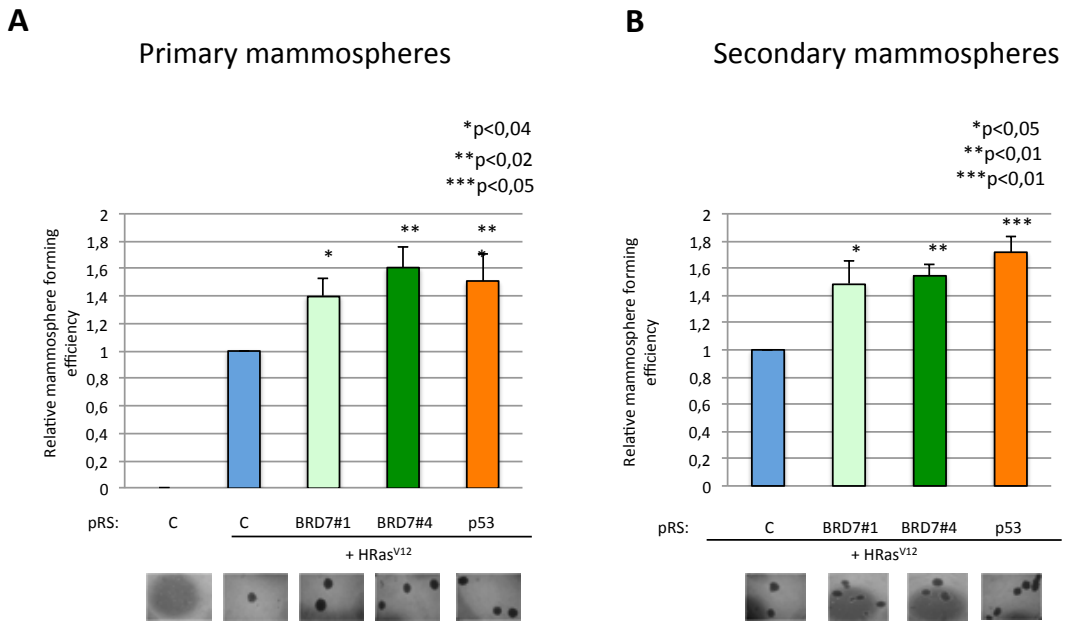


Figure 19. BRD7 loss increases oncogene-induced mammosphere formation. Mammosphere assays were conducted in normal MCF10A breast epithelial cells stably expressing the indicated knockdowns and HRas^{V12} or control empty vector; after one week of primary culture (panel A), mammospheres were disgregated, replated and allowed to form secondary mammospheres for an additional week (panel B). At each passage only mammospheres > 50μm were counted. The data are reported as mammosphere formation efficiency normalized to the control.

MCF10A mammary epithelial cells, when grown on a reconstituted basement membrane (3D culture), form polarized, growth-arrested acini-like spheroids that recapitulate several aspects of glandular architecture *in vivo*. Introduction of oncogenes into MCF10A cells disrupts this morphogenetic process (Debnath et al., 2003), and allows for modelling the biological activities of cancer genes, with particular regard to their ability to disrupt the epithelial architecture during the early steps of carcinoma progression (Debnath et al., 2003). We performed 3D culture assays on MCF10A cells transduced with Ras^{V12} and silenced for BRD7 expression; as can be seen in Figure 20, $HRas^{V12}$ expression *per se* is able to disrupt the acinar structure as expected. BRD7 depletion fosters the acquisition of a highly disorganized acinar morphology thus promoting the formation of markedly invasive stellate structures (Figure 20). We are currently investigating whether in the absence of oncogenic *stimuli* BRD7 can predispose the acini to oncogenic transformation by leading to disorganized cell polarity.

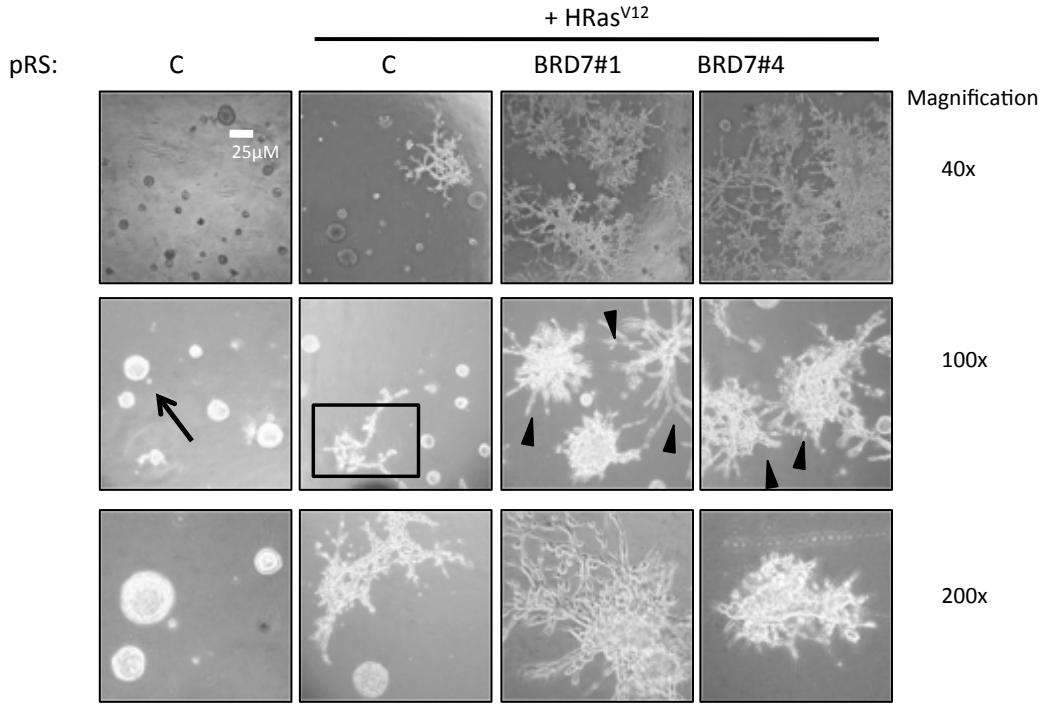


Figure 20. BRD7 loss fosters the disruption of the normal acinar structure of MCF10A cells grown in 3D culture after $HRas^{V12}$ infection. Normal MCF10A cells form organized spherical acinus-like structures when grown for 3 weeks in 3D culture (see arrow). After $HRas^{V12}$ infection the cells lose the acinar organization and this results in the acquisition of a "stellate" morphology (square). BRD7 loss fosters this phenotype by increasing the amount of cells that acquire the aberrant morphology and increasing the size of the outgrowths (arrow heads).

In sum, we demonstrated by different means that BRD7 acts as a negative regulator of the process of EMT and of associated aggressive features including migration/invasion and cancer stem cell expansion. Notably, we observed the same phenotypes also by inducing EMT and transformation through overexpression of oncogenes such as TWIST and c-MYC (data not shown). This suggests a general role of BRD7 in controlling the acquisition of EMT-related traits during the transformation process.

BRD7 impacts on the transcriptional program of MCF10A and MCF10A HRas^{V12} cells

At this point we wanted to get insight into the cellular pathways and mechanisms by which BRD7 may negatively act on transformed cell features. Given the well-assessed role of BRD7 as a regulator of transcription we decided to analyse the consequences of BRD7 knockdown on global gene expression in MCF10A and MCF10A HRas^{V12} cells. In the normal cells and in their transformed counterpart we silenced BRD7 expression by transfecting twice a specific BRD7 siRNA or a control siRNA and after 48h the RNA was collected for the analysis (Figure 21A). We performed a global sequencing of polyA^{+} transcripts in order to obtain a precise evaluation not only of mRNA expression but also of splice variants and lncRNAs with polyA-tail. After the sequencing, the data obtained have been uploaded on the server of the European Bioinformatics Institute (EBI) and analysed by imposing a fold enrichment cut-off of 1,5 and a p-value cut-off of 0,000001. As expected, overexpression of the oncogene HRas^{V12} alone was able to modulate the transcription of thousands of genes (Figure 24B). In addition, the analysis of the profile of polyA^{+} transcripts after BRD7 depletion, both in normal and oncogene-expressing cells, unveiled that BRD7 knockdown altered the expression of hundreds of genes. Interestingly, the majority of genes were upregulated upon BRD7 depletion, suggesting that BRD7 may normally act mainly as a transcriptional repressor. In particular, we identified 528 genes that were upregulated and 148 genes that were downregulated by depletion of BRD7 in normal MCF10A cells, and 423 genes that were upregulated and 149 genes that were downregulated in transformed MCF10A Ras^{V12} cells (Figure 21B). The number of BRD7 regulated transcripts was comparable and overlapping between normal and transformed conditions, even if the quantity of transcripts influenced by BRD7 knockdown was slightly

higher in the experiment performed in the absence of oncogenic stress. Moreover, it is noteworthy that an important fraction of genes upregulated by BRD7 silencing in normal conditions are also regulated in the same direction by $HRas^{V12}$ overexpression, thus suggesting that BRD7 loss can partially mimic the effects of oncogene overexpression in inducing processes that promote transformation.

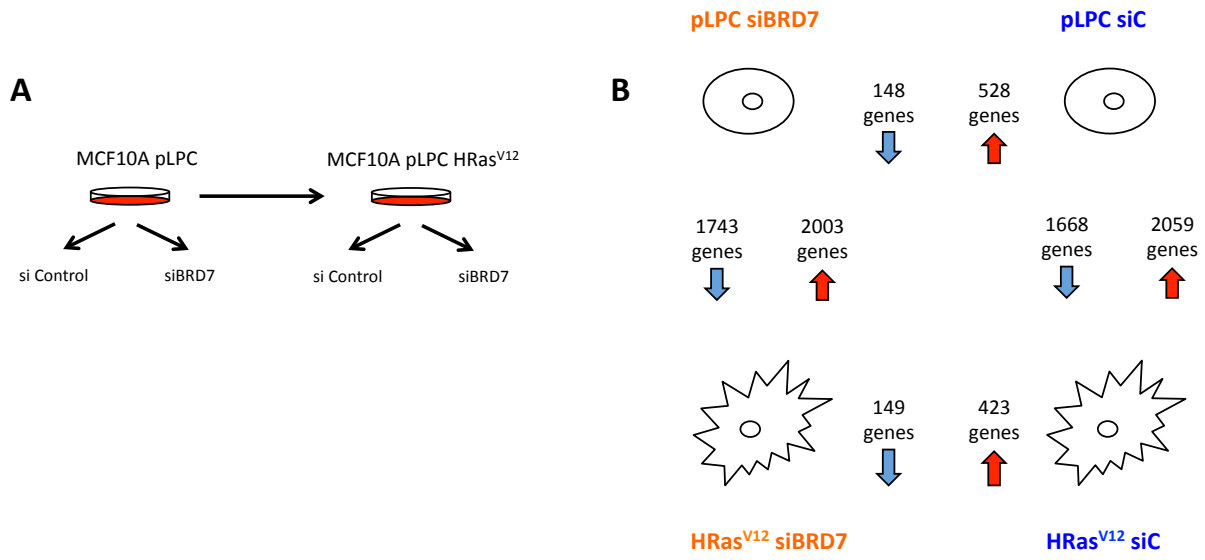


Figure 21. BRD7 silencing induces global alterations of gene expression. Normal MCF10A cells and $HRas^{V12}$ transformed cells were silenced for BRD7 expression as shown in panel A. The analysis of $polyA^+$ transcripts pointed out that BRD7 loss leads to differential expression of the indicated numbers of genes (panel B).

BRD7 regulates a transcriptional program in MCF10A cells that is linked to cancer progression

In order to understand which cellular processes BRD7 may putatively regulate in the conditions examined, we took advantage of a bioinformatic tool for gene annotation, Ingenuity Pathway Analysis (IPA – Ingenuity® Systems, www.ingenuity.com). This program is based on the Ingenuity Knowledge Base, a database that contains biological and chemical interactions and functional annotations created from millions of individually modelled relationships between proteins, genes, complexes, cells, tissues, drugs, and diseases. Each relationship originates from reported experimental facts from primary literature sources, including peer-reviewed journal articles, review articles and textbooks.
We loaded the whole results of our differential gene expression analysis (upregulated and downregulated genes with a 1.5 fold change) into IPA. The program is able to integrate the whole data from the transcriptome and create a list of processes modulated in the given experimental conditions along with their p-value and activation z score. Notably, as can be seen in Figure 22A, both in the normal and transformed context BRD7 loss results in the induction of many cellular processes, while the pathways that appear to be repressed are only few. Moreover, a detailed clustering of the single modulated transcripts in specific processes unveiled that BRD7 loss activates programs involved in tumorigenesis, abnormal cellular growth and proliferation, development and inflammatory response (Figure 22B). Of note, the only category significantly repressed by BRD7 relates to cell death. Interestingly, when comparing the list of processes regulated by BRD7 in MCF10A and MCF10A $HRas^{V12}$ cells, we can notice that they completely overlap, with the exception of metabolic pathways that appear to be regulated by BRD7 only in normal conditions.

