TRIM22/Staf50 - a novel target gene of the tumor suppressor p53

Obad, Susanna

2007

Division of Hematology and Transfusion Medicine
Lund University

TRIM22/Staf50
– A NOVEL TARGET GENE OF THE TUMOR SUPPRESSOR p53

Doctoral Thesis

by

SUSANNA OBAD

With approval of the Lund University Faculty of Medicine,
this thesis will be defended on March 8th , 2007, at 9:00 am
in the Segerfalkssalen, Wallenberg Neurocentrum, BMC, Sölvegatan 17, Lund

LUND UNIVERSITY
Faculty of Medicine

Faculty opponent:
Professor Silvia Soddu
Molecular Oncogenesis Laboratory
Regina Elena Cancer Institute
Rome, Italy

ISBN 978-91-85559-12-1
Printed by Media-Tryck, Lund, Sweden 2007

In Memory of my Father . . .

Nothing shocks me.
I´m a scientist.

Harrison Ford (1942 -),
as Indiana Jones

TABLE OF CONTENTS

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their respective roman numerals.

I **Susanna Obad**, Hans Brunnström, Johan Vallon-Christersson, Åke Borg, Kristina Drott and Urban Gullberg.
Staf50 is a novel p53 target gene conferring reduced clonogenic growth of leukemic U-937 cells.
Oncogene 23(23):4050-4059, 2004

II **Susanna Obad**, Tor Olofsson, Nadir Mechti, Urban Gullberg and Kristina Drott.
Expression of the IFN-inducible p53 target gene TRIM22 is down-regulated during erythroid differentiation of human bone marrow.
Leukemia Research In Press

III **Susanna Obad**, Tor Olofsson, Nadir Mechti, Urban Gullberg and Kristina Drott. Involvement of the interferon-inducible p53 target gene TRIM22 (Staf50) in human T lymphocyte activation.
Submitted

IV **Susanna Obad**, Stina Oredsson, Mattias K. Andersson, Pierre Åhman, Urban Gullberg and Kristina Drott.
The p53-target gene TRIM22 induces cell death and co-localises with PML.
Manuscript

SELECTED ABBREVIATIONS

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
APC	antigen presenting cell
APL	acute promyelocytic leukemia
ATRA	all-trans retinoic acid
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CML	chronic myeloid leukemia
EFP	estrogen responsive finger
FACS	fluorescence activated cell sorting
GFP	green flourescence protein
HERF1	hematopoietic RING finger 1
HSC	hematopoietic stem cell
ISG15	interferon stimulated gene of 15 kDa
ISRE	interferon stimulatory response element
LSC	leukemic stem cell
MPP	multipotent progenitor
NB	nuclear body
NK	natural killer cells
PBMC	peripheral blood mononuclear cell
PIG	p53 induced gene
PMA	phorbol 12-myristate 13-acetate
POD	PML oncogenic domain
RACE	rapid amplification of cDNA ends
ROS	reactive oxygen species
RING	really interesting new gene
STAF50	stimulated trans acting factor of 50 kDa
TCR	T cell receptor
TRIM	tripartite motif
TRAF	tumor necrosis factor receptor-associated factor
UBL	ubiquitin-like protein

INTRODUCTION

The general aim of my work was to identify candidate molecular pathways for targeting in novel therapies of leukemia and cancer. Therefore, a deeper understanding of the regulation of proliferation, differentiation and viability of leukemia cells is necessary. The tumor suppressor gene p53 is positioned in the centre of a complex of signaling pathways that prevent proliferation and survival of potentially malignant cells. Consequently, downstream events of p53 are potential targets for therapeutic manipulation.

Hematopoiesis

Normal hematopoiesis and stem cells

The bone marrow constitutes 4.5% of the body weight, and daily produces approximately 300 billions of red and white blood cells. This remarkable cell renewal process originates from a small population of multipotent bone marrow cells termed hematopoietic stem cells [1]. As early as 1917 Arthur Pappenheim found the first evidence of a hematopoietic stem cell (HSC) [2].

A normal adult stem cell is defined as a cell that has the ability to self-renew and to generate mature cells of a particular tissue through differentiation [3]. Hematopoietic development starts with long-term (LT)-HSCs, highly self-renewing cells that can reconstitute an animal for its entire life span, which further give rise to short-term (ST)-HSCs, with the ability to reconstitute an animal for a limited period (Figure 1) [4]. The ST-HSC differentiates into multipotent progenitors (MPP), which progressively lose their potential to self-renew, and develop into either common lymphoid progenitors (CLP) which are restricted to mature into B cells, T cells, NK cells or dendritic cells, or into common myeloid progenitors (CMP) which are progenitors for myeloerythroid lineages, i.e. dendritic cells, granulocytes, macrophages, platelets or erythrocytes. Thus, the hematopoietic development is a strictly regulated process, depending on control mechanisms regulating the expansion, differentiation and death of each hematopoietic cell [1].

Cancer

The process of transformation

Cancer is a multistep process where the accumulation of somatically acquired genetic changes disturbs the normal balance of controlled cell differentiation, proliferation and death [5]. When the normal balance is disturbed, tumor

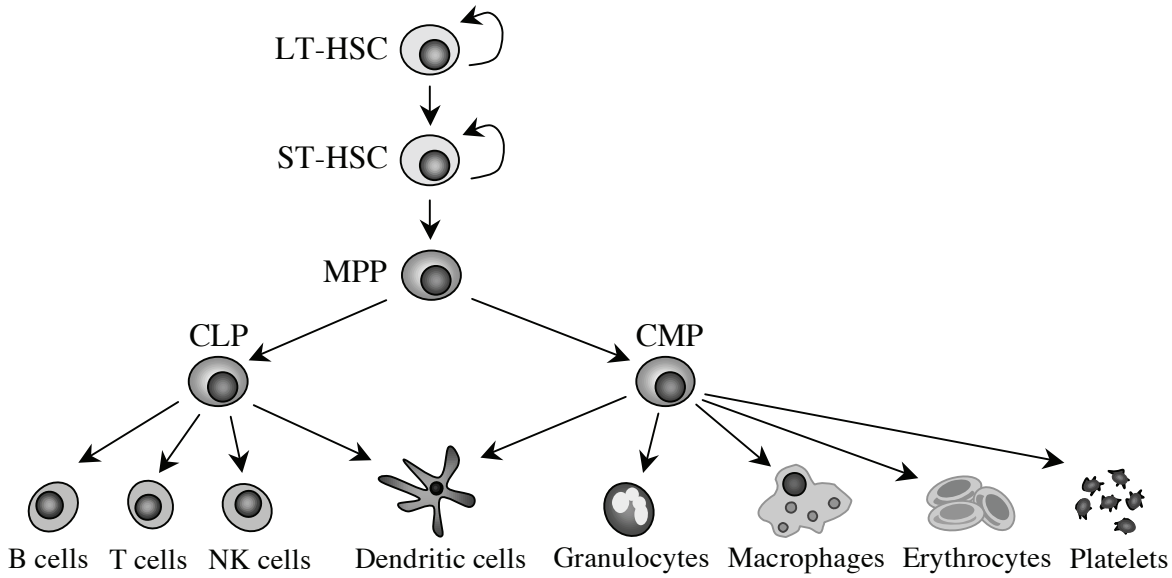
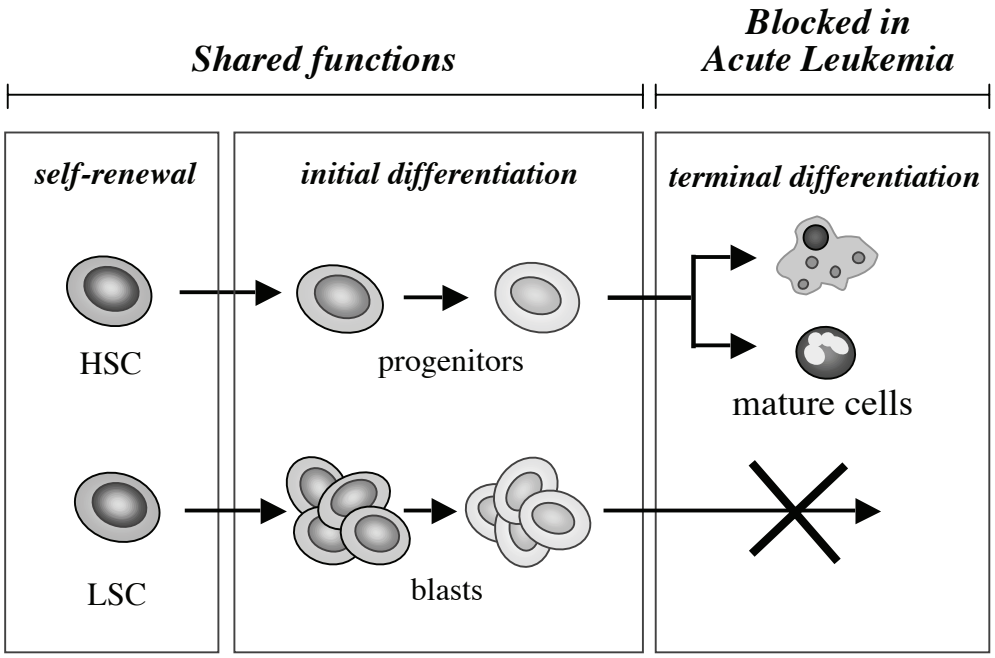


Figure 1. The hematopoietic tree consists of 8 lineages.
Schematic picture of hematopoietic development from HSCs via CLP and CMP to mature cells. LT-HCS: long-term hematopoietic stem cell, ST-HCS: short-term hematopoietic stem cell, MPP: multipotent progenitor, CLP: common lymphoid progenitor, CMP: common myeloid progenitor.

suppressor genes are activated, which are genes that reduce the probability for transformation. The tumor suppressor proteins usually have repressive effects on the cell cycle or are able to promote apoptosis. However, a mutation or deletion of a tumor suppressor gene increases the probability of transforming normal cells to malignant cells. During the course of cancer development, a normal cell progresses towards malignancy by acquiring series of specific mutations. It could be mutations or genetic rearrangements that activate proto-oncogenes, which are genes normally required for promotion of growth and survival, but after transforming events turns into oncogenes. Hence, the activated oncogenes often code for proteins that regulate cell growth, survival and differentiation. By acquiring these mutations a cell progressively alters its phenotype and thereby escapes the various controls that normally prevents malignant growth in an organism [5].