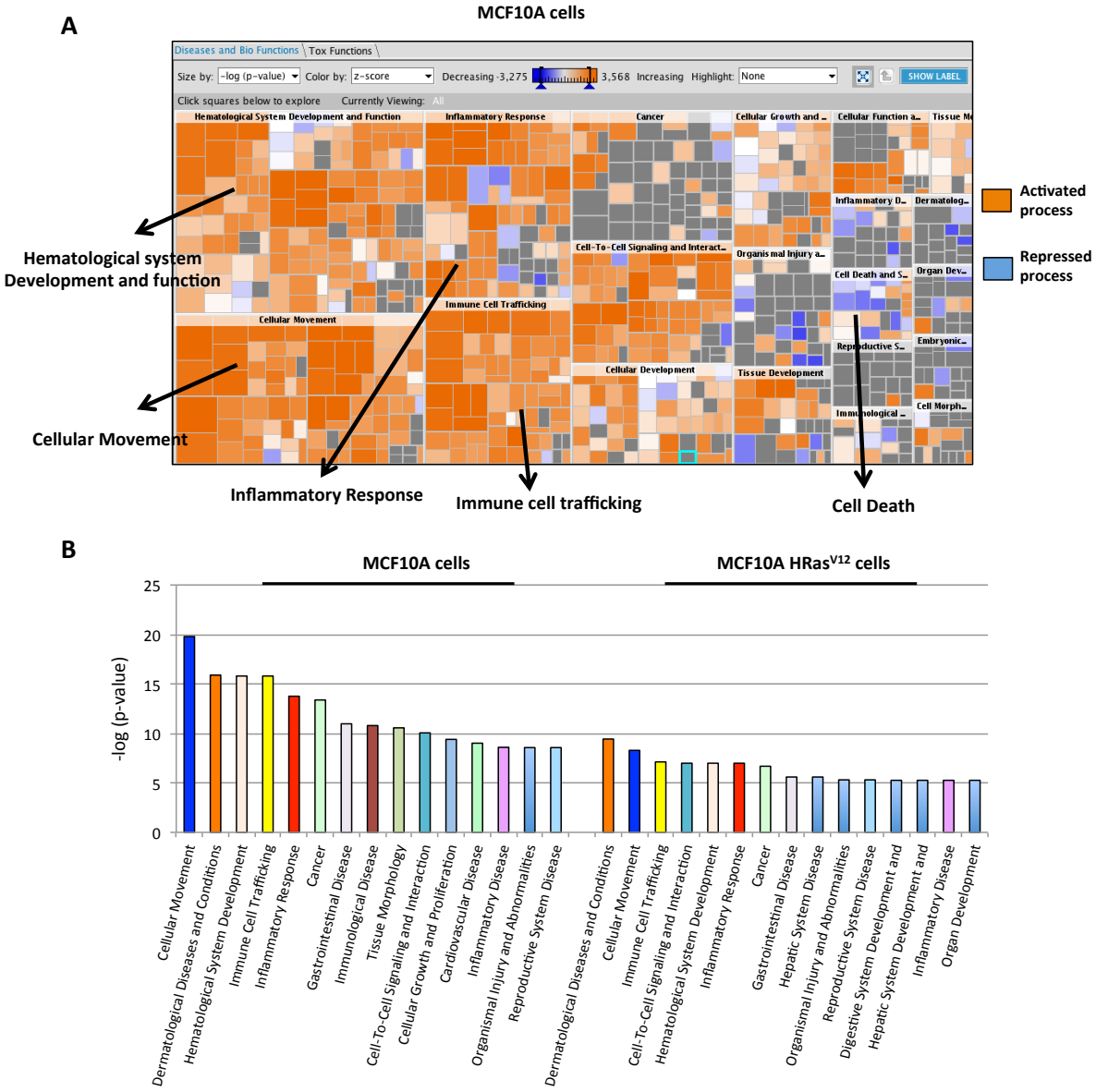


Figure 22. Global gene expression analysis unveils several cancer-related processes regulated by BRD7 in normal and transformed cells. Analysis of functional annotation of the genes differentially expressed upon BRD7 KD in normal and transformed contexts was performed by means of Ingenuity Pathway Analysis to obtain an indication of regulated processes.

A) Heatmap representing significative process activation/repression after BRD7 depletion. Many changes, especially in the upregulated functions, are shared between normal and transformed cells. Bigger squares correspond to smaller p-Value. Color coding bar based on the activation z score: orange, process activation; blue, function repression.

B) Gene ontology analysis of differentially expressed genes from siBRD7 vs siControl cells in normal and transformed conditions. Top categories include "cellular movement" and "inflammatory response". Bars represent the –Log of the over-representation p-value, as calculated by the IPA algorithm. For the sake of clarity only the first 15 results for each condition have been shown.

It is important to underline that this analysis revealed new processes putatively regulated by BRD7 that are likely to be a hallmark of its loss. In this context, inflammation appears

as an important gene ontology category that emerges as regulated by BRD7 also with other tools for gene annotation (e.g. David Functional Annotation Tool (Dennis et al., 2003)). A considerable part of the genes included in this group encode for secreted proteins, in particular cytokines and chemokines. Given that an emerging hallmark of cancer is the ability to evade from immune surveillance and induce pro-tumour inflammation (Hanahan and Weinberg, 2011) and considering that the inflammatory response can stimulate several pro-tumoral phenotypes that we demonstrated to be induced also by BRD7 loss, including proliferation, EMT, invasion and metastasis (Grivennikov et al., 2010; Porta et al., 2009) , we decided to focus on this process for further validation.

BRD7 regulates a subset of inflammatory genes that are overexpressed in cancer

In order to understand the mediators of the processes that we were investigating, we constructed a "BRD7 inflammatory signature" based on IPA output manually curated to include new hits possibly important for the process (Figure 23A). This signature comprises, among the others, several cytokines and chemokines such as CXCL1, CXCL2, IL6, IL1β and IL8. We analysed some of these inflammatory signature genes for the expression in breast cancer datasets taking advantage of ONCOMINE, a publicly available cancer microarray database and integrated data-mining platform that collects global genome DNA copy number alteration and microarray expression data from different human cancer patients' datasets (Rhodes et al., 2004). To date, ONCOMINE database includes 715 datasets comprising 86733 samples: through this tool it is possible to verify if a gene or group of genes results significantly differentially expressed within these datasets giving an hint for a functional significance *in vivo*. Data are compared between cancer and normal tissue or between different cancer subtypes or by clinical-based and pathology-based classifications. Interestingly, the inflammatory signature genes resulted significantly upregulated in several datasets (i.e. comparing gene expression between cancer and normal samples) belonging to different tumour types including brain and central nervous system, oesophageal, head and neck, liver, ovarian and pancreatic cancer. Focusing on breast cancer, that is the malignancy in which we constructed the signature, we found that it was strongly upregulated in basal-like breast carcinomas (Figure 23B) and triple negative breast cancers (Figure 23C), which represent breast cancer subtypes characterized by

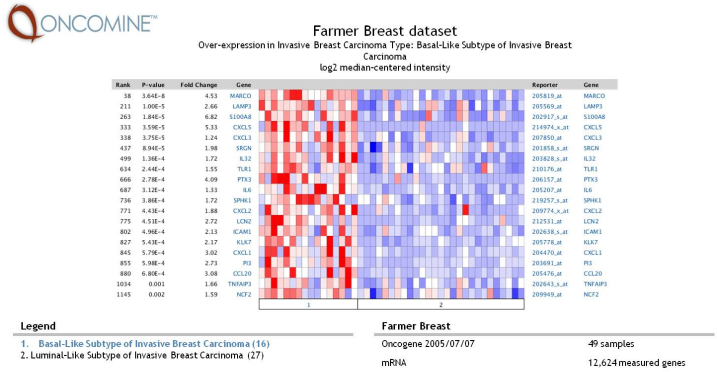
highly aggressive phenotype and poor prognosis due mainly to relapse from chemotherapy. Additionally, this signature was found upregulated in Grade 3 versus Grade 2 breast tumours and in samples derived from patients showing a metastatic event in 3 years after diagnosis. This holds true for inflammation signatures derived from both MCF10A and MCF10A HRas^{V12} cells.

Hence, this analysis suggests that BRD7 depletion induces a transcriptional program related to inflammation that is found enriched in human tumours, and particularly in highly aggressive cancer subtypes.

A

BRD7 inflammatory signature		
C3AR1	IL1RL1	S100A9
CASP10	IL32	SAA2
CCL20	IL6	SAA4
CD274	IL8	SAMSN1
CLCF1	KLK7	SCGB1A1
COX6B2	LAMP3	SERPINB1
CSF2	LCN2	SERPINB2
CSF3	LGALS9	SLAMF7
CXCL1	MARCO	SOCS1
CXCL2	NAV3	SOCS3
CXCL3	NCF2	SPHK1
CXCL5	NFKBIZ	SRGN
DAPP1	OASL	TLR1
DOK4	PI3	TNFAIP3
EBI3	PLSCR4	TNFRSF11A
ESM1	PRDM1	TNFSF14
GGT5	PTGS2 (COX2)	VTCN1
HSH2D	PTPRH	ZC3H12A
ICAM1	PTX3	ZEB1
IFIT2	RAB11FIP1	
IL1B	S100A7	
IL1R1	S100A8	

B



C

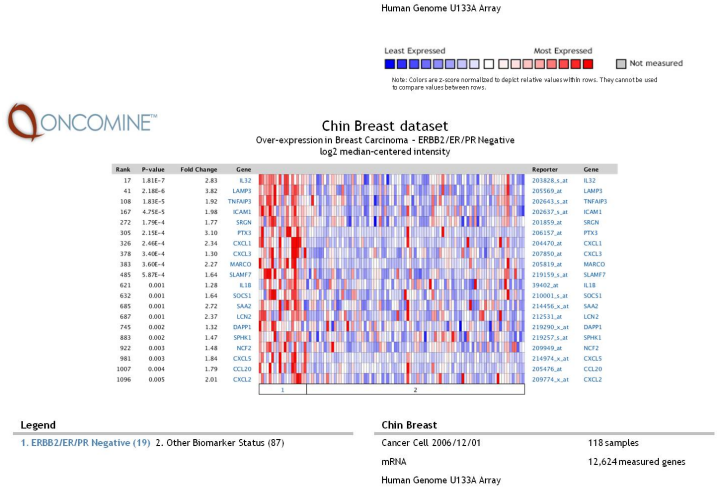


Figure 23. The "BRD7 inflammatory signature" is found upregulated in most aggressive breast cancer subtypes.

A) List of genes upregulated by BRD7 that belong to the inflammation category.

B) and C) Analysis of differential expression of the BRD7 inflammatory signature in human cancer datasets within the ONCOMINE database highlighted a significant enrichment of those genes in two datasets comparing basal versus luminal breast tumours (B) and triple-negative breast cancer versus other receptor status (C).

BRD7 regulates the expression of important cancer-related inflammatory genes in MCF10A cells

Prompted by the strong association found in ONCOMINE between the BRD7 inflammatory signature and high-grade breast cancer, we decided to better investigate the extent of the regulation exerted by BRD7 on inflammatory genes. As some of these genes are strongly induced by $HRas^{V12}$ overexpression *per se* and this robust induction could cover BRD7 effect, we chose to perform the experiments in non-transformed MCF10A cells. From the whole gene list (shown in Figure 23A) we first selected the genes that from literature emerged as relevant for the acquisition of aggressive cancer phenotypes. Among these there are genes that had been previously linked to cancer progression, in particular in the breast context; for example IL6 is linked to breast cancer stem cell survival (Marotta et al., 2011) while IL1β induces breast cancer cell migration and invasion (Ma et al., 2012). Moreover it has been reported that the IL8/CXCR1 (IL-8 receptor) axis plays an important role in regulation and survival of normal and malignant mammary epithelial stem/progenitor cell population (Ginestier et al., 2010), and in addition all the cluster of CXC chemokines (composed by IL8, CXCL1, CXCL2, CXCL3 and CXCL5) is found upregulated in breast cancer and this increased expression correlates with aggressiveness and bad prognosis (Bieche et al., 2007). Recent reports also associate overexpression of CCL20 with breast cancer, demonstrating that CCL20 induces migration and proliferation of breast epithelial cells (Marsigliante et al., 2013). Also S100A8 plays a pivotal role in this context; indeed it has been involved in a paracrine loop with CXCL1 and CXCL2 between cancer cells and myeloid cells and this communication has been shown to induce chemoresistance and metastasis of breast cancer cells (Acharyya et al., 2012). Moreover, the expression of other two BRD7 repressed genes, MMP9 and COX2, leads to the destruction of the normal acinar structure in three-dimensional culture of MCF10A cells (Chimal-Ramirez et al., 2013; Prasad et al., 2013).

We transfected MCF10A cells with two different siRNA oligonucleotides targeting the BRD7 coding sequence and performed qRT-PCR analysis of the selected transcripts to validate BRD7 effect. As shown in Figure 24 efficient BRD7 knockdown with either siRNA was able to induce a significant increase in the expression levels of all the genes analysed, confirming RNA sequencing data.

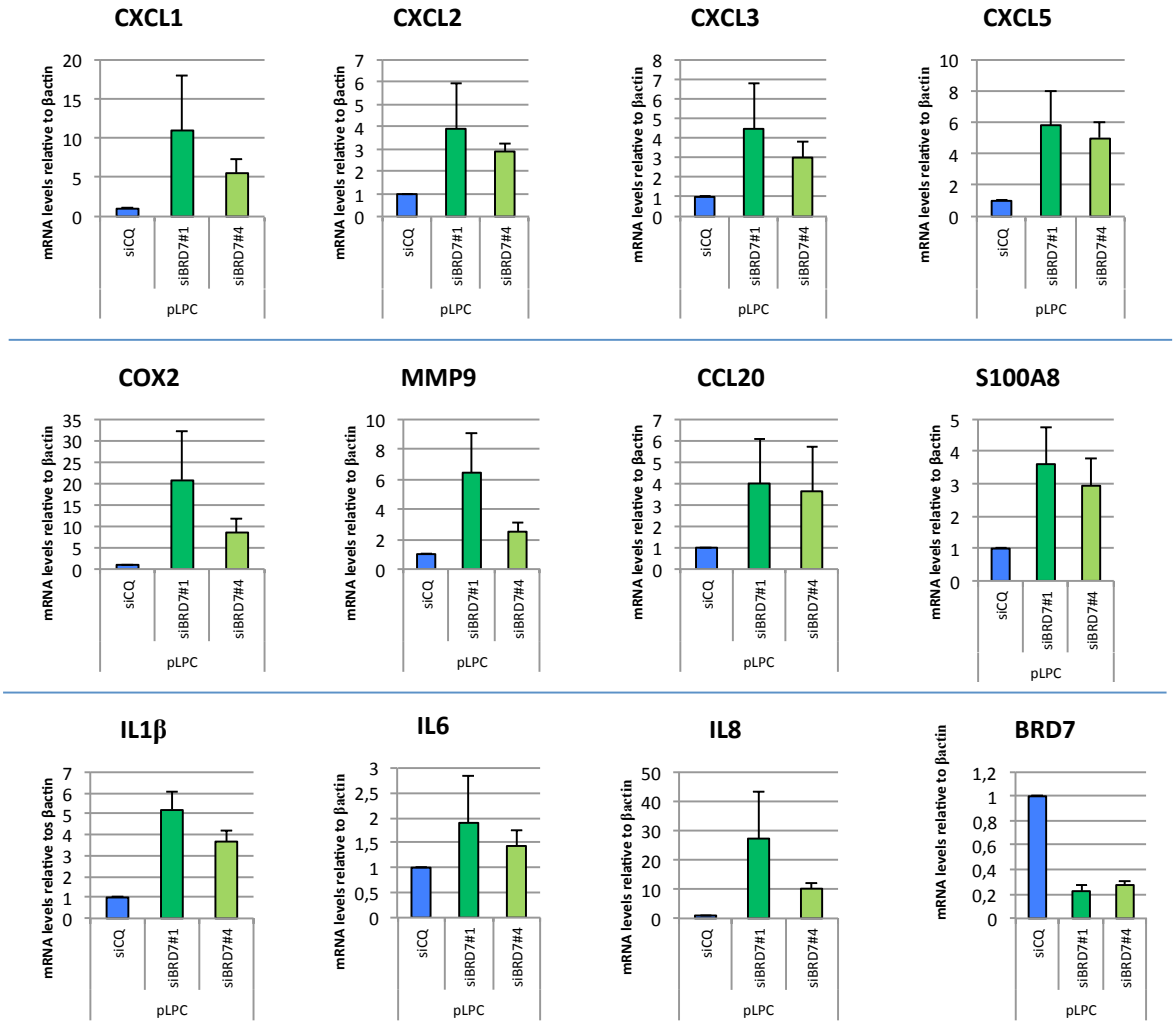


Figure 24. BRD7 silencing induces the expression of cancer-related inflammatory genes.
Validation of the effect of BRD7 depletion on induction of inflammatory-related genes in MCF10A and MCF10A HRas^{V12} cells.