Leukemic stem cells

Increasing amounts of data support the view of cancer as a stem cell disorder. Evidence for cancer stem cells are particularly strong in the case of leukemia, where there is considerable proof that certain types of leukemia can arise from mutations that accumulate in HSCs. Leukemia can be viewed as a newly formed abnormal hematopoietic tissue initiated by a few leukemic stem cells (LSCs) that undergo an abnormal and poorly regulated change comparable to that of the



Modified from Rosenbauer et al Blood 106; 2005

Figure 2. Origin of the leukemic stem cell (LSC), its shared functions with the hematopoietic stem cell (HSC) and the development of leukemia.

normal HSC (Figure 2) [4,6]. LSCs share important stem cell functions with normal HSCs, such as self-renewal, initial differentiation and increased survival. Both eventually differentiate into less pluripotent daughter cells. Nevertheless, while HSCs produce short-lived progenitors, such as CMPs or CLPs, which terminally differentiate into mature cells, LSCs give rise to leukemic blasts, characterised by a block in their terminal differentiation (Figure 2) [7]. Thus, although leukemias are heterogenous in terms of phenotypes, there are general mechanisms characterizing leukemic transformation, i.e. increased cell survival, increased proliferation capacity, increased self-renewal and impaired differentiation.

Therapies

At present, most treatment for leukemia in clinical practice uses chemotherapeutic agents that target leukemia cells. However, by targeting also normal cells, this treatment not only causes severe side effects, it is also possible that this approach spares the small population of LSCs, which are responsible for the continued expansion and propagation of the malignant cells. In many cases, this leads to recurrence of the disease.

If we possessed a greater knowledge of LSC biology it would allow us to design therapeutic agents that specifically would target the LSC population. Such therapies alone or in combination with conventional chemotherapeutic agents

could potentially reduce the expansion of malignant cells and could cause a more permanent disease remission [3]. A challenge to this approach lies in finding a way to specifically target cancer-cell self-renewal pathways without toxicity to normal stem cells. Although not targeting LSCs distinctively, still today, novel therapies targeted directly to molecular pathways within the malignant cell have been developed.

Several drugs are today in routine clinical use, among these are retinoic acid used in combination with conventional chemotherapy in the treatment of acute promyelocytic leukemia (APL), and imatinib utilized in chronic myeloid leukemia (CML) [8,9]. Interferons (IFNs) are sometimes used in treatment of CML, hairy cell leukemia and myeloma, however the molecular function of IFN in cancer treatment still remains unexplored. Takaoka *et al* have shown that one mechanism of IFNs involvement in tumor progression may involve activation and induction of the tumor suppressor p53 [10].

Therefore, the aim of this thesis is to better understand the cross talk between IFN and p53 pathways, utilizing the novel IFN-inducible p53 target gene TRIM22.

p53 - "the guardian of the genome"

The p53 gene, discovered in 1979, was one of the first tumor suppressor genes to be identified. It was originally believed to be an oncogene, but data obtained ten years after its discovery showed it to be a tumor suppressor gene and a transcription factor. It was also found that p53 does not function properly in most human cancers. p53 is usually inactivated directly as result of missense mutations in the DNA-binding domain of the p53 gene, inactivated indirectly through binding to viral proteins, or inactivated as a result of alterations in genes whose products interact with p53 [11]. Therefore, intact p53 pathways are of importance for preserving a benign phenotype [12].

p53 in leukemia

Inactivation of the p53 gene is a major event in human tumorigenesis. More than 60% of human primary tumors exhibit a mutation in the p53 gene. Hematological malignancies present a rather low incidence of genetic alterations in this gene (10-20%). Nonetheless, leukemia cells with mutant p53 show a strong correlation with unfavorable prognostic factors and resistance to chemotherapy [13,14]. p53 mutations are most often found in lymphoid malignancies such as Hodgkin´s disease, Burkitt´s ALL and adult T-cell leukemia, but also in AML and in the blast crisis of CML [15].

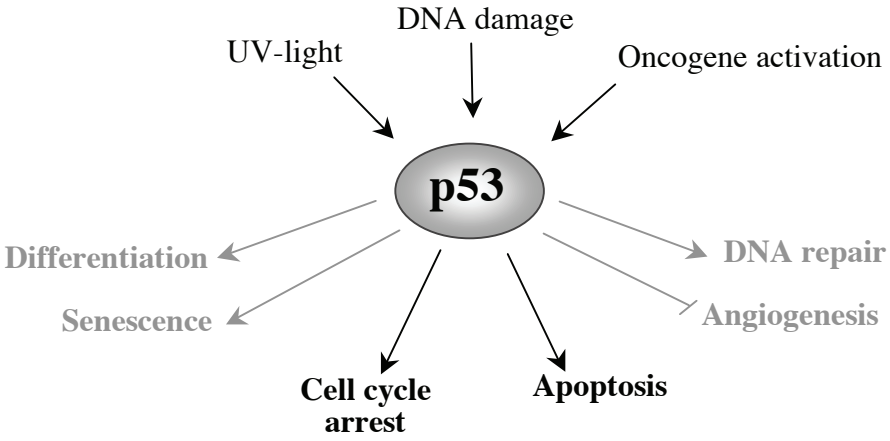


Figure 3. Schematic presentation of some activators and functions of p53. *The activation of p53 leads to the different functions of p53, with cell cycle arrest and apoptosis being the two best characterized.*

Patients that have a germline mutation of p53 often develop the so-called Li-Fraumeni syndrome [16]. The Li-Fraumeni syndrome is an autosomal dominant syndrome characterized by a multitude of neoplasms at many different sites in the body, where osteosarcoma, ovarian cancer and leukemia occur at an increased rate [17].

Since there is a low frequency of p53 mutations in hematological tumors, the potential alterations of p53 related genes has intrigued many. Gronbaek *et al* have shown a relationship between disrupted p16 and the ARF-p53 pathway in non-Hodgkins lymphoma, while Eischen *et al* showed disruption of the ARF-Mdm2-p53 pathway in lymphomagenesis [18,19]. The disrupted pathways result in additional growth advantage for the tumor, compared to alteration in only one gene. Data suggest that all these pathways are connected and among the most commonly inactivated in cancer [20,21].

p53 functions

The amount of p53 protein present in normal cells is low and is controlled by its rate of degradation rather than transcriptional activation or translation from mRNA. As will be described, the degradation is regulated by ubiquitination performed by, among others, the p53 target genes mdm2, Pirh2 and COP1 [22].

The posttranslational activation of p53 can occur by several independent pathways. One pathway is triggered by DNA damage, a second by aberrant growth signals such as those resulting from the expression of oncogenes, Ras or Myc. Other triggering events for p53 activation are chemotherapeutic drugs, ultraviolet light or protein-kinase inhibitors (Figure 3). All pathways described inhibit degradation of p53 protein, thereby stabilizing p53 protein at high levels[23].

The functions of activated p53 are modulated by phosphorylation or acetylation. Activated p53 has the ability to function as a sequence-specific transcription factor, which can act directly or indirectly to turn on or off various target genes. By these means, several responses in cells including differentiation, senescence, DNA repair and inhibition of angiogenesis can be induced [24]. Nevertheless, the best understood mechanisms are the abilities of p53 to induce cell cycle arrest or apoptosis. By manipulating these processes, p53 can prevent tumor formation by reducing the likelihood with which potentially transforming genetic lesions accumulate.

p53 mediates cell cycle arrest

One of the first effects after p53 activation in nearly all mammalian cell types is a block in the cell division cycle. Of genes identified to date as p53 target genes involved in cell cycle arrest, $p21^{WAF1}$ stands out as playing an important role in the induction of cell cycle arrest [25,26]. $p21^{WAF1}$ is a cyclin-dependent kinase inhibitor (CDK) that inhibits both the G1-to-S and G2-to-mitosis transitions [27]. Another target gene of p53 that contributes to the G2-arrest is 14-3-3-σ [28]. 14-3-3-σ can bind p53 and activate its sequence-specific DNA-binding after irradiation and thus represents a positive feedback loop to p53 to prevent cell cycle progression in damaged cells [29]. Additional mediators of G2 arrest downstream of p53 include GADD45 and Reprimo [30,31].