SWI/SNF PBAF and PRC2 activity represses a subset of BRD7 target genes

Since BRD7 is a component of the SWI/SNF PBAF chromatin remodeling complex (Kaeser et al., 2008) and it is also known to interact with the PRC2 subunit EZH2 affecting its methylation activity on target genes (Tae et al., 2011), we sought to investigate whether the effect of BRD7 on the gene signature analysed above may involve these epigenetic regulatory complexes. First of all we compared the list of BRD7 regulated genes with

genes that were previously shown to be bound by the SWI/SNF complex in ChIP-sequencing experiments (Euskirchen et al., 2011). This chromatin-remodeling complex localizes on a very large number of promoters and interestingly almost half of BRD7 regulated genes were found within this list; among the genes whose promoter is bound by the SWI/SNF complex we found also some of the genes validated above including CCL20, CXCL2, CXCL3, COX2, IL6 and IL8 (Figure 25A).

Therefore we proceeded with evaluating the impact of depleting some components of SWI/SNF PBAF and PRC2 complexes on the expression of a subset of BRD7 regulated genes. To this aim we separately knocked-down the expression of several of these proteins in MCF10A cells by means of siRNA oligonucleotide transfection, including the ATPase subunit of the SWI/SNF complex BRG1, the PBAF-specific subunits ARID2 and BAF180, and the PRC2 Polycomb catalytic subunit EZH2 (Figure 25B).

As shown in Figure 25C, there are genes showing dependence for different regulatory proteins among those tested: CXCL1 and COX2 expression appears to be regulated by both SWI/SNF and PRC2 complexes and in particular it is induced upon silencing of all the subunits analysed, instead IL8 expression appears to be influenced more by the SWI/SNF complex than by the PRC2 complex. Finally, IL1β levels do not change significantly upon silencing of either SWI/SNF complex subunits or EZH2.

Interestingly, IL8 and COX2 promoters were shown to be bound by SWI/SNF in HeLa cells (Euskirchen et al., 2011): the result of our experiment suggests that this binding could be associated to a repressive effect of the SWI/SNF complex on the transcription of these inflammation-related genes. The other two genes analysed, IL1β and CXCL1 instead did not emerge as SWI/SNF targets in the published ChIP-sequencing experiment: interestingly, here we have observed that IL1β expression is not significantly influenced by SWI/SNF. Instead, CXCL1, even if not previously shown to be a target of SWI/SNF, appears to be repressed by this chromatin-remodeling machinery in our experimental system.

Thus from the data presented above we can conclude that SWI/SNF and PRC2 complexes could act with BRD7 to repress transcription of a subset of its inflammatory signature genes.

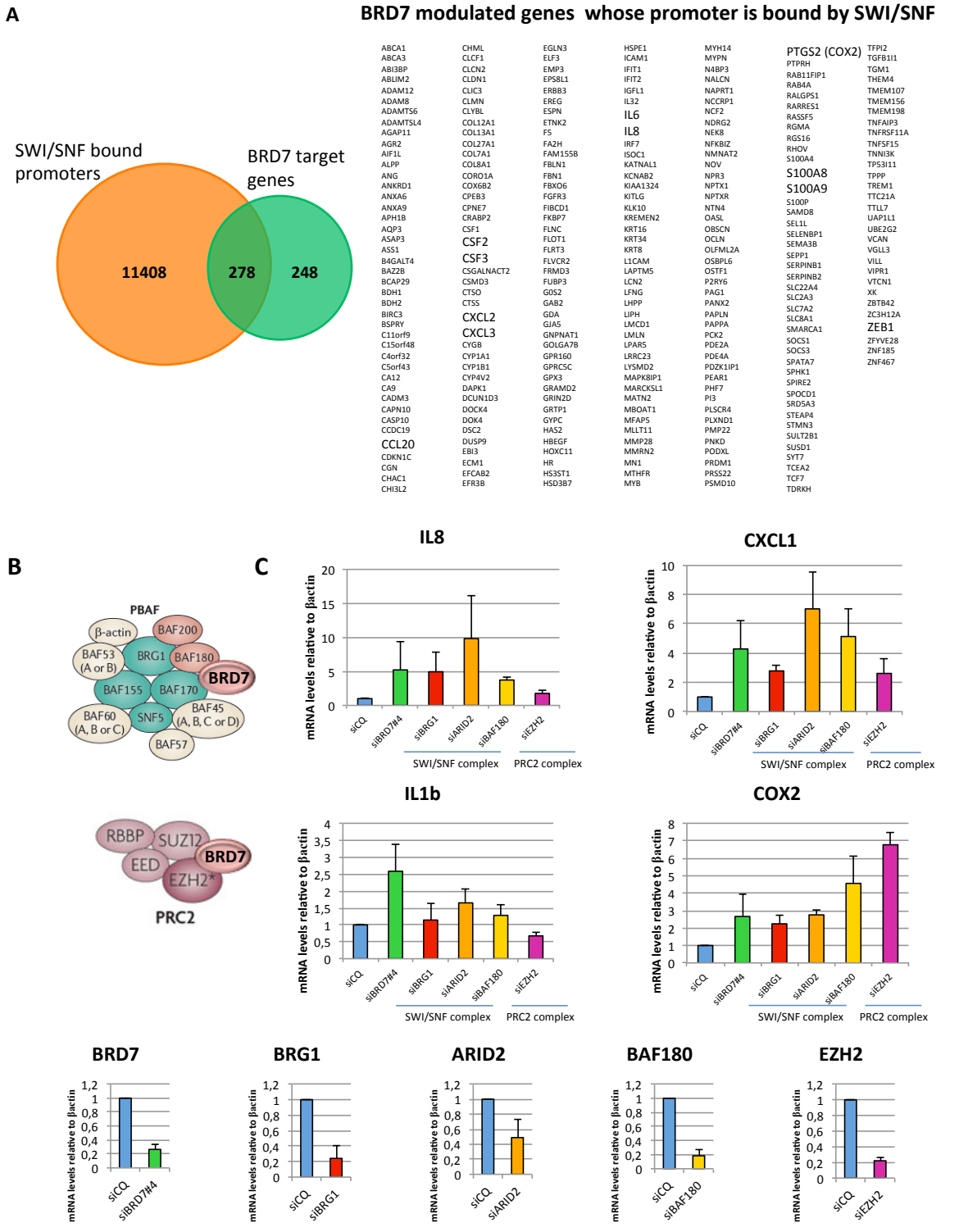


Figure 25. SWI/SNF PBAF complex and PRC2 cooperate with BRD7 in regulating several genes involved in the inflammatory process.

A) Intersection between published SWI/SNF ChIP-sequencing data and genes regulated by BRD7 in MCF10A cells. B) Schemes showing the composition of the SWI/SNF PBAF and PRC2 complexes.

C) qRT-PCR analysis of a subset of BRD7 regulated genes upon RNAi-mediated depletion of SWI/SNF subunits BRG1, ARID2 and BAF180 or PRC2 catalytic subunit EZH2. The analysis was performed in MCF10A cells; the mean and s.d. of three independent experiments are shown.

Transfac® and ExPlain™ analysis unveils possible interaction of BRD7 with important transcription factors

As BRD7 has been shown to act as a transcriptional cofactor for several master regulators of major tumorigenic processes, we sought to investigate which transcription factor(s) could be responsible for induction of the identified signatures and the observed phenotypes upon BRD7 depletion. Importantly, this analysis holds the potential to unveil novel unreported connections between oncogenic pathways. For this task we took advantage of ExPlain™, which is a unique data analysis system that combines promoter and pathway analysis tools. ExPlain™ employs TRANSFAC® derived transcription factor binding site positional weight matrices to identify transcription factors affecting gene expression in microarray and RNA sequencing experiments. First of all we annotated the promoters of the genes regulated by BRD7 in order to find common motifs (Figure 26A); after the annotation, we used ExPlain™ to identify transcription factors whose binding sites were enriched in the promoters of the genes affected by BRD7 knockdown both in normal and transformed conditions (Figure 26B). It is important to underline that for the analysis, we selected promoter regions (from 1000 bp upstream to the transcription start site) of genes belonging to the set upregulated and downregulated by BRD7 depletion. As a background dataset we used the promoters of genes whose expression was not altered by BRD7 depletion in the RNA sequencing data. As the diagram in figure 26B shows, Explain™ analysis identified a high number of transcription factors whose binding sites are predicted as enriched in the "BRD7 knockdown set" in respect to background set. Notably, most of these transcription factors are common between normal and transformed conditions. Among enriched transcription factors there are proteins involved in processes related to cancer progression such as inflammation (e.g. NF-κB), cell movement (e.g. ZEB1), metabolism (e.g. SREBP), differentiation and development (e.g. SMAD3) and stemness (e.g. OCT1).

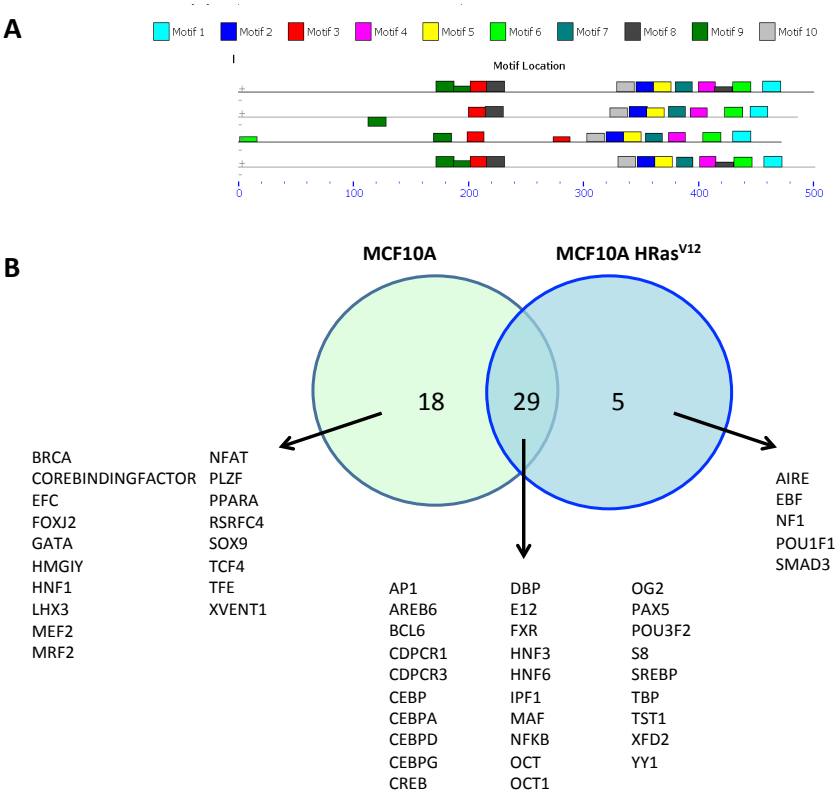


Figure 26. Unveiling transcription factors putatively involved in the activation of BRD7-regulated genes through analysis of their promoter sequences with Explain™ tool.
A) Graphical example of the alignment of promoter sequences and search for common transcription factors binding motifs.
B) Venn diagram showing transcription factors enriched in the promoters of genes regulated by BRD7 silencing in both normal and transformed conditions.

Interestingly, in our analysis we found proteins already reported as partners of BRD7 in transcription regulation, such as BRCA (Harte et al., 2010), while we found also a number of novel putative BRD7 interactors, master regulators of the processes of inflammation, cell cycle progression, cell movement, metabolism, differentiation/development and stemness. Importantly, our analysis also allowed us to find new putative activators of classes of genes (e.g. inflammatory genes), thus providing new regulators to processes connected to cancer development.

BRD7 loss unleashes the expression of inflammatory genes *via* NF-κB mediated transcription

The Explain™ analysis predicted an enrichment of NF-κB binding sites among BRD7 regulated genes in both normal and oncogene-transformed MCF10A cells. Moreover, also the analysis performed with IPA indicated NF-κB and TNF-α as strong upstream activators of the BRD7 signature. To confirm this result, we performed a comparison between the genes regulated by BRD7 in our RNA sequencing experiments and a list of all published NF-κB target genes: this analysis indeed highlighted a strong overlap between NF-κB targets and BRD7 regulated transcripts (Figure 27A).

Interestingly, it is also well established that the chromatin remodeling activity of the SWI/SNF complex is required for enhanced chromatin accessibility to NF-κB binding during activation of the "delayed subset" of its target genes (Natoli, 2009). It this therefore conceivable that BRD7 associates with SWI/SNF complex PBAF to regulate NF-κB transcriptional activity.

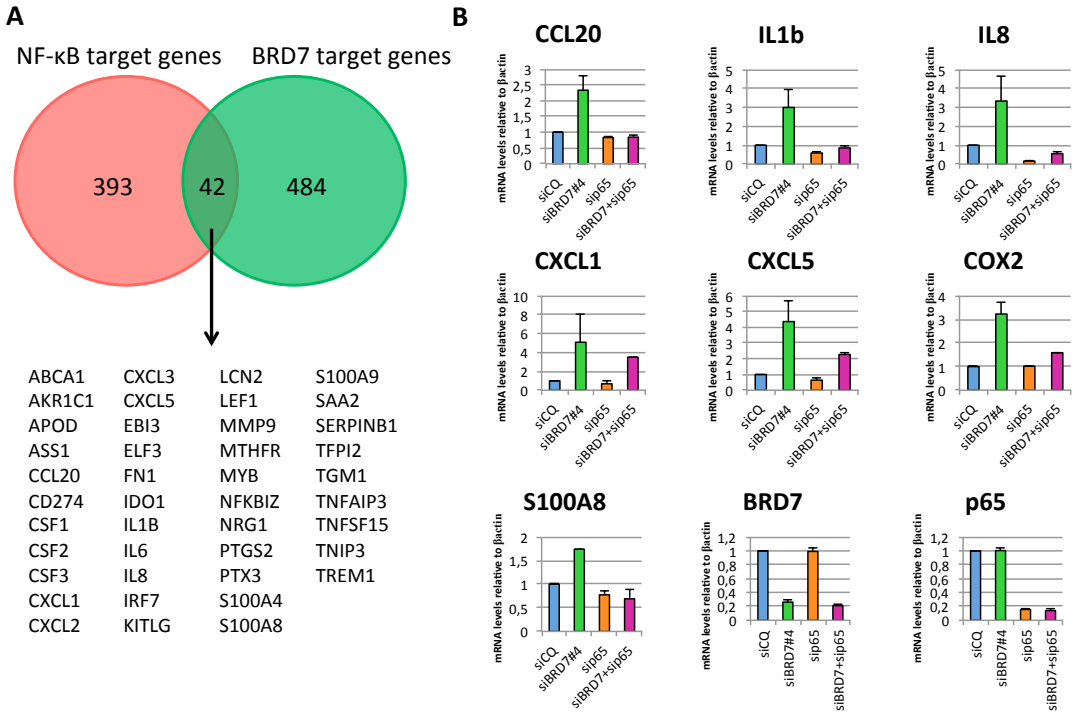


Figure 27. Induction of a subset of inflammatory genes regulated by BRD7 requires the transcription factor NF-κB.