The killer instinct of p53 – APOPTOSIS

Some cells in which p53 is activated undergo programmed cell death (apoptosis). How p53 facilitates the process of apoptosis is much less clear than for cell cycle arrest. Considerable evidence suggests that p53 functions as a transcription factor, where p53 is able to both activate and repress the genes that are involved in the process. There are several potential mediators of p53-induced apoptosis (Figure 4). The first identified apoptotic target of p53 was the bax gene, a pro-apoptotic member of the Bcl-2 family [32]. Since then several other pro-apoptotic genes have been identified, e.g. Noxa and PUMA [33,34]. These proteins localize to the mitochondria and promote loss of the mitochondrial membrane potential and cytochrome c release, thereby activating the Apaf1/caspase 9 apoptotic cascade[35]. Several genes coding for redox-controlling enzymes mediate pertubation of mitochondrial integrity. Some of these genes are identified as p53-induced genes (PIGs) [36]. The PIGs produce reactive oxygen species (ROS) that can cause damage to the mitochondria, which in turn initiates apoptosis [37]. Activation of IGF-BP3 by p53 leads to negative regulation of the IGF pathway and inhibits integrin-associated survival signalling, which induces cells into apoptosis [38].

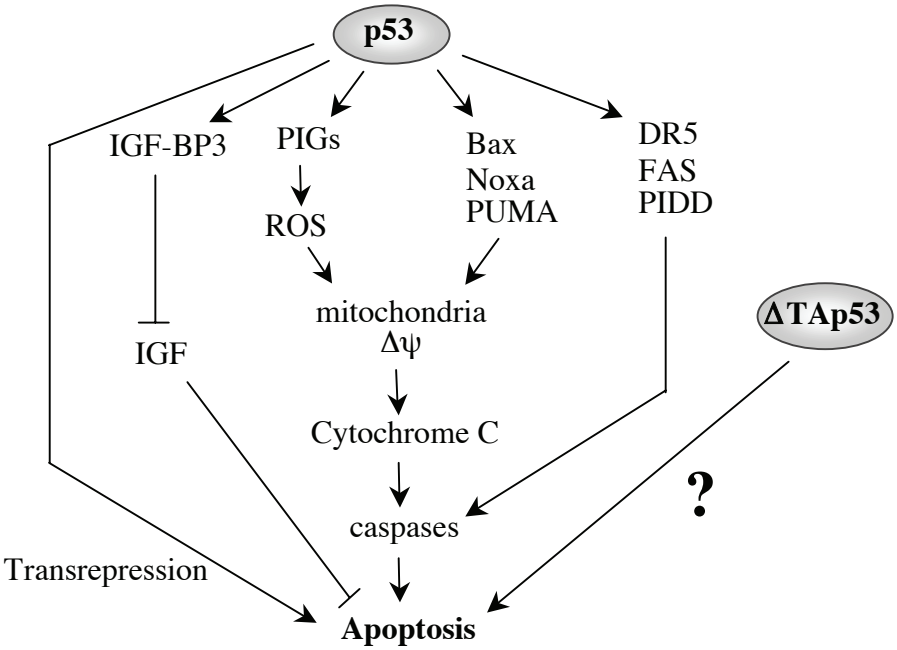


Figure 4. Mechanisms for p53 mediated apoptosis.
Apoptosis mediated by p53 can be induced through;
1.) death receptors (DR5, FAS, PIDD), 2.) proteins involved in cytochrome c release (Bax, Noxa, PUMA), 3.) genes coding for redox controlling enzymes (PIGs), 4.) negative regulation of survival signals, 5.) transrepression, 6.) ΔTA: deletion of transactivation domain.

p53 has also been implicated in the membrane death receptor induced pathway of apoptosis in several ways. Expression of FAS/APO, DR5/KILLER and one of the death receptor ligands, FASL, are up regulated by p53 [39,40]. The DR5 promoter is a direct target gene of p53 [41,42]. Another death domain containing protein, PIDD, is also activated by p53 and thereby induces apoptosis through the death receptor pathway [43].

The majority of the studies investigating p53 in apoptosis have focused on its transactivation functions. However, p53 has also transrepression capabilities that can contribute to apoptosis [44-46]. The mechanism behind p53-mediated transrepression is not fully established, however it appears to involve the ability of p53 to recruit histone deacetylases to certain genes [47]. It seems that under specific conditions (i.e. hypoxia), p53s ability to transrepress for induction of apoptosis may be more important than inducing it by transactivation function [48].

It has also been suggested that p53 can control apoptosis through transcription independent mechanisms. However, most studies connecting apoptosis to transcriptional independent functions of p53 involve forced expression of transcriptionally impaired p53 at non-physiological levels [49,50]. Hence, it still seems to be an open question whether wild type p53 possesses transcription independent features as well, although cell surface expression of FAS is

enhanced by p53 through promotion of the trafficking of FAS from the Golgi to the plasma membrane [42].

But what makes a cell decide whether to undergo apoptosis or cell cycle arrest ? The fate of the cells appears to depend on the nature of the stress signal, the state of the cell and its environment, the types and locations of the protein modifications on the p53 protein, the proteins associated with p53 and the pattern of gene expression at the moment in that specific tissue undergoing a p53 response.

The role of p53 in differentiation and development

The involvement of p53 in cellular differentiation and development is less characterized than its role in cell cycle arrest and apoptosis. Initial studies on $p53^{-/-}$ mice showed no effect on development, however more detailed studies have now revealed that these mice did suffer from developmental abnormalities, such as female-associated defects in neural tube closure [51-53]. The defects have been suggested to arise because p53 plays a physiological role at the time of neural tube closure, or because of an abnormally high frequency of mutations within the haploid gametes of p53 null parents [53]. However, as discussed below, knock out of the p53-family members p63 and p73 results in severe developmental defects, suggesting important functions of the p53-family during development.

In spite of the modest differentiation-related effects in p53-knock-out mice, introducing or overexpressing p53 in different tumor cell lines induces differentiation. For example, p53 has been shown to take part in the differentiation of muscle cells, neurons and pancreatic carcinoma cells [54-56]. In leukemia, p53 seems to be involved in differentiation of both lymphoid and myeloid lineages. Expression of p53 in the promyelocytic cell line HL60 induces differentiation along the granulocytic or the monocytic pathways [57,58], additionally, p53 has also been implicated in erythroid differentiation [55,59,60].

The molecular mechanisms behind p53-mediated differentiation are not yet understood. Nevertheless, it has been shown that p53 mediated differentiation of leukemic cells is mediated through its transcriptional activity [61]. The identification of differentiation-induced genes that are transactivated by p53, in reporter assays, lends further support to this concept [62-64].

Recent results have shown that p53 can suppress osteoblast differentiation and bone development both in vivo and in vitro [65,66]. In contrast to previous results, suggesting a differentiation-stimulatory role for p53, these observations established p53 as a negative regulator of osteoblast differentiation. Although the mechanisms are largely unexplored, considerable amounts of data favor a

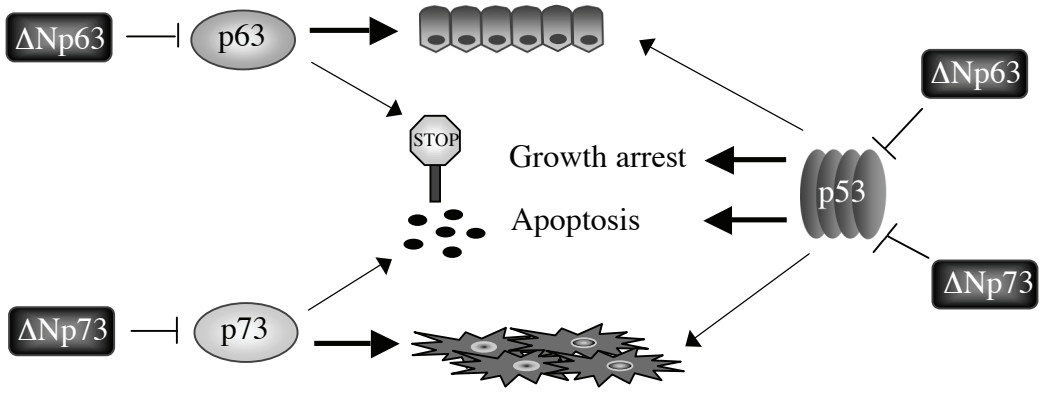


Figure 5. Schematic representation of p53, p63 and p73 functions. *p53, p63 and p73 are involved in cell cycle arrest and apoptosis, while p63 shows involvement in epithelial differentiation and p73 in neuronal differentiation. The dominant negative isoforms (ΔN) of p63 and p73 can suppress the expression from fullength p63, p73 and p53.*

role for p53 and its family members in differentiation and development. Nevertheless, it cannot be excluded that the differentiation inducing effect of p53 are mere reflections of its relationship to p63 and p73.

p63 and p73, members of the p53 family

p53 was long thought to be a unique gene with no genetic paralogues. In 1997 Daniel Caput identified the first p53 related gene, p73 [67]. Several groups have since then independently identified the third member of the family, p63 [68-72]. The high level of sequence similarity between p53, p63 and p73 proteins, particularly in the DNA binding domain, allows p63 and p73 to transactivate p53-responsive genes causing cell cycle arrest and apoptosis (Figure 5) [73,74].