A) Venn diagram showing the intersection between known NF-κB targets and BRD7 regulated genes.

B) qRT-PCR analysis of the mRNA expression levels of BRD7 regulated genes involved in the inflammatory response upon RNAi-mediated knockdown of BRD7, p65 or both.

In order to understand if BRD7 effect on transcription of inflammatory genes was dependent on NF-κB, we knocked down BRD7 and p65 expression either alone or in combination and performed qRT-PCR analysis of selected genes. The results, reported in Figure 27B, showed that the expression of some BRD7 signature genes (CCL20, IL1β, IL8 and S100A8) appeared to be dependent on the presence of NF-κB in our experimental system. Moreover, induction of these genes upon BRD7 depletion was lost in cells lacking p65. The same results were obtained for CXCL2 and CXCL3 genes (data not shown). This suggests that BRD7 may act by repressing NF-κB-dependent activation of these genes.
On the contrary, CXCL1, CXCL5 and COX2 are still induced upon BRD7 depletion in cells lacking p65, suggesting that in this case another transcription factor may be involved.
In summary, this set of experiments allowed us to demonstrate that a subset of BRD7 inflammatory signature genes is represented by NF-κB target genes and that p65 is functional for their induction upon BRD7 depletion in MCF10A cells.

DISCUSSION PART 1

In the first part of the work we have identified BRD7 as a potential tumour suppressor gene whose interaction with p53 is stimulated by Ras^{V12} expression, and that is required for activation of a subset of p53 target genes, in particular those involved in cell cycle arrest. p53 recruits several cofactors to activate its target promoters, including the p300/CBP acetyltransferases. We have shown that BRD7 interacts with p300 and is critical for its recruitment and for efficient histone acetylation at p53 arrest-specific target promoters. It is conceivable that BRD7, which binds acetylated histones through its bromodomain, helps to sustain a proper acetylation status of histones surrounding the p53 binding sites and could thereby contribute to efficient transcriptional regulation during oncogene-induced senescence. Theoretically, tumours that have inactivated the p53 pathway by loss of BRD7 should still be sensitive to apoptosis. It is therefore likely that these tumours have to complement BRD7 loss with genetic events affecting the apoptotic response, for example by overexpression of Bcl-2 or Bcl-xL. Intriguingly, on chromosome 16q12, next to BRD7, the cylindromatosis (CYLD) gene is encoded. CYLD is a well-known pro-apoptotic factor and tumour suppressive activity has been ascribed to it.

Therefore, deleting 16q12 would endow tumours with the means to interfere with both OIS and induction of apoptosis by loss of BRD7 and CYLD, respectively. The frequent loss of 16q12 observed in breast cancer and its correlation with retention of wild-type p53 support such notion. In addition to p53, BRD7 could also serve as a transcriptional cofactor for other proteins. It was shown that BRD7 transcriptionally regulates components of the RAS/MEK/ERK and Rb/E2F pathways (Peng et al., 2007; Zhou et al., 2004). Therefore, besides its important role in the p53 pathway, BRD7 may also modulate transcription of other genes involved in cell cycle regulation. Furthermore, many differentially expressed genes in $BRD7^{KD}$ cells are not p53 target genes.

Driven by the evidence of BRD7 loss in a subset of breast cancers and by our preliminary indications that this loss is associated with high-grade tumours and thereby with aggressive phenotypes, in the second part of this work we analysed the impact of BRD7 loss on a model of mammary cell transformation *in vitro* generated taking advantage of the immortalized MCF10A cell line and its transformed counterpart expressing $HRas^{V12}$. Thanks to this tool, we were able to assess that BRD7 loss is able to foster the process of epithelial-to-mesenchymal transition and also impacts on cancer stem cell number. By performing RNA sequencing analysis of $polyA^+$ transcripts differentially expressed upon BRD7 silencing, we determined that BRD7 appears to normally repress the expression of a high number of genes, connected to different processes involved in cancer progression. One of most prevalent processes was the inflammatory response. Interestingly, the "inflammatory signature" regulated by BRD7 was found to be enriched in highly aggressive breast cancer subtypes, namely triple negative breast cancer, and to be associated with negative clinical outcomes. We validated the effect of BRD7 depletion on the regulation of the most relevant genes by means of qRT-PCR; to ascertain that BRD7 has a repressive effect on the transcription of these genes we will rescue BRD7 expression in silenced cells. In addition, since BRD7 has been identified as a component of a Brg1-specific SWI/SNF PBAF (polybromo BRG1-associated factor) and PRC2 chromatin-remodelling complexes (Kaeser et al., 2008; Tae et al., 2011), we demonstrated that some of these BRD7 target genes are upregulated also by knockdown of SWI/SNF and PRC2 subunits, suggesting that BRD7 may act on these genes through epigenetic regulating complexes. It will be interesting to verify if the induction of the genes that are targets of SWI/SNF or PRC2 complexes is accompanied by modulation of epigenetic marks on their

promoters; therefore we will evaluate the changes of histone modifications after BRD7 depletion through chromatin-immunoprecipitation of specific histone marks.

To explore which transcription factors may be involved in the regulation of BRD7 target genes we obtained a bioinformatics prediction of transcription factor enrichment on selected promoters. By ExPlain™ tool we found both known and novel transcription factors that may be responsible for regulating the gene signatures that we identified as controlled by BRD7; we are currently validating these findings by ChIP and qRT-PCR experiments. As a proof of principle of the reliability of the predictions made by ExPlain™, we compared the results with a list of experimentally validated targets of the master transcription factor of inflammatory genes NF-κB. Indeed we were able to validate the involvement of NF-κB in inducing the "inflammatory signature". Further experiments will be needed to evaluate if the effect of BRD7 on NF-κB activity occurs at the target genes' promoters or rather relies on the regulation of NF-κB stability or subcellular localization.

We have also performed a proteomic analysis of BRD7 interactors in MCF10A $HRas^{V12}$ cells that highlighted a clear binding of BRD7 to the protein PML. This discovery is particularly interesting because it has recently emerged that PML plays a dual role in breast cancer both as tumor suppressor and as an oncogene impacting on metabolism of breast cancer cells (Carracedo et al., 2012). Our discovery opens the intriguing possibility that BRD7 could also play an oncogenic role in the subset of tumours that display augmented levels of the protein.

We are also inspecting the data about $polyA^+$ non-coding transcripts regulated by BRD7. Interestingly some of these transcripts are involved in the maintenance of chromatin structure and this finding suggests that BRD7 loss may contribute to breast carcinogenesis not only via transcriptional regulation of selected gene signatures but also by globally impacting on chromatin structure, as it was previously reported for the protein BRCA (Zhu et al., 2011).

Interestingly, a very recent report showed that BRD7 is required to repress Phosphoinositide 3-kinase (PI3K) signalling (Chiu et al., 2014) after serum or insulin stimulation. This finding is in line with the tumour suppressive roles that we propose for BRD7, and is also particularly intriguing because we demonstrated that BRD7 is lost in a percentage of high-grade breast cancers. It is conceivable that ablation of BRD7 could concomitantly impair p53 tumour suppressive response and activate PI3K oncogenic

activity, thus fostering the acquisition of highly transformed traits. We will verify the status of PI3K pathway activation in our cellular system and its possible contribution to the observed phenotypes.

One main open question is whether BRD7 exerts the activities highlighted via its participation in the chromatin remodelling complexes or by an independent function. SWI/SNF complexes constitute a highly related family of multisubunit complexes that remodel nucleosome structure and are capable of mobilizing nucleosomes both by sliding and by catalysing the ejection and insertion of histone octamers (Saha et al., 2006). In mammals, multiple complexes falling in the SWI/SNF class exist, including the BRG1-associated factor (BAF) complexes and the polybromo BRG1-associated factor (PBAF) complexes. BRD7 was found to belong to the PBAF complex, that bears BRG1 as catalytic subunit (Harte et al., 2010). Interestingly, recurrent mutations in subunits of the complex have been defined in various cancers, providing a novel link between chromatin remodelling and tumour suppression. Interestingly, many of these alterations have been reported to affect the same processes controlled by BRD7, suggesting a possible common regulation of the same cellular functions. For example BRG1, whose expression is absent in 10-50% of human primary non-small-cell lung cancer (NSCLC) samples and whose heterozygosity in mice results in mammary tumours in 10% of the cases, has been reported to be involved in the processes regulated by BRD7. First of all BRG1 knockdown results in decreased binding of p53 to the p21 promoter (Xu et al., 2007); moreover, BRG1 has been reported to bind BRCA1 (Bochar et al., 2000) and to modulate its response to UV radiation (Zhang et al., 2013). Interestingly, it was demonstrated that the interaction between BRG1 and BRCA1 was regulated during Ras-induced senescence (Tu et al., 2013). In this context the interaction is disrupted during senescence, and this correlates with the dissociation of BRCA1 from chromatin and an enhanced association of BRG1 with chromatin. Moreover, knockdown of BRG1 inhibited the formation of SAHF induced by oncogenic Ras^{V12}. However, BRG1 did not dissociate BRCA1 from chromatin but acts downstream of BRCA1 to promote SAHF formation during senescence.

Also another specific subunit of the SWI/SNF PBAF complex, BAF180, has been found involved in the process of senescence along with BRD7 (Burrows et al., 2010). Interestingly, mutations in PBRM1 (which encodes for BAF180) were identified in 41% of renal cell carcinomas (Varela et al., 2011) and also in a small minority of breast cancers

(Xia et al., 2008).

Importantly, the SWI/SNF complex PFAF could be involved also in the regulation exerted by BRD7 on the processes of EMT and inflammation. Indeed although *S. cerevisiae* SWI/SNF complexes were identified on the basis of their roles in the activation of transcription, evidence indicates that mammalian SWI/SNF complexes contribute to both repression and activation. In embryonic stem (ES) cells, BRG1 most commonly acts as a repressor to inhibit programmes that are associated with differentiation, but it also facilitates the expression of core pluripotency programmes (Ho et al., 2009). Contributing to the mechanism of repression, SWI/SNF complexes are capable of recruiting histone deacetylases (HDACs), which remove activating acetyl marks from histone tails. For example, SNF5, a PBAF core subunit, represses cyclin D1 (CCND1) in an HDAC1-dependent manner (Zhang et al., 2002b). These seemingly opposing activities may actually be similarly achieved, by positioning nucleosomes away from binding sites to facilitate factor binding or by moving nucleosomes over sites to prevent binding.

Evidence from SWI/SNF-mutant cancers suggests that the aberrant activation of gene programmes that are involved in cellular motility may contribute to invasion and metastasis. SWI/SNF complexes have roles in the regulation of the actin cytoskeleton. The SNF5 subunit of SWI/SNF is inactivated via biallelic mutations, including deletion, nonsense, missense and frameshift mutations, in nearly all malignant rhabdoid tumours (RTs), which are aggressive cancers that occur in young children (Versteege et al., 1998). In particular, SNF5 regulates RHOA-mediated control of cytoskeletal structure, so knockdown of SNF5 increases RHOA activity and stimulates migration (Caramel et al., 2008). RHOA regulates cell migration by stimulating stress fibre formation and contractility, and its overexpression in cancer has been shown to correlate with poor prognosis (Karlsson et al., 2009). Similarly, alterations in cytoskeleton function have also been implicated in BRG1-deficient tumours. Specifically, reducing BRG1 levels in a BRG1-expressing pancreatic carcinoma cell line led to an increase in the number of actin stress fibres (Rosson et al., 2005).

Moreover, the SWI/SNF complex has been also involved in the inflammatory response. Indeed both NF-κB recruitment on target genes and their activation depend on SWI/SNF (Hargreaves et al., 2009; Kayama et al., 2008; Ramirez-Carrozzi et al., 2006). It was demonstrated, for example, that BAF180 is a repressor of IL10 transcription in T helper

lymphocytes (Th2) (Wurster et al., 2012), where it binds directly to regulatory elements in the IL10 locus but is replaced by BAF250 containing BAF complexes when absent, resulting in increased histone acetylation and CBP recruitment to the IL10 locus. This suggests that the differential recruitment of different SWI/SNF subtypes can have direct consequences on chromatin structure and gene transcription.

Recent findings have provided new hints on the mechanisms by which SWI/SNF complexes contribute to the fine control of gene expression by uncovering a functionally antagonistic relationship with Polycomb group (PcG) proteins. These proteins covalently modify histones and have roles in regulating gene expression during essential cell fate decisions. They have been divided into polycomb repressive complex 1 (PRC1) and PRC2. The catalytic subunit of the PRC2 complexes EZH2 mediates tri-methylation of histone H3 at lysine 27 (Cao and Zhang, 2004), a silencing mark, which in turn is a histone binding site for PRC1 complexes, cooperatively leading to the formation of a repressive chromatin environment. SWI/SNF was found to suppress defects that were conferred by mutations in PcG proteins (Kennison and Tamkun, 1988; Tamkun et al., 1992). The functions of PRC2 complex have been recently linked to cancer; indeed the expression of EZH2 is frequently elevated in a wide range of cancers, including breast cancers, prostate cancers and lymphomas, and this overexpression is often correlated with advanced stages of disease progression and a poor prognosis (Simon and Lange, 2008).