Similarly to p53, p63 and p73 can give rise to several mRNA isoforms that, when translated, produce distinct protein isoforms. Transactivating isoforms, such as TAp63/p73, show tumor suppressor gene properties similar to p53, while isoforms lacking N-terminal transactivating domain such as ΔNp63/p73, induce a functional block against p53 as well as TAp63/p73 activities (Figure 5).

In contrast to $p53^{-/-}$ transgenic mice, which show extensive tumor formation, severe developmental abnormalities are observed in $p63^{-/-}$ and $p73^{-/-}$ mice and in humans with germ-line p63 mutations, but no spontaneous tumors [68,75]. $p63^{-/-}$ mice are born alive but display severe deformations of limbs as well as of epithelia including skin, breast, urothelia and prostate [68]. Neuronal defects were the first phenotypic trait observed in $p73^{-/-}$ mice. Beside neurological and cognitive impairments, $p73^{-/-}$ mice show severe defects in pheromone-based behavior, infection control and reproduction [75].

Interferons

Interferons (IFNs) constitute a large family of cytokines that share antiviral, antitumor and immunomodulatory activities [76]. IFNs, classified as α, β, ω and γ, on the basis of their structure and antigenic properties, are grouped into two families; type I and type II IFNs. IFN α, β and ω, which belong to type I family, are produced by most cell types in response to stimuli like viral infection or double-stranded RNA [76]. IFNγ is the only member of the type II family and is secreted by T lymphocytes and NK cells in response to specific antigens or mitogens [77]. Type I and II IFNs interact with cell surface receptors, resulting in the activation of specific Janus protein kinase (JAK)/signal transducers and activators of transcription (STAT) pathways, leading to the transcription of a distinct set of genes that mediate the biological responses of these cytokines. IFN-stimulated response elements (ISREs) drive the expression of most IFN α/β regulated genes and a few IFNγ regulated genes [76].

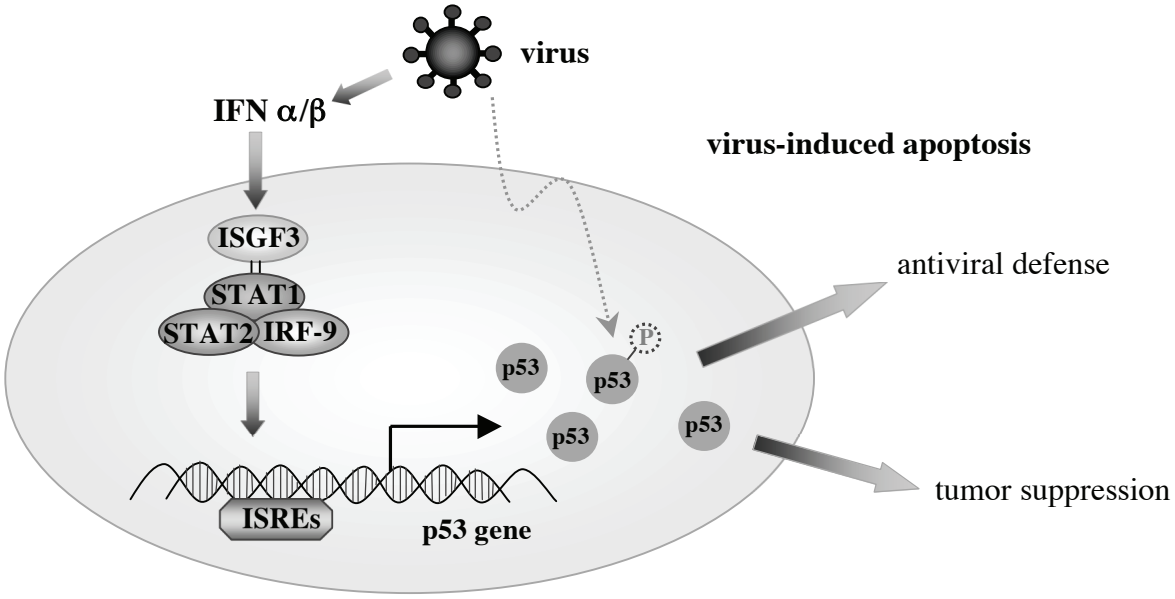
IFNs are essential for the survival of higher vertebrates because they provide an early line of defense against viral infections, hours to days before immune responses. The best-characterized IFN-induced antiviral pathways are the dsRNA-dependent protein kinase (PKR), the 2-5A system, and the Mx proteins [78]. Any stage in viral replication appears to be a point of attack for inhibition by IFN-regulated pathways; entry, uncoating, RNA stability, initiation of translation, maturation or assembly and release.

IFNs inhibit cell growth and control apoptosis, activities that are important for the suppression of cancer and infection [76]. Cells in culture exhibit varying degrees of sensitivity to the antiproliferative activity of IFNs. Growth arrest may be due to differentiation, particularly when IFNs are used in combination with other agents, i.e. retinoids [79,80]. IFNα has been shown to target specific components of the cell-cycle control, including c-myc, pRb, cyclin D3 and cdc25A [76].

The IFNs are not the only cellular defense proteins that can influence apoptosis to prevent viral propagation. Among others, the tumor suppressor protein p53 can also induce apoptosis in response to virus infection.

The action of interferons and virus on p53

Takaoka *et al* have demonstrated that IFN treatment of cells induced p53 gene expression and p53-mediated apoptosis, connecting tumor suppression and antiviral immunity [10]. This was further supported by Chelbi-Alix and colleagues who showed that the apoptosis by IFNα is dependent upon presence of wild-type p53 but not apoptosis induced by IFNγ [81]. p53-induction by IFN α/β is initiated by the binding of IFN α/β to the Type I receptor on target cells. This induces the formation of ISGF3, composed of activated STAT1, STAT2 and



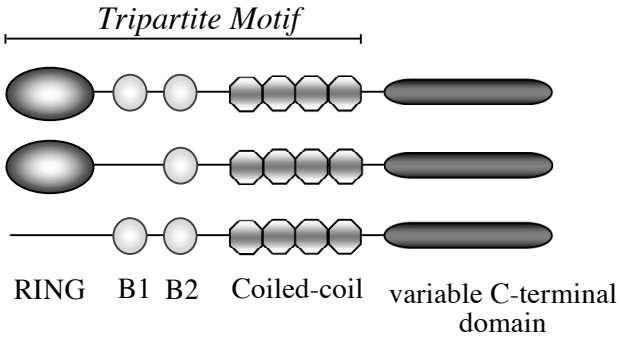
Modified from Vilcek, Nature 2003

Figure 6. Connection between viral infection, secretion of IFNα/β, and transcription and activation of p53.
Cells infected with virus activates p53 by phosphorylation and thereby the target genes of p53. Virus causes also IFN production, mediating formation of the ISGF3 complex, which binds to the ISREs in the p53 gene and starts transcription of p53.

IRF-9, a complex that binds to the p53 promoter, which contains two ISRE sites, and by these means activates transcription of p53 (Figure 6) [10].

Furthermore, Takaoka *et al* showed that virus infection also could lead to posttranscriptional activation of p53, which could produce apoptotic cell death of the virus-infected cells, resulting in the restriction of virus replication (Figure 6). In support of this, mice with "super p53" (extra copy of p53), have been shown to be more resistant to viral infection as compared to WT mice [82]. One early line of defense after virus infection is induction of apoptosis, since it severely limits virus production in the cell or even eliminates the spread of virus. Many viruses have therefore evolved strategies to avoid early apoptosis, including suppression of IFN and p53 inhibition [83,84]. The antiviral activity of this tumor suppressor is reinforced by the fact that p53 frequently affects the expression from viral promoters.

Takaoka *et al* suggest that chemotherapeutics should be tried together with interferons in order to minimize the doses of chemotherapy and thereby mitigate the side effects [10]. However, the doses used in the experiments described above were as high as 10,000 U/ml, rendering the experiments impossible to translate to clinical treatment because of severe IFN related side effects. Although high doses of exogenous IFNα in patients with advanced myeloma have been



Modified from Meroni and Diez-Roux, BioEssays 2005

Figure 7. Schematic structure of the domains of the TRIM/RBCC family. *The TRIM/RBCC proteins all share RING, B1, B2 and coiled-coil domain, while the C-terminals are variable.*

successful, dose related side effects constitute a practical problem. Therefore, a solution could be to deliver the interferons to the malignant cells locally, thereby overcoming the problems with side effects.

The story of the TRIM/RBCC family

The TRIM/RBCC protein family is characterized by a tripartite motif (TRIM), consisting of a RING-finger, B-box type 1 and/or 2 (B), a coiled-coil (CC) region and a variable C-terminal domain (Figure 7) [85]. To date, in the human genome, 68 genes encoding TRIM proteins have been identified [86]. It has also been shown that TRIM proteins often are expressed as multiple splicing isoforms. However, the splicing variants usually share the same RBCC motif but differ in their C terminus [85].