Interestingly, EZH2 expression is elevated in primary SNF5-deficient tumours, suggesting the hyperactivation of PcG function in these tumours (Kia et al., 2008). The antagonistic relationship is further supported by increased levels of trimethylation of lysine 27 of histone 3, the epigenetic mark that is added by EZH2, in the absence of SNF5. Interestingly, overexpression of EZH2 has also been implicated in the pathogenesis of ovarian and renal carcinomas, two tumour types that have recurrent SWI/SNF mutations (Lu et al., 2010; Wagener et al., 2008). Such findings suggest that there could be a complex interplay between SWI/SNF and PRC2 complexes in normal and cancer cells and that BRD7, being part of both complexes, could drive their interplay at promoters. Interestingly it was shown that EZH2 regulates NF-κB dependent transcription in breast cancer (Lee et al., 2011), in particular promoting the transcription of the NF-κB target genes *IL6* and *TNF* in basal-like breast cancer and repressing their transcription in ER-positive breast cancers. In this scenario, it may not be unlikely that the presence of

bromodomain-containing proteins such as BRD7 could be essential to drive the choice to either transcribe or not NF-κB target genes.

All these evidences point towards a transcriptional cooperation between BRD7 and both SWI/SNF and Polycomb complexes. This may open the possibility to extend the knowledge gained through the study of BRD7 to all the tumours and also other diseases bearing alterations in the chromatin remodelling machinery, thus providing new prognostic markers and targets for therapy.

6. RESULTS PART 2

Pin1 mediated isomerization stimulates p53-dependent apoptosis induced by mutant Huntingtin

In this part of the thesis we sought to investigate the role of common events that activate p53 under oncogenic stess, namely Ser46 phosphorylation and Pin1-mediated prolyl-isomerisation, in regulating activation of p53 apoptotic function in Huntington's Disease.

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder that is caused by an unstable expansion of a CAG repeat within the coding region of the IT-15 gene (The Huntington's Disease Collaborative Research Group (1993). The gene encodes for a protein called Huntingtin, and the mutation results in an elongated stretch of glutamines near the NH_2 terminus of the protein. Prevalence of the mutation is 4–10 cases per 100,000 in populations of Western European descent, with many more at risk of having inherited the mutant gene. Over time, the consequence of carrying the HD mutation is a massive brain neurodegeneration characterized by the prevalent loss of efferent medium spiny neurons in the striatum (caudate nucleus and putamen) of the basal ganglia, which is primarily responsible for the typical HD symptoms, characterized by motor abnormalities and cognitive decline (Vonsattel et al., 2011).

Toxic properties of mutant Huntingtin (mHtt) are believed to cause HD and as a demonstration the introduction of the mutant gene into non-human primate, mouse, fly, fish, and worm has generated disease models (Zuccato et al., 2010). Several mechanisms have been proposed by which mutant Huntingtin (mHtt) may trigger striatal neurodegeneration, including mitochondrial dysfunction, oxidative stress and apoptosis.

Interestingly, expression of full-length mHtt protein and N-terminal fragments containing the polyQ expansion, that are produced during the pathogenic process through cleavage of the protein by caspases and calpains, elicit a DNA damage response, with activation of the ATM/ATR pathways (Illuzzi et al., 2009) and their downstream effectors, including the tumour suppressor p53 (Bae et al., 2005; Illuzzi et al., 2011). Mutant Huntingtin was found to bind p53 and increase its levels and transcriptional activity, leading to the upregulation of two pro-apoptotic downstream targets, Bax and Puma, as well as to mitochondrial membrane depolarization. Indeed p53 mediates mitochondrial dysfunction and cytotoxicity in HD cells and in transgenic animal models, whereas its inhibition prevents these

phenotypes (Bae et al., 2005). Given the pivotal role of p53 pro-apoptotic pathway for the neuronal loss in HD, we sought to investigate the signalling network responsible for p53 activation in neuronal cells, a topic that is still largely unknown. From cancer biology it is known that regulation of p53 activities relies on a complex network of post-translational modifications and protein interactions (Vousden and Prives, 2009) and entails site-specific phosphorylation by several DNA damage-activated protein kinases, including, among others, ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), homeodomain-interacting protein kinase 2 (HIPK2), and PKCδ. The subsequent transduction of stress-dependent phosphorylation into specific conformational changes of p53 that fully unleash its apoptotic activity is performed by the prolyl-isomerase Pin1. Upon genotoxic insults, Pin1 binds multiple sites on p53, promoting its accumulation in stressed cells, the activation of its transcriptional functions, and the induction of its apoptotic activity (Zacchi et al., 2002; Zheng et al., 2002a). Based on the importance of specific p53 phosphorylation and Pin1-catalyzed isomerisation for p53 tumour suppressive functions, we investigated whether this pathway may be aberrantly activated also in neuronal cells upon genotoxic stress produced by mHtt activities.

Expression of mutant Huntingtin promotes the interaction of p53 with Pin1

Previous work performed in collaboration with the group of F. Persichetti at SISSA Trieste was aimed at analysing p53 content and post-translational modifications in post-mortem brains of HD patients. This analysis revealed high levels of p53 in HD brains relative to healthy controls, in agreement with previous reports (DiFiglia et al., 1997). To study the stress pathways responsible for p53 activation in HD neurons, p53 phosphorylation was then analysed. Interestingly, in HD brains p53 was phosphorylated on Ser46, a modification that has been associated with activation of its apoptotic function upon stress (Mantovani et al., 2007).

Nuclear accumulation of mHtt N-terminal fragments is observed in HD brains and animal models (DiFiglia et al., 1997). Expression of these truncated forms recapitulates many molecular and neurological HD phenotypes (Schilling et al., 1999). The N-terminal fragment (residues 1–171) of either wild-type or mutant Htt (bearing 21 and 150 polyQ, respectively – Figure 28A) were thus expressed in p53-null H1299 cells along with wild-

type p53 to verify whether p53 Ser46 phosphorylation was a consequence of mHtt expression. Interestingly, mutant but not wild-type Htt induced the phosphorylation of transfected p53 on Ser46 (Figure 28B). Because Ser46 phosphorylation generates a target site for the prolyl isomerase Pin1 (Mantovani et al., 2007; Zacchi et al., 2002), we asked whether Pin1 might play a role in mediating activation of p53 upon mHtt expression. Strikingly mHtt expression stimulated direct interaction of p53 with Pin1 as demonstrated by GST-Pin1 pull-down assays (Figure 28C), and this effect was proportional to the amount of mHtt. To confirm that the phosphorylation pathway was active also in neuronal cells, we took advantage of SH-SY5Y human neuroblastoma cells in which we transfected either the wild-type or mutant Htt. Also in this cellular system mutant but not wild-type Htt induced the phosphorylation of endogenous p53 on Ser46 (Figure 28D), in addition to the previously reported phosphorylation of Ser15(Illuzzi et al., 2011). Of note, we were able to reproduce this result also in other neuronal (SK-N-SH) and cancer (U2-OS) cell lines bearing endogenous wild-type p53 (data not shown).

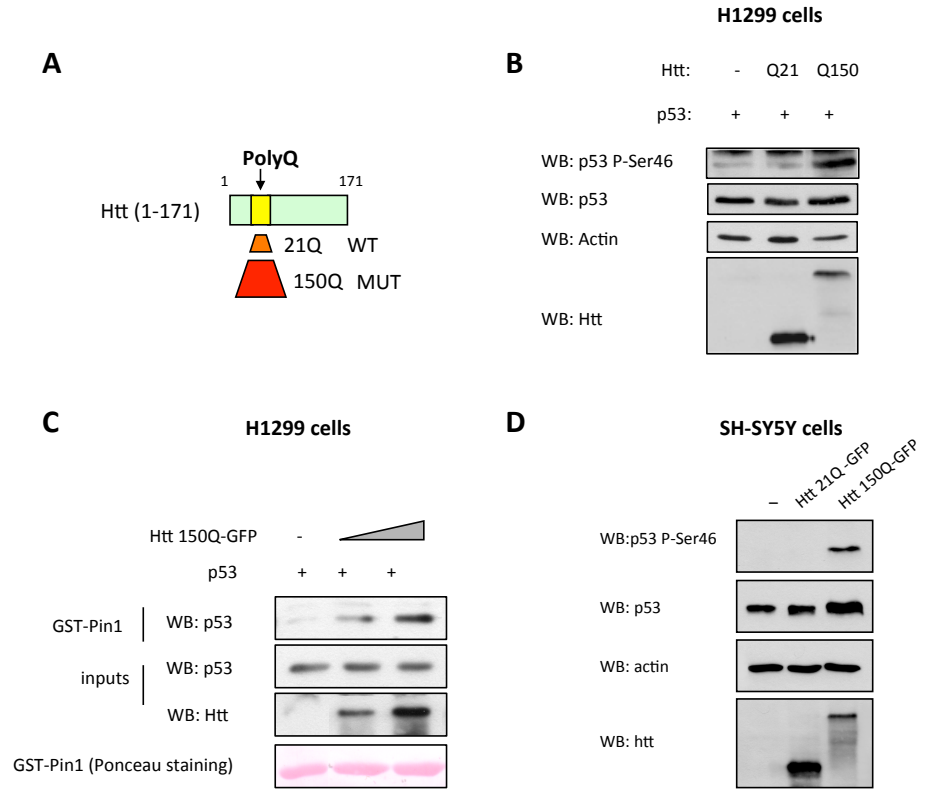


Figure 28. Expression of mutant Huntingtin induces p53 phosphorylation on Serine 46 and its interaction with Pin1.

A) Schematic representation of the wild-type and mutant forms of the amino-terminal fragments of Huntingtin used for this study.

B) H1299 cells were transfected with constructs expressing p53 and Htt(1–171) fragments bearing either 21Q

or 150Q. Total cell lysates were analyzed by Western blot with antibodies specific for Ser46-phosphorylated and for total p53.
C) H1299 cells were transfected with indicated constructs, and the interaction of p53 with recombinant Pin1 protein was analyzed by GST pull-down of cell lysates normalized for p53 levels.
D) SH-SY5Y cells were transfected with constructs expressing the N-terminal 1–171 Htt fragment with either 21Q or 150Q. p53 was immunoprecipitated from equal amounts of total cell lysates and analyzed by Western blot with antibodies specific for phosphorylated Ser46 and total p53. The levels of actin and Htt proteins in input lysates are shown.

Pin1 mediates the activation of the p53 pathway by mHtt

It had been previously shown that expression of mHtt in SH-SY5Y cells triggers a p53-dependent response involving the activation of apoptotic genes, including Bax and Puma (Bae et al., 2005). As shown in Figure 29A and in accordance with previous reports, expression of mHtt in SH-SY5Y cells provoked apoptosis, which was reduced by 50% upon silencing p53 expression. Importantly, the same effect was observed upon silencing of Pin1, and this points towards a functional role of Pin1 in promoting p53-dependent apoptosis. Importantly, mHtt induced apoptosis could be re-established in Pin1-depleted cells by overexpression of a siRNA-resistant Pin1 construct; this was not effective in cells depleted of p53, suggesting that the effect of Pin1 relies on p53. It is noteworthy that expression of a catalytically inactive Pin1 mutant was unable to rescue knockdown of endogenous Pin1, proving that the prolyl isomerase activity is essential for transducing mHtt-dependent stress into p53 activation.
We then analysed activation of the p53 response in the brains of HdhCAG knock-in mice in which the glutamine tract of mouse Htt is extended to 111 residues (HdhQ111)(White et al., 1997). These mice show striatal neurodegeneration, reactive gliosis, and gait abnormalities at older age (after 24 months)(Wheeler et al., 2002). However, we observed stabilization of p53 in brain extracts and the consequent transcriptional induction of the p53 target gene p21WAF1 in the striatum of 12-mo-oldHdhQ111 mice compared to their wild-type littermates, HdhQ7 (Figure 29B). This finding suggests that activation of the p53 pathway by mHtt-associated stress is an early event in HD pathogenesis and could precede neurological symptoms. HdhQ111 mice were then crossed with Pin1KO mice (Atchison and Means, 2003) to verify whether Pin1 is required for p53 activation. Importantly, in contrast to HdhQ111/Pin1WT mice, p53 transcriptional activity was not induced in HdhQ111/Pin1KO mice (Figure 29B). These results indicate that Pin1 plays a critical role

for p53 activation in response to mHtt expression in striatal neurons of a mouse model of HD pathogenesis.

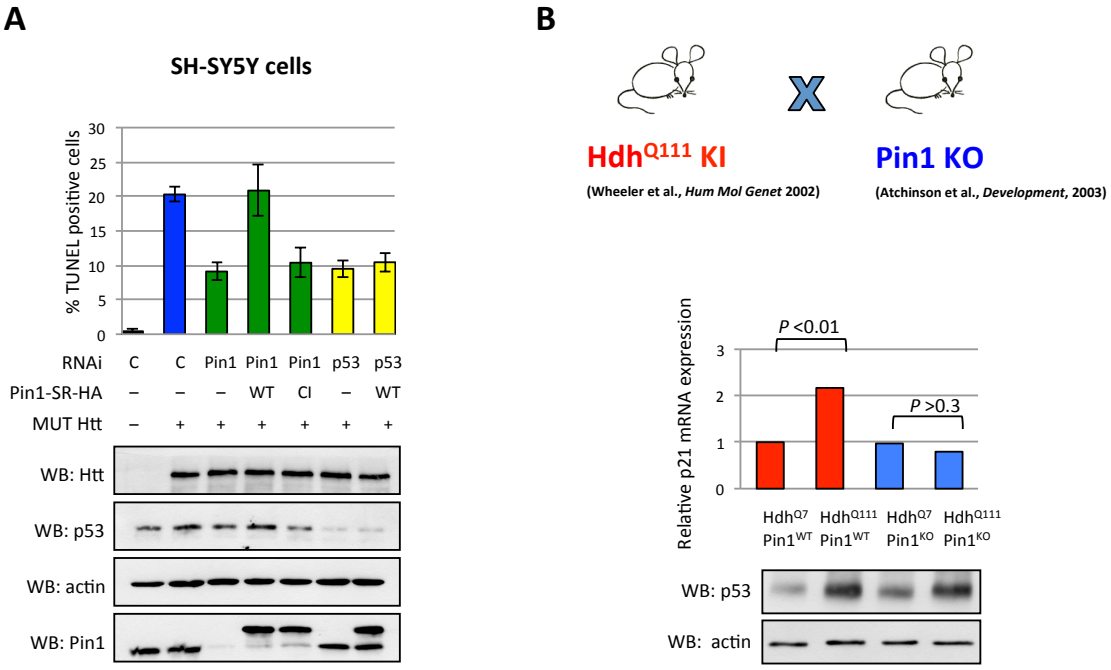


Figure 29. Pin1 activity is required for induction of p53-dependent apoptosis by mHtt.
A) SH-SY5Y cells were transfected with the indicated combinations of constructs expressing mHtt(1–171)150Q-GFP, Pin1 siRNA oligonucleotides, and siRNA-resistant wild-type Pin1-HA (WT) or the catalytically inactive mutant S67E (CI). Apoptosis of mHtt GFP-expressing cells was evaluated by TUNEL assay after 48 h. The histograms show mean and SD of three independent experiments.
B) Expression of p21WAF1 mRNA was analyzed by qRT-PCR from the striatum of 12-mo-old mice of the indicated genotypes (at least three mice for each genotype), normalizing for expression of β-actin. A t-test was performed using homoschedastic variance through groups and one tail parameter. p53 was immunoprecipitated from equal amounts of brain lysates of the same mice and analyzed by Western blot. Actin protein levels in input lysates are shown.