The RING domain is defined by a specialized zinc finger of 40-60 amino acid residues that binds two zinc atoms and seems to be involved in protein-protein interactions[86,87]. Interestingly, RING finger domains have been found to play an essential role in mediating the transfer of ubiquitin to substrates. This domain is therefore a characteristic feature of many E3 ubiquitin ligases [88]. In the TRIM family ubiquitin ligase activity has been shown for the TRIM5δ isoform, TRIM18/MID1, TRIM25/EPF, TRIM32/HT2A and TRIM35/ARD1 [89].

Characteristic B-boxes (B1 and B2) with two zinc-finger motifs each follow the RING domain. B boxes are found exclusively in TRIM proteins and are probably a critical determinant of the tripartite motif. If both B-boxes are present, B1 always precedes B2, but if only one is present it is always B2. Nonetheless, so far no function has been clearly assigned to these domains [85].

The coiled-coil domain has the capacity to merge with other coiled-coils and by these means mediate homo- and hetero- interactions. The homo interactions are much more common than the hetero interactions among the TRIM family members. This propensity for homo interactions stimulates the formation of TRIM high-molecular-weight complexes. These are compartmentalized either in the nucleus or the cytoplasm, suggesting that TRIM proteins might define subcellular compartments [85].

While the RBCC-structure of TRIM proteins appear to be rather conserved within the family, the C-terminal is more variable. The most common C-terminal motifs are the B30.2/SPRY-PRY domain, the NHL repeats and the associated PHD-domain, but there are many others i.e. MATH, ARF, BROMO and TRAF [90]. The B30.2 domain has been identified in 11 protein families and has been shown to be involved in for example developmental processes and the immune defense[91,92]. The TRAF (tumor necrosis factor receptor-associated factors) proteins are not exclusive for TRIM proteins, as there are 6 non-TRIM TRAF proteins, which constitute their own family. They are suggested to be important regulators of cell death and cellular responses to stress [92].

Recently, Short and Cox subclassified the TRIM/RBCC family on the basis of their varied C-terminal domain compositions and revealed a novel motif, COS box, which was shown to be necessary for microtubule binding, adding further evidence for the involvement of the TRIM/RBCC family in subcellular compartments [90].

TRIM proteins and viral defense

Among the large family of TRIM proteins, less than 20 members have been individually characterized, showing involvement in cell proliferation, development, differentiation, oncogenesis or apoptosis. Interestingly, 3 of the 68 TRIM proteins have been identified as IFN-inducible (TRIM19/PML, TRIM22/Staf50, TRIM5), which might suggest involvement of TRIM proteins in viral defense [93-95]. Indeed, apart from the IFN-inducible PML (promyelocytic leukemia), TRIM22 and TRIM5, other TRIM proteins have been found to interfere with viral replication, including TRIM1 and TRIM32 [96,97].

The antiviral activity of PML is probably the best documented. PML expression is induced by type I IFNs, and it has been demonstrated that in the absence of IFN, constitutive overexpression of PML mediates resistance to infection by vesicular stomatitis virus (VSV) and influenza A virus, indicating that PML participates in the antiviral state induced. PML has an inhibitory effect on both VSV mRNA and protein synthesis, and can also bind the human foamy virus transactivator Tas, thereby inhibiting retroviral transcription [98,99]. Moreover, using a yeast two-hybrid system, TRIM32 was shown to bind to the activation

domain of Tat proteins of HIV-1, giving further evidence for the role of TRIM proteins in inhibition of viral replication [97].

TRIM5α is the largest isoform coded by the TRIM5 gene and was recently shown to block the replication of several retroviruses (e.g. HIV-1 and MLV). TRIM5α blocks the replication at a stage in the viral cycle following reverse transcription but before integration of viral DNA into the host chromosome [100]. During the characterization of TRIM5α, other TRIM members were tested for antiviral activity, and thus TRIM1 was identified to restrict infection of MLV in a similar fashion to TRIM5α.

Very recently, Bouazzaoui *et al* demonstrated that the IFN-inducible TRIM22 inhibits the HIV-1 replication in human monocyte-derived macrophages [101]. Although the precise mechanisms for this effect remain to be discovered, the observation that TRIM22 can act as a potent inhibitor of transcription from the LTR promoter of HIV-1 could suggest a role as a transcriptional regulator and a negative role on HIV replication cycle [101,102].

Together this information suggests that TRIM proteins could play a thus unexpected role in innate immunity and viral defense.

Differentiation effects among the TRIM members

Interestingly, several recent studies have indicated roles in differentiation and development for a number of TRIM-family members. For example, TRIM10/HERF1, TRIM22 and TRIM25/EFP are some of the TRIM members that have involvement in differentiation.

HERF1 was identified when searching for components of the downstream pathways initiated by the transcription factor AML1/CBFβ, which is essential for definitive hematopoiesis. Inhibition of HERF1 expression blocked terminal erythroid differentiation, whereas its overexpression induced erythroid differentiation [103]. The estrogen responsive finger (EFP) is developmentally regulated during mammalian development, expressed in osteoblasts, and has been suggested to function in osteoblast differentiation [104,105]. As will be discussed later in this thesis, TRIM22 is also suggested to have a role in the erythroid differentiation, as downregulation of TRIM22 expression was linked to normal erythroid differentiation [106]. It is remarkable that all the mentioned proteins have a B30.2 domain in their C terminal.

There are even more TRIM proteins, which have been shown to be involved in differentiation and also belong to the same C terminal family of RFP-like/B30.2/SPRY-PRY. TRIM27/RFP (SPRY) prevents degradation of EID-1 which is an inhibitor of histone acetylation and differentiation, Bloodthirsty (B30.2) which is required for erythropoiesis in zebrafish and the novel PU.1

binding factor Pub (B30.2) which is responsible for inhibiting the transcriptional activity of PU.1 which in turn is necessary for normal hematopoiesis [107-109]. However, there are exceptions, since TRIM members with other C terminal domains are involved in differentiation. For example, TRIM32 that have a NHL domain is induced in myogenic differentiation [110].

TRIM/RBCC proteins as E3 ubiquitin ligases

As briefly discussed above, the involvement of TRIM proteins in such diverse cellular processes as viral response and differentiation, could suggest that their conserved structure might underlie a common biochemical function. There is a strong association between the presence of a RING domain and ubiquitin ligase activity [111]. The covalent attachment of ubiquitin to selected cellular proteins is used by eukaryotic cells to control different cellular functions, the most well known being proteasome mediated degradation [112].

The ubiquitination process involves at least three classes of enzymes, and begins with the formation of a thiol-ester linkage between the C-terminus of Ubq and the active site cysteine (Cys) of the Ubq activating enzyme (E1) (Figure 12). Ubq is then transferred to an Ubq conjugating enzyme (Ubc or E2), after which the complex is transferred to the E3 ligase. The E3 ligase interacts with E2 and substrate and mediates formation of isopeptide bonds between the C terminus of Ubq and lysines on the target protein [113]. E3 ligases are considered to be the primary specificity determining factors in ubiquitination, since they can specifically recognize different substrates. The large number of proteins regulated by ubiquitination demands high specificity and accounts for the growing number of discovered E3 ligases, on the other hand there are only a few E1s but at least 20 E2s known to date [112,114].

Ubiquitination affects the function and location of targeted proteins either through monoubiquitination (regulating for example endocytic processes, chromatin remodelling and budding of retroviruses from plasma membrane) or polyubiquitination (regulating proteasomal degradation) [115]. Moreover, experimental evidence has shown that the RING domain can be one of the responsible domains for E3 ligase activity. The strong connection between the RING domain and ubiquitination has led to the proposal that the TRIM/RBCC family represents a subclass of RING finger E3 ligases [89]. This is supported by evidence that some of the family members do possess E3 ubiquitin ligase activity. For example TRIM25/EFP has been shown to regulate the protein levels of the cell cycle regulator 14-3-3-σ [116]. Moreover TRIM33/Ecto can modulate the TGFβ signaling pathway by ubiquitination of Smad4 [117].

In addition, the nature of the modifier does not have to be the classical ubiquitin peptide. Lately, a growing number of ubiquitin-like (UBL) peptides have been

discovered and found to determine different cellular events, i.e. SUMO, Nedd8 and ISG15 [114].

TRIM/RBCC E3 ligases in cancer

The TRIM proteins are involved in a broad range of biological processes and are therefore, when altered, implicated in several pathological conditions. There are several TRIM members that have been associated with human disease. In many cases the pathogenic mechanism is connected to their E3 ubiquitin ligase activity.

TRIM25/EFP, as previously discussed, is predominantly expressed in various female reproductive organs, uterus, ovary and mammary glands, as well as in breast cancer, and is thought to be essential for estrogen-dependent cell proliferation and organ development [118,119]. TRIM32 is associated with skin carcinogenesis. When overexpressed in keratinocytes, TRIM32 protects cells from apoptosis induced by TNFα/UVB. Interestingly, after TNF treatment the E3 ligase activity of TRIM32 increases [120].

There are four family members, TRIM19/PML, TRIM27/RFP, TRIM24/TIF1α, TRIM33/TIF1γ, that form oncogenic fusion proteins upon chromosomal translocations in several types of malignancies [121-124]. Of these four, PML is the most known. PML is normally localized in PML nuclear bodies (NBs), but in acute promyelocytic leukemia the creation of the fusion protein PML-RARα (t15:17) disrupts the integrity of PML nuclear bodies. The important role of these subnuclear organelles in preserving a non-transformed phenotype is suggested by the fact that treatment of the leukemia and remission induction with retinoic acid restores PML nuclear bodies [125]. Interestingly, one of the components of PML NBs is TRIM27/RFP. Although the presence of TRIM27/RFP is probably through a direct interaction with PML, the functional impact of this interaction is still unknown [126].

The discovery of the interferon inducible gene TRIM22/Staf50

TRIM22 (Staf50=Stimulated Trans-acting Factor of 50 kDa) was originally identified in a screening for interferon inducible genes, where it was observed that TRIM22 mRNA and protein levels could be induced with both type I and type II IFN. In addition, when determining endogenous expression of TRIM22 in different tissues, it was shown that, in the absence of exogenous IFN treatment, TRIM22 was mainly expressed in peripheral blood leukocytes, in lymphoid tissue such as spleen and thymus, and in the ovary [102]. Further characterization of the TRIM22 expression demonstrated that the high levels

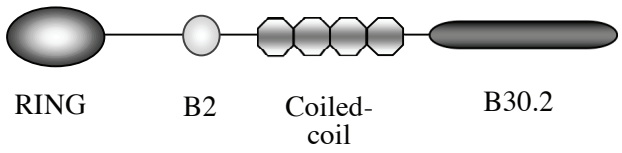


Figure 8. Schematic presentation of TRIM22 structure.

decreased during T lymphocyte activation with CD3/CD2/CD28, which could indicate antiproliferative effects for TRIM22 [94].

As will be described in this thesis, in search for novel p53 target genes, the transcription of TRIM22 was found to be upregulated in response to p53, and subsequently direct binding of p53 to the p53 response element in intron 1 of the TRIM22 sequence was identified [127]. When Wei *et al* performed a global screening of p53 binding sites in the human genome, TRIM22 was confirmed as a p53 target gene [128].

As discussed under "TRIM proteins and viral defense", recently published results have shown that overexpression of TRIM22 mediates inhibition of HIV-1 replication in monocyte-derived macrophages, which is consistent with Tissot and Mechtis discovery that TRIM22 down-regulates the transcription regulated by the LTR promotor region of human HIV-1 [101,102]. This notion is further supported by the fact that the TRIM22 mouse ortholog Rpt-1 gene is selectively expressed in resting helper/inducer T cells and has been shown to down-regulate gene expression directed by the HIV-1 promoter [129].

Hence, increasing evidence suggest a role for TRIM22 in viral defense. In sections to follow, the role of TRIM22 at the crossroads of p53 and IFN signaling pathways will be further investigated.

Negative and positive feedback regulation of p53

In response to potentially transforming stimuli, levels of active p53 quickly rise, providing a rapid protection against transformation. On the other hand, inadequate activation of p53 could have deleterious effects on a cell, with the inappropriate induction of apoptosis. Therefore, the levels of active p53 are tightly regulated.

So far, seven negative and three positive feedback loops in the p53 pathway have been identified. Molecules responsible for down modulation of p53 activity are Mdm2, COP1, Pirh2, cyclinG, Wip1, Siah1 and ΔNp73, while molecules

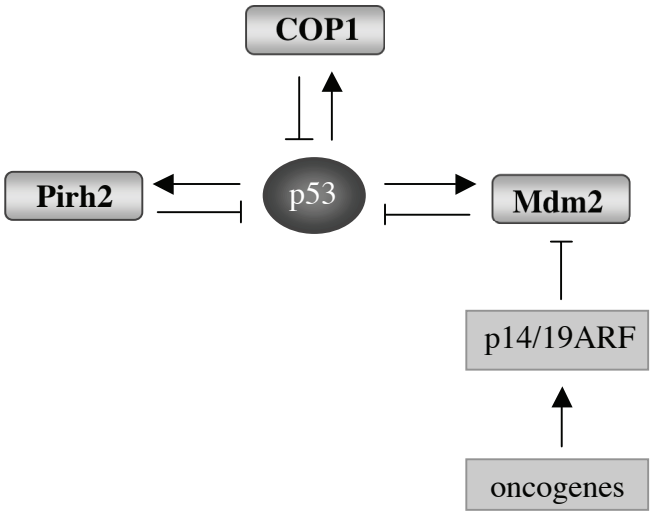


Figure 9. Control of p53 levels by p53 activated ubiquitin ligases.
p53 target gene ubiquitin ligases (bold) act by negative feedback loops and thereby controlling p53 levels. Activation of oncogenes (e.g. Myc) increases levels of p14/p19ARF, which suppresses mdm2 levels, leading to increased p53 levels.

stimulating it are PTEN-AKT, p14/p19ARF and Rb [22]. Mdm2, Pirh2 and COP1 are three ubiquitin E3 ligases transcriptionally activated by p53 that have been shown to regulate p53 levels by ubiquitination and degradation (Figure 9)[130,131].

Mdm2 is the most well characterized negative regulator of p53. Mdm2 can either bind to p53 where it interferes with the ability of p53 to transactivate target genes, or most importantly, the ubiquitin ligase activity of mdm2 contributes to p53 degradation [132-134]. The levels of mdm2 can be regulated by p14/19ARF which binds to Mdm2 and in this manner inhibit the ubiquitin ligase activity of Mdm2, thus increasing p53 levels (Figure 9)[135]. In turn, the levels of p14/p19ARF can be regulated by oncogenes (eg, Myc or Ras) [136].

Howcome there are so many feedback loops in the p53 pathway ? One answer could be that they are not all activated at the same time, i.e. different feedback loops are functional in different cells, depending on development and tissue type. Another answer might be that p53 needs many networks. If one safety net is disrupted another one takes over and prevents uncontrolled p53 activity, which would be devastating for the cell.

THE PRESENT INVESTIGATION

General aim

To understand the mechanisms for the p53 phenotype by identification and characterization of novel p53 target genes.

Tools/Method

- To identify novel p53 target genes in myeloid cells, in order to find candidate genes responsible for p53 differentiation effects, by cDNA microarray analysis in the monoblastic cell line U-937-4, expressing a temperature inducible-form of p53.

- To understand the role of the p53 target gene TRIM22 during hematopoietic maturation and in T lymphocyte activation by studying the levels of TRIM22 in different stages of hematopoietic differentiation and T lymphocyte activation.

- To investigate the consequences of TRIM22 overexpression on the viability of cells with and without expression of wild-type p53.

- To study the subcellular distribution of TRIM22 and its relationship to p53 and the interferon-inducible TRIM protein PML.

EXPERIMENTAL CONSIDERATIONS

The general principles of the most important methods used in this thesis are briefly discussed as well as their significance, advantages and disadvantages. For more detailed descriptions of the methods used, see Papers I - IV.

Cell lines versus primary cells

A cell line is a defined population of cells that has been immortalized and is maintained in culture for extended periods of time. Cell lines have usually undergone a spontaneous process of transformation, conferring an unlimited culture lifespan on the cells. The transformation process is a disadvantage compared to primary cells, since the cells might change characteristics. For that reason, an advantage with primary cells is that the experiments are performed in a more authentic environment. Nevertheless, with primary cells, the limited access of cells is a major disadvantage, while working with cell lines, large amounts of cells can be obtained. Primary cells can also be more difficult to transfect as compared to cell lines, the former showing lower transfection efficiency and higher amount of cell death. The low transfection efficiency was the limiting factor in *Paper II*, when we attempted to overexpress TRIM22 in human bone marrow cells.

Overexpression of TRIM22 in cell lines

Experimental systems in which a specific gene is overexpressed are artificial but can nevertheless indicate a function of the gene and are therefore a common way to study cellular functions. A plasmid containing the cDNA and a strong promoter is usually transfected to the cells. The protein is often produced at abnormally high levels compared to the physiological levels, which is an aspect that has to be considered when interpreting the results. For example, when overexpressing a gene, high expression levels are achieved, which could cause unspecific DNA-binding to sequences it normally would not bind to.

Tagging of TRIM22

Due to lack of a specific TRIM22 antibody, in *Paper III* and *IV*, TRIM22 was tagged and overexpressed. The experiments were performed with an expression plasmid encoding TRIM22 tagged with a V5-epitope or with GFP. Since the GFP sequence is exceptionally long compared to the TRIM22 sequence and could give rise to effects not caused by TRIM22, e.g. deviant localization or

abnormal clusters, parallel experiments with TRIM22-V5 and GFP-TRIM22 were performed to confirm that the fusion proteins behaved similarly.