Phosphorylation of p53 on Ser46 by HIPK2 is an upstream event in the mHtt-Pin1-p53 pathway

Our observations indicated that phosphorylation of p53 on Ser46 is triggered by mHtt. To define whether this or other phosphorylations are responsible for activating p53 apoptotic response upon expression of mHtt, we used p53 phosphorylation mutants with single and multiple substitutions of Ser/Thr with Ala residues within Pin1 binding sites(Mantovani et al., 2007) (Figure 30A). Expression of these proteins in p53-null H1299 cells demonstrated that Ser46 is required for mHtt induced apoptosis, because a Ser46-Ala p53 mutant was

unable to cause apoptosis in response to mHtt expression (Figure 30B). In contrast, a p53 mutant (p53 3M-S46wt) that lacked the remaining three major Pin1 binding sites (i.e., Ser33, Thr81, and Ser315) could efficiently induce apoptosis downstream to mHtt expression. This protein was phosphorylated on Ser46 in cells expressing mHtt, and Pin1 potentiated its apoptotic activity (Figure 30B). Both p53 WT and p53 3M-S46wt were then able to induce the pro-apoptotic p53 target Puma upon transfection of mHtt, whereas p53 S46A was almost inactive (data not shown). Therefore, we concluded that phosphorylation of p53 on Ser46 is a crucial event in the pathway leading to neuronal death induced by mHtt in human and mouse cells. Our data also indicate that modification at this site is sufficient for Pin1 to enhance p53's apoptotic function in cells expressing mHtt. Among the protein kinases that catalyse phosphorylation of p53 on Ser46, HIPK2 plays a key role in unleashing p53's apoptotic activity upon DNA damage (Di Stefano et al., 2004). HIPK2 is induced by cytotoxic stimuli through the ATM/ATR pathway(Winter et al., 2008), which becomes activated upon mHtt-dependent stress (Illuzzi et al., 2009). Interestingly, expression of mHtt was sufficient to up-regulate HIPK2 protein levels in SH-SY5Y cells, and this effect required ATM kinase activity because it was prevented by treatment with the ATM-specific inhibitor KU55933 (Figure 30C). We thus inhibited HIPK2 expression by RNAi, which dampened mHtt-dependent phosphorylation of p53 on Ser46 with concomitant decrease of apoptosis (data non shown). This result indicates a major role for HIPK2 in the activation of p53 by mHtt. Interestingly, depletion of HIPK2 did not fully prevent Ser46 phosphorylation triggered by mHtt, implying the involvement of other kinases in inducing apoptosis downstream of mHtt.

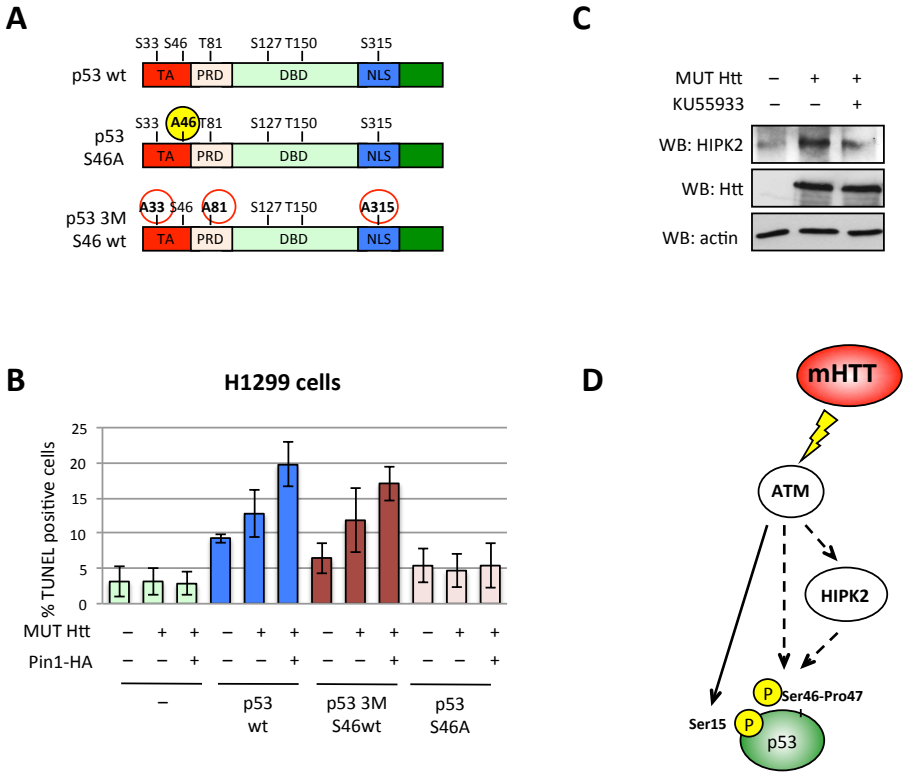


Figure 30. p53 Ser46 phosphorylation promoted by mHtt through ATM and HIPK2 is required for apoptosis induction by Pin1.

A) p53 scheme indicating Pin1 consensus sites (phospho–Ser/Thr-Pro). DBD, DNA binding domain; NLS, nuclear localization signal; TA, transactivation domain. p53 mutants have Ser/Thr-to-Ala substitutions in Pin1 consensus sites at residue 46 (p53 S46A) or at the three other major Pin1-binding sites at residues 33, 81, and 315 (p53 3MS46wt).

B) p53-null H1299 cells were transfected with the indicated constructs (see also A). Apoptosis of mHtt GFP-expressing cells was evaluated by TUNEL assay after 48 h. The histograms show mean and SD of three independent experiments.

C) SH-SY5Y cells were transfected with mHtt(1–171)150Q-GFP and treated with the ATM inhibitor KU-55933 10 μM for 24 h. HIPK2 protein levels were evaluated by Western blot.

D) Model of the phosphorylation events that may occur following mHtt expression.

Interfering with p53 activation by Pin1 prevents apoptosis downstream of mHtt

Given that p53 activation mediates the cytotoxic effects of mHtt, we hypothesized that pharmacologic inhibition of catalytic activity of the kinases that phosphorylate p53 on the Pin1 target site Ser46 could prove effective in preventing mHtt-induced apoptosis. Because specific inhibitors of HIPK2 are not available, we attempted to pharmacologically interfere with this pathway by inhibiting the upstream kinases. Treatment of SH-SY5Y cells with caffeine, a well-known inhibitor of ATM/ATR activities, strongly reduced mHtt-dependent

cellular toxicity and Ser46 phosphorylation (Figure 31A). We then focused on ATM, which, besides inducing HIPK2, directly phosphorylates p53 on Ser46, in addition to Ser15 (Kodama et al., 2010). Strikingly, the ATM-specific inhibitor KU55933 was effective in preventing mHtt-induced apoptosis by reducing phosphorylation of p53 on both Ser46 and Ser15 (Figure 31B). It is important to underline that we also demonstrated that specific inhibition of either the PKCδ kinase, that also phosphorylates p53 on Ser46 after mHtt stress, or of Pin1 catalytic activity was able to strongly reduce mHtt-induced apoptosis (data not shown).

Together, all the experimental evidences support a model where stress generated by mHtt triggers activation of ATM, HIPK2, and PKCδ kinases, which lead to phosphorylation of p53 on Ser46. This process preludes Pin1-dependent prolyl-isomerization and consequent induction of p53 pro-apoptotic genes (Figure 31C).

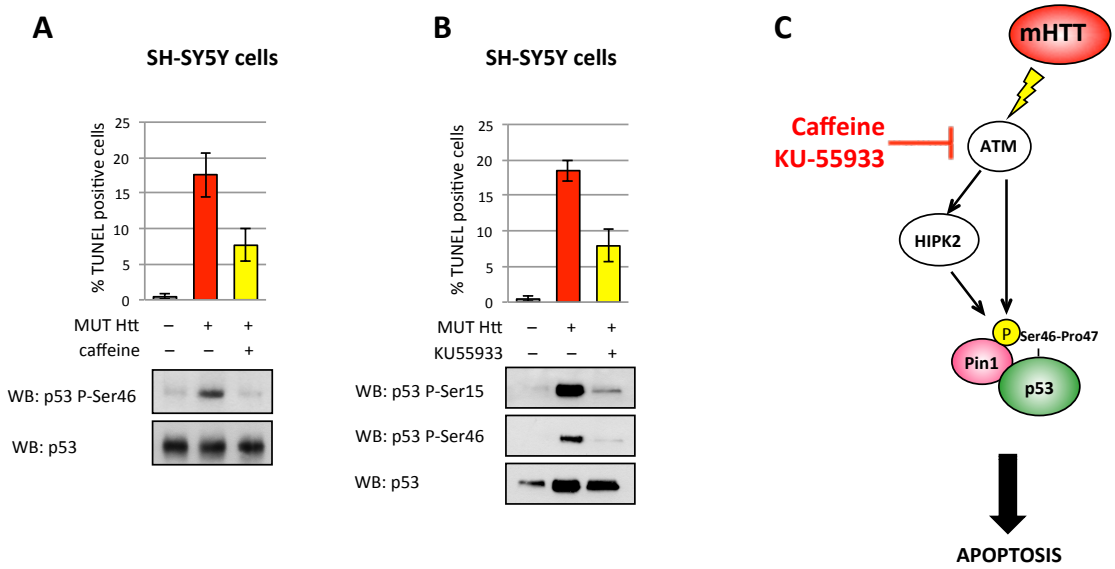


Figure 31. Inhibition of p53 phosphorylation on Ser46 reduces mHtt-dependent apoptosis.
A and B) SH-SY5Y cells were transfected with a construct expressing mHtt(1–171)150Q-GFP and treated with the ATM/ATR inhibitor caffeine, 3 mM (A), the ATM-specific inhibitor KU-55933,10 μM (B), or with same amount of solvent as a control (–). Apoptosis of mHtt GFP-expressing cells was evaluated by TUNEL assay after 24 h. Graphs show means and SD of three independent experiments. To detect p53 phosphorylation, p53 was immunoprecipitated from equal amounts of total cell lysates and analyzed by Western blot. The protein levels of Pin1, actin, and mHtt in input lysates are shown.
C) Model for regulation of p53 by Pin1 upon cellular stress generated by mutant Huntingtin. In cells expressing mHtt, the activities of ATM and HIPK2 lead to phosphorylation of p53 on Ser46. Subsequent prolyl isomerization of the phospho–Ser46-Pro47 site by Pin1 leads to induction of apoptotic genes. Pharmacologic interference with this pathway can be accomplished by use of small-molecule inhibitors that target ATM, thereby preventing p53 cytotoxic activity.

The results reported in this part have been published in the original article:

Grison, A.*, Mantovani, F.*, Comel, A., Agostoni, E., Gustincich, S., Persichetti, F., and Del Sal, G. Ser46 phosphorylation and prolyl-isomerase Pin1-mediated isomerization of p53 are key events in p53-dependent apoptosis induced by mutant huntingtin. PNAS 2011 : 1106198108v1-201106198. *Equal contribution

DISCUSSION PART 2

The majority of therapeutics currently used to treat HD patients are designed to ameliorate the primary symptoms of the HD condition itself, i.e., psychiatric agents for the control of behavioural symptoms, motor sedatives, cognitive enhancers, and neuroprotective agents (Handley et al., 2006). These drugs have limited benefits, however, and do not alter the inexorable disease progression. Unluckily, as discussed previously, neuronal dysfunction and cell death in HD are due to a combination of interrelated pathogenic processes and targeting only one aspect may not be sufficient to avoid neuronal loss; this is particularly true if considering that the vast majority of the therapies that have been proposed so far are designed to target either the phenotypic manifestations of the disease or single downstream events such as excitotoxicity, caspase cleavage of mHtt and aggregates or mitochondrial dysfunction. In this context, deciphering the codes of activation of cell death pathways from the upstream events to the final outcome becomes a main goal of research as it may provide important hints for HD treatment.

In this work, we decided to focus on an emerging model of HD pathogenesis, where mHtt evokes a canonical DNA damage response in neuronal cells, with induction of ATM/ATR kinases and consequent activation of p53 (Illuzzi et al., 2009). Therefore, we took advantage of the vast knowledge available on the p53 pathway to dissect possible upstream pathogenic events in HD. We demonstrated that p53 activation entails its phosphorylation on Serine 46 driven by ATM/ATR activated kinases and is necessary and sufficient for p53-dependent induction of apoptosis. Importantly, we also examined the contribution of the prolyl-isomerase Pin1, a well-known modulator of p53-dependent stress responses, to the observed phenotype. We observed that mHtt expression in neuronal cells is able to trigger the binding of p53 to Pin1 and that Pin1 catalytic activity is required to efficiently unleash p53 apoptotic response.

The model emerging from our data is particularly intriguing because it unveils as

mediators of mHtt induced pathogenesis important enzymes that could represent novel druggable targets for HD therapy. Indeed we demonstrated that inhibiting the activity of apical kinases, which lead to Ser46 phosphorylation either directly or indirectly, in an *in vitro* model of neurodegeneration, is able to prevent cell death. Indeed protein kinases are a growing drug target class for diseases of peripheral tissues, and several candidate therapeutics targeting CNS kinases are now in various stages of preclinical and clinical development. For instance, specific inhibitors of PKCδ have shown preclinical *in vivo* efficacy in treatment of PD(Chico et al., 2009).

Moreover in the same *in vitro* experimental setting small-molecule inhibitors of Pin1 can protect neuronal cells from mHtt-induced apoptosis and may therefore be effective as a therapeutic strategy for treatment of HD, although it is arguable that development of clinically useful inhibitors of Pin1 awaits further improvement.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the strong effort posed in trying to understand the molecular pathways underlying two of the most life-threatening pathologies worldwide, namely cancer and neurodegenerative diseases, in order to find effective therapies, adequate cures are still lacking for most of the subtypes of each category. Indeed, many targeted therapies based on drugs targeting key proteins with tumour- or neurodegeneration-driving functions have partially failed due to either development of resistance or strong side effects. Nowadays, it is emerging the concept of developing drugs or drug combinations that may target complex multicomponent cellular machineries – including chromatin modifiers, different kinase cascades or the proteasome – thus simultaneously inhibiting multiple signalling intermediates (an approach referred to as "polypharmacology" (Knight et al., 2010)). This approach may overcome the plasticity of tumours and enhance inhibitory effects in neurodegenerative disease, thus decelerating disease progression.

Data obtained from epidemiologic studies in the last two decades have unveiled an inverse comorbidity between cancer and CNS disorders, and single-genes studies (Plun-Favreau et al., 2010) but also recent high-throughput analyses suggest that this inverse correlation is due to the deregulation of the same signalling pathways but in opposite direction (Ibanez et al., 2014), e.g. the proteasome activity that is hyperactivated in tumours but downregulated in neurodegenerative diseases. These observations unveil the intriguing possibility that knowledge and strategies developed for the treatment of one disease could be exploitable also for the other pathology and *vice versa*. Prompted by this remark, in this thesis we focused on p53, a transcription factor best known for its tumour suppressive activities but recently found activated also in the most frequent neurodegenerative diseases. We aimed at dissecting the involvement of p53 and of the signalling pathways and machineries that modulate its activation and functions in both neurodegeneration and cancer, with the purpose of exploiting the knowledge acquired in a field for the other.

As a model of neurodegeneration we employed Huntington's Disease, in which neuronal death was previously reported to largely depend on p53 (Bae et al., 2005). We have focused on an emerging model of HD pathogenesis, where the expression of mutant Huntingtin (mHtt) protein, that is the causative agent of HD, evokes a canonical DNA damage response in neuronal cells, with induction of ATM/ATR kinases and consequent

activation of p53 (Illuzzi et al., 2009). In this context we demonstrated that mHtt-induced signalling events, converging on p53 also under genotoxic stress, namely phosphorylation of Serine 46 and Pin1-mediated prolyl-isomerization, drive p53-dependent neuronal cell death. Importantly, we also demonstrated that concerted inhibition of these pathways was able to avoid neuronal cell loss in an *in vitro* system.

On the other hand, in the context of oncosuppressive p53 activities, we analysed the contribution of a protein belonging to the chromatin remodelling family, BRD7, to one of the main tumour suppressive activities driven by p53 *in vivo*, oncogene-induced senescence (OIS). In this context we discovered that BRD7 was not only required for p53-dependent OIS induction thus imposing a barrier to tumorigenesis, but also to restrain the acquisition of highly malignant phenotypes, such as the ability to migrate and acquire cancer stem cell-like traits, in response to active oncogene expression but independently of p53. Moreover concomitantly we observed also a strong induction of inflammatory genes after BRD7 depletion in presence or absence of oncogenic stress, whose contribution to the observed processes and mechanism of regulation is still under investigation. Interestingly, BRD7 was shown to belong to important epigenetic regulatory complexes such as SWI/SNF and PRC2, whose multicomponent composition will be the ideal target for a polypharmacological approach. Thus, the knowledge generated in the cancer context would be exploitable also for neurodegenerative diseases; indeed global alterations of chromatin have been found in Alzheimer's Disease (Frost et al., 2014) and in other CNS disorders. For example recent studies point to aberrant histone acetylation status as a key mechanism underlying acquisition of inappropriate alterations of genome structure and function in post-mitotic neurons during neurodegenerative processes (Pirooznia and Elefant, 2013). It is therefore conceivable that targeting or restoring the functions of some members of the class of chromatin modifiers may provide benefit not only for cancer patients but also for people affected by neurodegenerative disorders.

8. MATERIALS AND METHODS

DNA manipulation techniques

DNA and siRNA transfection: Cells were transfected with Lipofectamine 2000 (Invitrogen). For RNAi, cells were transfected with Lipofectamine RNAiMAX (Invitrogen).

Calcium phosphate-mediated transfection of 293GP cells: In order to obtain the retroviruses for the generation of MCF10A stable clones 293GP cells were transfected with the vector of interest and the vector carrying the envelope sequence of the vesicular stomatitis virus. For calcium-phosphate transfection of 293GP cells a solution of calcium-chloride and DNA in water is prepared and added drop by drop to an equal volume of HBS: after 30 minutes of incubation DNA-salt precipitates were added to the cells.

Protein manipulation techniques

SDS-PAGE (SDS-polyacrylamide gel electrophoresis): For the electrophoretic separation of protein samples in SDS-PAGE the running gel was composed by 12,5% acrylamide, 0,1% bisacrylamide, 0,374M Tris-HCl pH 8,7, 0,1% SDS. The stacking gel composition was: 5% acrylamide, 0,14% bisacrylamide, 0,125M Tris-HCl pH 6,9, 0,1% SDS. The electrophoretic run was performed applying a constant potential difference of 80V while the samples were in the stacking gel, increasing it to 180V for the running gel run.

Electrotransferring of proteins from gel to nitrocellulose membrane: For the transfer of proteins on nitrocellulose membrane the *Wet transfer cell* (BioRad) was used: the blotting buffer is composed by 00,2 M Tris, 0,2M Glycine). The protein transfer is achieved applying a constant potential difference of 100V for 1 hour and 30 minutes.

Western blot: After incubation of nitrocellulose membrane for 30 minutes in blotto tween (5% milk, 0,2% Tween20 in PBS), the membranes were incubated with the primary antibodies diluted in blotto tween for 1 hour or overnight. After three washes the membranes were incubated with the secondary antibodies (conjugated with HRPO) that target the constant region of the species of the corresponding primary antibody. After 30

minutes the membranes were washed three times with blotto tween and twice with PBS. For the western blot development *ECL plus* (Pierce) or *ECL* (Amersham) were used.

RNA manipulation techniques

Total RNA extraction: For total RNA extraction the Qiagen lysis reagent was used: it is composed by phenol and guanidine isothiocyanate and allows the extraction of RNA, DNA and proteins. After chloroform addition there is the separation in three phases: taken the supernatant isopropanol addition allows RNA precipitation. Removed the supernatant the RNA pellets were washed with ethanol 75%, air-dried and resuspended in water.
To guarantee an increased RNA quality the steps with chloroform and ethanol were repeated twice.

RNA retrotranscription: The retrotranscription was performed using the *QuantiTect Reverse Transcription* kit (Qiagen). This kit allows the retrotranscription of extracted RNA through two sequential steps, a first step of genomic DNA removal and a second one of retrotranscription with a mix containing poliT and random primers. cDNA obtained were then diluted 1:10 in water in order to proceed with qRT-PCR.

Immunofluorescence

Cells grown on coverslips for one day were washed with PBS, fixed with 3% paraformaldehyde in PBS for 20 minutes and incubated with glycine for 5 minutes. Cells were then permeabilized through incubation with TritonX-100 0,1% in PBS for 5 minutes, washed with PBS and incubated for 20 minutes with BSA 10% in PBS. Antibodies were diluted in PBS-BSA 10% and the incubation was performed at 37°C for 45 minutes. After washing with PBS, cells were incubated for 30 minutes with secondary antibodies conjugated to FITC or TRITC. After washing with PBS nuclei were stained with Hoechst (2μg/ml in PBS) for 5 minutes. After two washes with PBS and one with water the coverslips were mounted through Mowiol application on a glass slide. The epifluorescence microscope Leica DM4000B was used with a 630X magnitude.

MATERIALS AND METHODS PART 1

Cell culture, treatments and transfections

Cell culture conditions: BJ/ET and U2-OS cells were cultured in DMEM (Sigma) supplemented with 10% FCS and antibiotics. MCF10A cells were grown in DMEM/F12 Ham's (1:1, Sigma) medium supplemented with 5% horse serum (Gibco), insulin (10 μg/ml; Sigma), hydrocortisone (0.5 μg/ml), EGF (20 ng/ml; Peprotech), penicillin (100 U/mL) and streptomycin (100 μg/mL). To detach cells it has been used Trypsin/EDTA (0.05% Trypsin, 0.53 mM EDTA - GIBCO).

Transient silencing: Cells were transiently silenced using Lipofectamine RNAiMAX reagent (Invitrogen): siRNAs duplex and Lipofectamine were resuspended in Optimem medium, mixed together and after 20 minutes added to the cell medium. The final concentration of siRNAs in the cell medium was 36 nM.
siRNAs sequences are listed in the Table:

Target	siRNA sense sequence
Control	AllStars Neg.Control siRNA Qiagen #1027281
BRD7 (siRNA#1)	5'-GUACUAAUGCCAUGAUUUA-3'
BRD7 (siRNA#4)	5'-GCACGUAUGGAGUUCGAAA-3'
p53	5'-GACUCCAGUGGUAAUCUAC-3'
ARID2	5'-CGUACCUGUCUUCGUUUCC-3'
BAF180	5'-UAUAGAGUUCAUGGCACACGGC-3'
BRG1	5'-GCAGAGAAGCUGUAGGACU-3'
EZH2	5'-AAGACUCUGAAUGCAGU-3'
p65	5'-GCCCUAUCCCUUUACGUCA-3'

For expression data 650.000 cells were plated on a 10 cm dish, silenced twice (the second transfection 24 hours after the first) and RNA was isolated 48 hours after the second siRNA transfection.

Constructs for DNA transfection. pRS-p53#2 containing the short hairpin for p53 silencing was previously described (Voorhoeve and Agami, 2003). BRD7 shRNA sequences were cloned into pRS-YFP, pRS-hygro, or pRS-blast. Targeting sequences are the same shown in the Table above.

pcDNA3-Flag-BRD7 has been previously described (Kzhyshkowska et al., 2003). From these, pEGFP-BRD7 was obtained by subcloning. pMSCV-BRD7 was obtained by PCR amplification on pcDNA3-Flag-BRD7 and put into pMSCV. This was used as a template to obtain pMSCV-BRD7Δ1/128, and pMSCV-BRD7Δ361/651. The plasmids for bacterial expression of MBP-BRD7 fusion proteins were all obtained by PCR amplification of the entire BRD7 ORF, or its portions using pCDNA3-Flag BRD7 as a template, and cloning in frame with Maltose binding protein (MBP) gene of pMAL-C2X vector (NEB).

Quantitative RT-PCR

qRT-PCR was performed using the *SsoAdvanced SYBR Green Supermix* (BIORAD) reagent. The qRT-PCR program is composed of a first step of denaturation (30 seconds at 95°C) and then 40 cycles of denaturation (95°C for 5 seconds), annealing and extension (60°C for 30 seconds) and dissociation. The instrument used is the BIORAD CFX96 Touch™ Real-Time PCR Detection System termocycler. The quantification is based on the $2^{-\Delta\Delta Ct}$ method using the housekeeping gene βactin as normalizer. Primers sequences are listed below.

βactin	Fw	TTTACAAGCTGCCTTCACCACA
	Rev	TTCACACCATTAAGGCTGGCACAC
ARID2	Fw	TCCGGACGAGCGGAGAAAGGG
	Rev	AAAGGCGACCCTCTGCTGTGGT
BAF180	Fw	ACCACCTCCATATCCCGGCCC
	Rev	GCGTCTTCGAGCTGCCAGTGT
BRD7	Fw	CTGGAGATGCCGAAGCACAC
	Rev	TGGGATCCACAGGATGGAGA
BRG1	Fw	GCGGCTGACCTGTGAGGAGGA
	Rev	TGCAGAACTGAAGGCCACGCT
CCL20	Fw	CAGTGCTGCTACTCCACCTC
	Rev	AAAGTTGCTTGCTGCTTCTGA
COX2	Fw	GTTCCACCCGCAGTACAGAA
	Rev	AGGGCTTCAGCATAAAGCGT
CXCL1	Fw	TTGCCTCAATCCTGCATCCC
	Rev	TTGGATTTGTCACTGTTCAGCA
CXCL2	Fw	TCACAGTGTGTGGTCAACAT
	Rev	ACACAGAGGGAAACACTGCAT
CXCL3	Fw	ACCGAAGTCATAGCCACACTC
	Rev	ACCCTGCAGGAAGTGTCAAT
CXCL5	Fw	ACGCAAGGAGTTCATCCCAA
	Rev	TCTTCAGGGAGGCTACCACTT
EZH2	Fw	TGCTTCCTACATCGT
	Rev	GGACGTTTTGGTGGG
IL1b	Fw	GCCTGAAGCCCTTGC
	Rev	GCGGCATCCAGCTACGAAT
IL6	Fw	CTGCACAGCTCTGGCTTGTTCCT
	Rev	AAAGCAGCAGCAAAGAGGCACTGGCA
IL8	Fw	GGCAGCCTTCCTGATTTCTG
	Rev	CTTGGCAAAACTGCACCTTCA
MMP9	Fw	GCCTTTGGACACGCACGACGT
	Rev	GCAGGACGGGAGCCCTAGTC
p65	Fw	GGGGACTACGACCTGAATGC
	Rev	TTGGGGGCACGATTGTCAAA
S100A8	Fw	TGTCTTTCAGAAGACCTGGTGG
	Rev	GAGGACACTCGGTCTCTAGC
Vimentin	Fwd	GAGAACTTTGCCGTTGAAGC
	Rev	GCTTCCTGTAGGTGGCAATC
E-cadherin	Fwd	TGCCCCCAATACCCCAGCGT
	Rev	ACGGTGGCTGTGGAGGTGGT
Fibronectin1	Fwd	CAGTGGGAGACCTCGAGAAG
	Rev	TCCCTCGGAACATCAGAAAC
N-cadherin	Fwd	ACAGTGGCCACCTACAAAGG
	Rev	CCGAGATGGGGTTGATAATG

Protein Production and *In Vitro* Binding Assays

MBP-BRD7 fusion proteins and MBP were extracted in Column buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA pH8, and 10 mM b-mercaptoethanol) and purified with amylose resin (NEB E8021S) following the manufacturer's instructions (NEB). *In vitro* translation of proteins was performed according to the standard protocol (Promega) in the presence of [^{35}S]methionine. The proteins were diluted in MBP-PD buffer (150 mM

Tris pH=7.5, 1% NP40, 10% glycerol) and incubated with 2.5 μg of MBP-BRD7 fusion constructs or MBP.

Immunoprecipitation

Co-immunoprecipitation of BRD7 and p53 was performed in 50 mM Tris-HCl pH 8, 100 mM NaCl, 1% NP-40, 1mM EDTA, 5% glycerol with protease inhibitor cocktail (Sigma), 1 mM PMSF, 5 mM NaF, 1 mM Na_3VO_4, 1 μM TSA and 5 μM Nicotinamide, using 2 mg of total lysate and 3 μg of anti-BRD7 polyclonal antibody or anti-HA antibody as control. Antibodies were covalently bound to protein A–Sepharose (Amersham) using 5 mg/ml dimethylpimelimidate (Pierce).