Retroviral Transduction

Use of retroviral packaging cell lines is an additional option to transfect cells and study overexpression of a protein, usually less toxic to the cells than use of transfection agents or electroporation. However, it has not been possible to establish retroviral packaging cell lines for retroviral transfer of TRIM22, consistent with recently published results showing that TRIM22 inhibits retroviral replication in human monocyte-derived macrophages [101]. Neither has it been possible to establish stable cell clones because of extensive cell death. A probable cause could be the observed cytotoxic/antiproliferative effects of TRIM22 (*Paper I and IV*) that would contribute to the difficulties in establishing stable cell clones.

Inducible expression

To attain a permanent (constitutive) expression of TRIM22, the GeneSwitch™ System from Invitrogen was utilized, using a mifepristone-inducible mammalian expression system. The human kidney cell line 293 was used together with the GeneSwitch™ System when trying to discover a TRIM22 phenotype. Regrettably, no obvious phenotype was detected although TRIM22 showed elevated levels on both mRNA and protein level, indicating that TRIM22 might show tissue-specific functions.

p53 cell models

Temperature-inducible tsp53

When performing the microarray in search for novel p53 target genes in *Paper I* the human monoblastic cell line U937-4 and the subclone U937-4/tsp53/A2 were utilized. U937-4 cells lack endogenous p53 expression, while its subclone U937-4/tsp53/A2 is expressing a temperature sensitive mouse p53 mutant (pLTRp53cGval135;tsp53) [137]. The p53 gene adopts an inactive conformation at 37°C, but at 32°C the tsp53 protein adopts an active conformation and can mediate p53 effects. The tsp53 contains valine instead of alanine at position 135 in the DNA-binding domain, as previously described [138,139]. When working with the cell line one should not forget to question weather the tsp53 protein is truly comparable with wt p53, in particular since tsp53 is a murine p53 in a human cell line. Nonetheless, when shifting the cells from 37°C to 32°C, sequence

specific DNA binding is restored and transactivation of all p53 target genes occurs, mediating p53 effects, i.e. proliferation arrest and apoptosis [138,140].

Transient overexpression in SAOS2 and U2OS

The human osteosarcoma cell lines U2OS and SAOS2 are frequently used together as a model system when studying p53 effects, since U2OS expresses wild-type p53 protein and SAOS2 lacks expression of endogenous p53 [141]. The endogenous levels of p53 in U2OS are quite low but can be increased by exposing the cells to DNA damage inducing agents like etoposide (a topoisomeras II inhibitor) [142]. In *Paper IV*, SAOS2 cells were used to study the effect of transiently overexpressed p53. Plasmid amount was optimized to obtain the lowest concentration mediating an effect. This was performed to prevent unspecific toxicity to the cells and to prevent effects obtained from abnormally high exogenous overexpressed levels, far above the levels that would be expressed endogenously.

Flow Cytometric Analysis and Cell Sorting

Flow cytometry may be defined as a technology to study individual cells in liquid suspension. Different properties of cells can be measured by fluorescent labeling of markers of the cells. The advantage with flow cytometry and cell sorting is the ability to handle very few cells for analysis, since cell number often is a limiting factor. The technique was used in *Paper II* and *III* to isolate small populations of interest from human bone marrow or peripheral blood. As few as 500 cells were sorted and TRIM22 mRNA expression analyzed with RealtimePCR.

RealtimePCR

Realtime Polymerase Chain Reaction is the ability to observe and collect data throughout the PCR process as it occurs, in contrast to an ordinary PCR that measures only the amount of accumulated PCR product at the end of the PCR. There are two developed types of chemistries commonly used to detect PCR products; TaqMan and SYBR Green. TaqMan probes, that were utilized in *Paper II* and *III*, use a fluorogenic probe to enable the detection of a specific PCR product as it accumulates throughout the PCR cycles. The most important difference between TaqMan and SYBR Green is that the SYBR Green will detect all double-stranded DNA, including non-specific reaction products. A well-optimized reaction is therefore essential for accurate results. An advantage with TaqMan is that a specific hybridization between probe and target is required in order to produce a fluorescent signal. The probes utilized can be

labeled with different distinguishable reporter dyes, which allows amplification of two different distinct sequences in one tube [143].

Analysis of RealtimePCR

When determining the results of the RealtimePCR, either absolute or relative quantification can be used. The absolute quantification assay is used to determine unknown samples by interpolating their quantity from a standard curve. In *Paper II* and *III* relative quantification was used where changes in gene expression is analyzed from a given sample relative to another reference sample (e.g. untreated sample versus treated). The calculation methods used for relative quantification are standard curve method or comparative C_T method. Theoretically, all the methods give equivalent results, but when using the comparative C_T method, following aspect has to be considered; a validation experiment has to be run that shows that the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal. When this was proven for the TRIM22 probe and the endogenous control 18S, the $\triangle C_T$ calculation for the relative quantification of target without running standard curves on the same plate could be applied [144]. The endogenous control used was 18S, since it was the most stably expressed endogenous control in all cell types, compared to for example huβ_2-microglobulin (our unpublished results).

However, in some occasions (*Paper II* and *III*) it was still difficult to perform normalization according to the $\triangle\triangle C_T$ calculation because different cell populations that were compared contained unequal amounts of 18S RNA [145]. Instead many repeated experiments were done, and 18S was analyzed in all populations only to ensure presence and integrity of RNA, but normalization against 18S was excluded. Because of the differences no internal control was used, instead the experiment relied on that RNA was extracted from equal amounts of cells, i.e. 500.

Determination of transcription start

For investigating a gene at the level of transcription it is essential to identify its promoter. The promoter includes DNA elements in the proximity of the transcription start site that can determine activation or repression of transcription. In order to identify the promoter region one has to establish the identity of the transcription start site, which is determined by identifying the 5´end of the encoded mRNA. Therefore it is of importance to isolate cDNA containing the entire 5´ untranslated region. The transcription start site, for mammalian cDNAs, is usually located 50-200 bp upstream of the translation initiation site. There are four techniques that are frequently used for

transcription start identification; S1 nuclease method, primer extension, RNase protection and RACE (rapid amplification of cDNA ends) [146].

I have used the 5´-RACE technique in order to establish the transcription start site. The advantage with the technique is that it is highly sensitive, since it is the only technique based on PCR amplification. The disadvantage is that the PCR can amplify products that do not represent the entire 5´ends of the transcript, since the reverse transcriptase might have terminated before reaching the 5´ends of the transcript. Because these products are shorter, they have a preference for being amplified, which is why it is of importance to continue working with the longest product obtained and not the product mostly amplified. Another disadvantage with the technique, as mentioned previously, is the degradation of mRNA.

In the RLM RACE kit the 5´-cap structure is used to separate intact mRNA from degraded mRNA. In order to minimize the possibility of degraded mRNA, the mRNA was isolated from two different cell lines (K-562 and U-937-4) (*Paper I*). In addition, the mRNA utilized in the RACE experiments was isolated with two different techniques (TRIZOL and RNeasy Mini) in order to avoid mRNA degradation. By sequencing many clones, the location of the potential transcription start could be derived. To confirm that the correct transcription start site has been identified, further experiments, (i.e. reporter assays), has to be made to establish that the surrounding DNA contains a functional promoter.

Luciferase reporter assay

In order to functionally characterize the identified p53 response element in intron 1 of the Staf50 gene and the putative Staf50 promoter, luciferase-reporter transfections were performed. The luciferase reporter gene is one of the most commonly used reporter genes and was used in *Paper I* and *IV*.

The luciferase reporter gene originates from the American firefly *Photinus pyralis* and encodes an enzyme that oxidizes firefly luciferin in the presence of ATP, oxygen and Mg^{2+}, resulting in a fluorescent product (560 nm) that can be quantified by measuring the released light. One major drawback with the model system is that the measurement of light should be initiated directly in front of the light detector using an injection system, since light emission is very rapid [147]. In our experiments we used a luminometer and manually injected the cell lysis, but to solve this problem, coenzyme A was included in our reactions, which enhances the sensitivity of the assay giving a sustained light emission. An advantage of using a reporter gene instead of the gene that is normally regulated by the promoter is that the reporter gene eliminates problems in distinguishing transcripts derived from the transfected plasmid and the endogenous gene [146].

In transient expression assays, an internal control (*Renilla*), is usually co-transfected. *Renilla* is used to normalize experimental variations in transfection data caused by changes in cell density and viability, cell lysis and the recovery of samples at various stages of the experiment [148]. However, previously published results and our own experience show that p53 affects *Renilla* expression, therefore in the reporter assays performed in U937-4 and U937-4/tsp53/A2 in *Paper I*, *Renilla* was not utilized [148]. Nonetheless, in *Paper IV*, the reporter assays executed on overexpressed p53 in SAOS2 showed no p53-related effects on *Renilla*. It is possible that the low levels of p53 plasmid utilized explains absence of p53-related effects. Therefore, *Renilla* was in this case used for normalization.

EMSA versus ChIP

The two main techniques for studying protein:DNA interactions at promoter level are the electrophoretic mobility shift assay (EMSA) and chromatin immuno precipitaion technique (ChIP). One main difference between the techniques is that EMSA shows binding in vitro, while ChIP demonstrates binding in vivo.