Antibodies

Antibodies against p53 (DO1), p21 (F5), Ras (F235) 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 and affinity purified by standard procedures. Anti-GFP rabbit polyclonal serum was raised against GST-GFP fusion protein expressed in bacteria and affinity purified by standard procedures. Other antibodies were anti–acetyl-Lys382-p53 (Cell Signaling Technology and Abcam), anti-p300 (NM11; BD-Pharmingen), anti-acetyl-Histone-H3 (Lys 9) (#07-352, Millipore), anti-Histone-H3 (ab1791, Abcam), anti-E-cadherin (BD Pharmaceutic) and anti-vimentin (ab8069, Abcam).

Chromatin immunoprecipitation assay

To exclude the possibility that differences in promoter binding occur in BRD7 and p53 knockdown cells because of their effect on the cell cycle, we performed the ChIP experiments within the first 48 hrs upon Ras^{V12} induction when the differences in proliferation rate between control and knockdown cells are minimal.

Cells were crosslinked with 1% formaldehyde for 15 min., neutralized with 125 mM glycine pH 2.5 and washed in PBS. For BRD7 ChIP, FA crosslinking was preceded by protein-protein crosslinking with 2 mM DSG (disuccinimidyl glutarate) for 30 min. Nuclei were prepared by hypotonic lysis (5mM Pipes pH 6.8, 85 mM Kcl, 0.5% NP40) and centrifugation, and resuspended in RIPA-100 buffer (20 mM Tris HCl, pH 7.5, 100 mM

NaCl 1 mM EDTA 0.5% Nonidet P-40 0.5% deoxycholate 0.1% SDS) with protease inhibitor cocktail (Sigma), 1mM PMSF, phosphatase inhibitors (NaF 5mM and Na_3VO_4 1mM), and deacetylase inhibitors (TSA 1μM and Nicotinamide 1μM). Chromatin was sonicated with Bioruptor (Diagenode) to 500-1000 bp average fragment size and cleared by centrifugation. IP was performed overnight at 4°C with the indicated antibodies. A negative control was performed in the presence of isotype-specific unrelated Ab. DNA–protein complexes were recovered by protein A/G PLUS-Agarose (Santa Cruz Biotech.) and washed sequentially with RIPA-100 buffer, RIPA-250 buffer (20 mM Tris HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS) and LiCl solution (10 mM Tris-HCl pH 8, 1mM EDTA, 250 mM LiCl, 0.5% Na-Deoxycholate, 0.5% NP40), then resuspended in TE, digested with 2U Dnase-free Rnase (Calbiochem) for 30 min. at 37°C, and incubated o/n at 68°C with 300 mg/ml Proteinase K (Invitrogen) in 0.5% SDS, 100 mM NaCl to digest proteins and reverse crosslinks. After purification by phenol-chloroform extraction and ethanol precipitation, DNA was resuspended in H_2O and 1/10 volume was used for quantification. Antibodies were as follows: anti-p53 FL-393 (Santa Cruz Biotechnology); anti-BRD7 polyclonal antibody (homemade); anti–acetyl-Lys382-p53 (Abcam); anti-p300 NM11 (BD-Pharmingen); anti-acetyl-Histone-H3 (Lys9) (#07-352, Millipore), anti-Histone-H3 (ab1791, Abcam). DNA–protein complexes were recovered with protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Real-time PCR was performed on an StepOne Plus cycler (Applied Biosystems), using SYBR Green Universal PCR Master Mix (Applied Biosystems). Promoter occupancy was calculated as the percentage of input chromatin immunoprecipitated using the $2^{-\Delta\Delta Ct}$ method. Primers used are listed below.

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

Bisulphite sequencing and methylation specific PCR

The upstream regulatory region of BRD7 was searched for CpG islands using EMBOSSCpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpgplot/). A CpG island was identified between nucleotides -273 to +581, relative to the transcription start site of BRD7. Primer sequences for methylation specific PCR (MSP) were designed using methprimer (http://www.urogene.org/methprimer/). Primer sequences and location relative to the transcriptional start site are as follows: unmethylated forward 5' TTGTTTTTTTGAGAGGGGTATTG 3', unmethylated reverse 5' ACCCTCATAAAAATATTTATCCAAC 3'; methylated forward 5' TCGTTTTTTTGAGAGGGGTATC 3'; methylated reverse 5' ACCCTCGTAAAAATATTTATCCGAC 3'. Genomic DNA was isolated from 10 micron tissue sections using proteinase K. Bisulphite conversion of genomic DNA was carried out using the Epitect Bisulfite kit (Qiagen). This process converts unmethylated cytosine residues to uracil while methylated cytosine residues remain unchanged. MSP was then carried out to determine the methylation status of BRD7. Bisulphite modified DNA was used as a template for MSP with primers specific for methylated or unmethylated sequences. Totally methylated DNA and normal human unmethylated DNA (Control DNA Set, Qiagen) were used as positive and negative controls, respectively. Cycling conditions were as follows: 95°C for 2 min followed by 40 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min, then a final extension at 72°C for 5 min. PCR products were electrophoresed through 2% agarose gels, stained with ethidium bromide and visualised using a transilluminator.

Immunohistochemistry of tissue samples

Tissue sections were pretreated in citrate buffer (10mM, pH 6.0) in a pressure cooker (96°C) for 40 min. Non-specific staining was blocked by incubation with BSA and slides were treated with primary anti-BRD7 homemade antibody (1:250) overnight at 4°C. Super Sensitive Polymer HRP IHC Detection System from BioGenex was used during the second day of staining.

Soft agar assay

Normal MCF10A breast epithelial cells stably expressing BRD7 or p53 knockdowns were

transduced with pLPC-puro-Ras^{V12} or with the empty vector. After selection the cells were resuspended in a top layer of culture medium with 0.25% agarose (Gellyphor, Euroclone), at 20,000 cells per 6 cm dish and plated on a bottom layer of culture medium containing 1% agarose. Every 2 days fresh growth factors were added. After two weeks the colonies were counted with a 20X objective on an Olympus CK30 microscope.

Transwell migration and invasion assay

For the transwell migration assay 100.000 cells were seeded on 8 μm pore transwell filters and allowed to migrate towards the bottom side for 15 hours. Inserts were then washed with PBS, cells that did not migrate were eliminated and migrated cells were fixed through incubation with 3% paraformaldehyde in PBS for 20 minutes. After two washes with PBS cells were stained with Crystal Violet 0,05%. After some hours inserts were washed with water, air-dried, cut and mounted on glass slices. Migrated cells were counted using the optical microscope Olympus CK30 with a 200X magnitude.

The same protocol was used for transwell invasion assay, where cells were seeded on transwell filters coated with Matrigel.

Mammosphere formation assay

Cells grown in 2D-colture were trypsinized and passed through a cell strainer with 40 μm pores in order to obtain a single cell suspension. Viable cells were counted after staining with Trypan blue and plated on ultra-low attachment (Corning) 24-multiwells. 5.000 cells were seeded in each well. Cells were then grown in non-adherent conditions for 15 days. The culture medium is constituted by MEBM (Mammary Epithelial Basal Medium-Serum free) (Lonza), supplemented with B27 (Invitrogen), 20 ng/ml EGF (PROSPEC), 20 ng/ml β-FGF (BD Biosciences), 4 μg/ml heparin (StemCell Technologies Inc), 0.5 μg/ml hydrocortisone (Sigma) and 5 μg/ml insulin (Sigma). In this way primary mammopheres (diameter ≥ 50μm) were obtained: they were then counted, collected, enzymatically disgregated and plated in the same conditions described above in order to obtain secondary mammospheres. Mammosphere formation efficiency is calculated as the number of secondary mammospheres divided for the number of plated cells, expressed then as a percentage. Mammospheres were counted using the Olympus CK30 microscope.

3D Morphogenesis assay

Cells grown in 2D-colture were trypsinized and passed through a cell strainer with 40 μm pores in order to obtain a single cell suspension. Viable cells were counted after staining with Trypan blue. 1.500 cells were seeded in a 24-multiwell covered with BD basement matrix according to (Debnath et al., 2003) and allowed to grow for 14 days.

Bioinformatical analysis

IPA functional analysis: The entire RNA sequencing data with their associated annotation (when present) with overall FDR, and the post-hoc P-value of each comparison were uploaded into IPA. The entire RNA sequencing was used as background or reference dataset for enrichment calculation of enriched functions. Each annotated gene was mapped to its corresponding gene object in the IPA Knowledge Base. The analysis was run using the following setting in IPA: all defaults setting for the selection of dataset, 1.5 fold-change cut-off, P-value = 0.001 and no FDR cut-off. The simple P-value for the enrichment was considered and reported. All functions were used except the one related to diseases.

ONCOMINE analysis: The gene lists were submitted in ONCOMINE for evaluation of their expression levels in different datasets. The gene expression data were log transformed, median cantered per array and the standard deviation was normalized to one per array. The threshold selected for the analysis were the following: odd ratio >2,0 and p.value ≤1E-4.

Explain™ promoter analysis: Promoter analysis was performed using the online tool Explain 3.0 (http://explain.biobase-international.com/) for detection of overrepresented transcription factor binding sites. For the analysis, we selected regions from 1,000-bp upstream to the transcription start site of each BRD7 target gene (Yes set) and the same promoter region of an equal amount of genes whose expression did not result influenced by BRD7 silencing in RNA sequencing data (No set). The entire vertebrate non-redundant set of transcription factors matrix from the TRANSFAC database was used for scanning potential binding sites.

MATERIALS AND METHODS PART 2

Cell culture, treatments and transfections

Cell culture conditions. SHSY5Y cells were maintained in MEM (Sigma #M4655) and F12 Ham's medium (Sigma #N6658) 1:1, supplemented with 15% foetal bovine serum (Euroclone #ECS0180L), 0,5% GlutaMAX (Gibco #35050), 1% Non-essential ammino acid solution (Sigma #M7145), 1% Pen-Strep (Lonza #DE17-602E). H1299 human lung carcinoma cells were cultured in RPMI with 10% FCS and antibiotics. Caffein and KU-55933 were purchased form Sigma.

Transient silencing. For RNA interference, cells were transfected with 36 nM of siRNA oligonucleotides using Lipofectamine RNAiMax (Invitrogen).
siRNAs sequences are listed in the Table:

Target	siRNA sense sequence
Control	AllStars Negative control siRNA Qiagen #1027281
Pin1	5'-CGGGAGAGGAGGACUUUGA-3'
p53	5'-GACUCCAGUGGUAAUCUAC-3'
Murine Pin1	siGENOME SMART POOL PIN1 M-040655-01-005
HIPK2	Hs_HIPK2_9 Qiagen #SI04439386
PKCδ	5'-CUCUACCGUGCCACGUUUU-3'

Constructs for DNA transfection. pcDNA3-htt(1-171)21Q-GFP and pcDNA3-htt(1-171)150Q-GFP encode the amino-terminal 171 aminoacids of human Huntingtin protein with 21 and 150 glutamines respectively, fused in frame at the carboxy-terminus with GFP. pGEX-Pin1, pcDNA3-Pin1-HA and pcDNA3-p53-S46A were described previously (Zacchi et al., 2002). pcDNA3-HAPin1SR and pcDNA3-HAPin1SR-S67E were generated introducing silent mutations in the region targeted by Pin1 siRNA by site directed mutagenesis of pcDNA3-HAPin1 or pcDNA3-HAPin1S67E (Rustighi et al., 2009). pcDNA3-p53-3M-Ser46 wt was generated from pcDNA3-p53 4M (Mantovani et al., 2007) by site-directed mutagenesis.

Quantitative RT-PCR

Total RNA was extracted with Qiazol and cDNA was transcribed using QuantiTect Reverse Transcription kit (Qiagen). To determine mRNA expression levels, real-time PCR was performed on a StepOne Plus instrument (Applied Biosystems) with QuantiFast SYBR Green PCR Kit (Qiagen). Primer sequences are reported below.

Gene	Forward	Reverse
Murine p21	GTGGGTCTGACTCCAGCCC	CCTTCTCGTGAGACGCTTAC
Murine β-actin	CACACCCGCCACCAGTTC	CCCATTCCCACCATCACACC

***In vitro* binding**

For GST pull-down, H1299 cells were transfected with 1 μg of pcDNA3-p53 along with increasing amounts (4, 6, 8 μg) of pcDNA3-Htt(1-171)150Q-GFP or empty vector. After 48h cells were lysed and protein extracts were incubated with 2.5 μg of GST-Pin1 protein or GST to detect p53 binding as described (Zacchi et al., 2002).

Antibodies

The used antibodies were: anti-Pin1 rabbit polyclonal antiserum(Zacchi et al., 2002), anti-Huntingtin MAB5490 (Millipore), anti-p53 DO-1 and FL-393, anti-p53 Pab240 monoclonal antibodies and anti-HSP90 F-8 (Santa Cruz Biotech); anti–phospho-Ser15-p53 and anti–phospho-Ser46-p53 (Cell Signaling Technology); anti–phospho-Ser46-p53 (BD Pharmingen^{TM}), rabbit polyclonal anti-Puma ab-9643 (Abcam), rabbit polyclonal anti-actin (Sigma). Anti-HIPK2 monoclonal antibody was a gift of L. Schmitz (University of Giessen, Giessen, Germany).

Mice strains

HdhQ111 knock-in mice expressing the complete endogenous Htt gene with 111 polyQ and their wild-type littermates (HdhQ7) in C57Bl background were provided by Dr. M.MacDonald (MGH, Boston). A colony of Pin1 KO mice in C57Bl background (Atchison and Means, 2003) was obtained from Dr. A. Means (Duke University, Durham NC). Mice containing one knock-in allele for HdhQ111 (HdhQ111/Q7) were crossed with Pin1$^{+/-}$ mice to generate cohorts of HdhQ111/Pin1wt or HdhQ111/Pin1$^{-/-}$ mice; mouse

genotype was determined by PCR on tail DNA. For detection of RNA levels in mouse, total brains of 12-months-old HdhQ111 mice were homogenized in Qiazol (Qiagen) and RNA was extracted with standard protocol.

TUNEL assays

Cells seeded on polylysine-coated coverslips were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. TUNEL assays were performed with TMR red In Situ Cell Death Detection Kit (Roche) and analysed with Leica DM4000B fluorescence microscope.

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10. APPENDIX

During the period of my PhD I have been collaborating in the following publications:

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

Grison, A.*, Mantovani, F.*, Comel, A., Agostoni, E., Gustincich, S., Persichetti, F., and Del Sal, G. Ser46 phosphorylation and prolyl-isomerase Pin1-mediated isomerization of p53 are key events in p53-dependent apoptosis induced by mutant huntingtin. PNAS 2011: 1106198108v1-201106198. *Equal contribution

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