The EMSA technique is based on the fact that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide electrophoresis. When studying DNA:protein interactions by an electrophoretic assay one has the ability to resolve complexes of different conformation. The EMSA technique can therefore be used qualitatively to identify sequence-specific DNA-binding proteins (i.e. transcription factors) in crude lysates and in conjunction with mutagenesis, to identify the important binding sequence within a given genes regulatory region (*Paper I*). The principle behind the ChIP technique is that DNA bound proteins in living cells can be cross-linked to the chromatin on which they are bound. However, the major disadvantage with ChIP is the requirement for highly specific antibodies for each protein to be tested, but also that it is based on PCR amplification, resulting in a risk for false positive results.

RESULTS AND DISCUSSION

TRIM22/Staf50, yet another p53 target gene ?

Why do we search for additional yet unidentified p53 target genes ? The answer is simple, there are still many out there which can help us in the identification of the different pathways of p53. It has been shown by many that p53 is involved in differentiation [55,60]. However, the mechanisms are unknown. In order for p53 to mediate differentiation it is dependent on its transcriptional activity, which indicates that one or several target genes must be involved [61]. Therefore, the aim of this thesis was to identify novel p53 target genes involved in differentiation. As a result, the interferon inducible TRIM22 that was identified, was shown to be involved in differentiation, proliferation and cell death of leukemic cells.

Microarray results

To identify potential differentiation inducing p53-target genes, a cDNA microarray was performed in the leukemic monoblastic cell line U-937-4. p53 cannot be detected in U-937-4 cells, while U-937-4/tsp53/A2 cells express the temperature inducible p53 mutant tsp53. U-937 was chosen because when incubated at 32°C, U-937-4/tsp53/A2 cells show signs of differentiation, reflecting the differentiation inducing capacity of wild type p53 [137]. However, the usage of these cells did not secure that the target genes identified would be differentiation related. That would have to be further explored.

To identify p53 responsive genes in U-937-4/tsp53/A2 cells, a series of three different cDNA microarrays was performed. RNA extracted from U-937-4/tsp53/A2 cells incubated at 32°C and 37°C was compared (array number 1), whereby 33 potential p53 target genes were identified (Table 1). The definition of up-or downregulation was set to a factor 2; upregulated genes were defined as at least twice the fluorescence in the experimental sample as compared to the control sample, and downregulated genes as half the fluorescence in the experimental sample as compared to the control sample. Because the cDNA microarray plates consisted of 4000 genes in duplicate, only genes that were found to be up-or down regulated on both of the gene settings were chosen. To assure that the identified genes were not induced by a temperature shift from 37°C to 32°C, a second cDNA microarray was performed, comparing mRNA from U-937-4 wt cells incubated at 32°C, to mRNA from U-937-4/wt cells incubated at 37°C (array number 2). By these means, 24 temperature inducible genes were identified. 3 of these were identical to the genes found in array number 1, and were therefore excluded from the study. To verify the results from array number 1, mRNA from U-937-4/tsp53/A2 cells incubated at 32°C was compared to mRNA from U-937-4 wt cells incubated at 32°C (array

number 3). This array resulted in 45 genes, out of which 14 were identical to the potential p53 response genes found in array number 1 (Table 1).

Which potential target gene to choose ?

Thus, 14 genes up- or downregulated more than twice in response to wild type p53 activity remained after a serie of arrays. Out of these, the three well-known p53 target genes, APO-1, Mdm2, and TRAIL receptor were upregulated 2.5, 4.0, 3.3 times respectively [149-151]. The remaining 11 potential p53 target genes were investigated by northern blot analysis utilizing RNA from U-937-4 wt cells and U-937-4/tsp53/A2 cells incubated at 37°C or 32°C (*Paper I*). However, all but two turned out to be false positive, namely TRIM22/Staf50 and NF-E2. TRIM22 was up-regulated while NF-E2 down-regulated. At that time TRIM22 had been shown to be interferon inducible and to have antiviral properties, while NF-E2 was an erythroid transcription factor [102,152]. Both were very attractive for further characterization, however since it is more difficult to verify a novel direct p53 target gene that is negatively regulated from low levels, TRIM22 was chosen.

The characterization of TRIM22 expression was continued with RACE, which identified the transcription start of TRIM22, making it possible to obtain the TRIM22 promoter for reporter assays, and thereby verify the promoter activity of TRIM22. The binding of p53 to the p53 response element in TRIM22 sequence was confirmed with EMSA, showing TRIM22 as a novel direct p53 target gene.

Table 1. *p53-target genes identified by cDNA microarray in U-937-4/tsp53/A2 cells. Genes were chosen on the basis of their expression level. Upregulated genes were set to a factor of at least 2 (i.e. twice the flourescense in the experimental sample compared to the control sample) and downregulation of genes to 0.5 (i.e. half the fluorescence in the experimental sample as compared to the control sample).*

Microarray plate (experimental – control)			***Number of up- or down regulated genes***
Nr. 1	32°C tsp – 37°C tsp	p53 regulated genes	33
Nr. 2	32°C wt – 37°C wt	temperature effect	24
Nr. 3	32°C tsp – 32°C wt	verification of nr. 1	45
potential p53 target genes			**14**

The first clues to the involvement of TRIM22 in differentiation

TRIM22 shows ATRA specificity

Since the aim was to identify differentiation related target genes, TRIM22 expression was analyzed in different leukemic cell lines that had been stimulated with various differentiation inducing agents (*Paper I*). Interestingly, TRIM22 mRNA levels were increased in NB4 and HL60 cells stimulated with ATRA [153]. By adding VitaminD3 to the cells, TRIM22 expression was however not affected. K562 cells treated with PMA (phorbol 12-myristate 13-acetate), showed also no change in TRIM22 expression, neither did U-937-4 cells treated with ATRA or VitaminD3 [154-156]. An explanation for the ATRA mediated induction of TRIM22 expression could be that ATRA stimulates IFN production in cells and increased IFN levels could therefore contribute to the increased expression of the interferon inducible TRIM22 [157]. In spite of this, the positive correlation between TRIM22 expression and ATRA induced differentiation of myeloid cell lines could suggest that TRIM22 might facilitate ATRA induced granulocytic/monocytic differentiation. Interestingly, RFN8, another RING finger protein, can interact with and enhance the transcription-stimulating activity from the retinoic X receptor [158]. We therefore hypothesize that TRIM22 potentiates the ATRA response via the retinoic acid receptor.

TRIM22 is down regulated in mature cells

The observed effects in the promyelocytic cell lines NB4 and HL60 prompted us to investigate TRIM22 expression in normal hematopoiesis. Therefore, CD34+ cells from human bone marrow was isolated and cultured towards erythroid and granulocytic differentiation. TRIM22 mRNA and protein levels decreased during differentiation in both lineages, however with a biphasic pattern in granulocytic differentiation and a more pronounced decrease in erythroid differentiation. The marked decrease during erythroid culture was confirmed when isolating different human bone marrow populations, since in the erythroid nucleated cells, TRIM22 expression was undetectable (*Paper II*). Nucleated cells were utilized because they still contain RNA, contrary to mature erythrocytes that lack nucleus, suggesting that the absence of TRIM22 expression depends on something else than complete lack of RNA [1,159]. Presence of RNA was verified by using the endogenous control 18S, which was always included in the experiments.

These results show intriguing resemblance with another TRIM protein, HERF1/TRIM10, one of several TRIM proteins involved in differentiation. As mentioned in the introduction, HERF1 (Hematopoietic RING finger 1) is necessary for terminal erythroid differentiation. If HERF1 expression is inhibited, terminal erythroid differentiation is blocked [103]. By analogy, the strong

down regulation of TRIM22 expression that was observed in *Paper II* is hypothetically required for terminal erythroid differentiation. In order to see if this hypothesis was correct, i.e. that TRIM22 could inhibit erythroid differentiation, experiments were done trying to overexpress TRIM22 in CD34+ cells. Due to technical difficulties it was not successful. Attempts were done with both electroporation (AMAXA) and retroviral transfection. However, recent results showed that TRIM22 have the ability to inhibit retroviral replication, explaining our problems with viral infections [101].

T lymphocytes

Broadly outlined, T lymphocytes can be divided into CD4+ and CD8+ T lymphocytes. CD4+ T lymphocytes have a specialized function in cytokine production in order to activate CD8+ T lymphocytes, B lymphocytes and APC (antigen presenting cells), while CD8+ T lymphocytes act together with natural killer cells (NK cells) as professional killers of target cells [160-162]. While the surface receptor CD28 is expressed on all CD4+ T lymphocytes, only 50% of CD8+ T lymphocytes express CD28. Moreover, CD28 expression is constitutive, but can be increased upon T lymphocyte stimulation [163,164]. CD4+ T lymphocytes produce large amounts of cytokines, for example IL2, TNFβ and IFNγ, in contrast to CD8+ T lymphocytes, which produce only low amounts of cytokines [165,166].

TRIM22 does not regulate CD25 (IL2Rα) levels

T cell activation is characterized by IL2 production, cell cycle progression and the expression of cytokine receptors as well as anti-apoptotic molecules. The IL2 production is directed through CD28 signaling via induction of NF-kB. The IL2 promoter contains one NF-kB site [167]. Expression of CD25 (IL2Rα) is repressed by Rpt1, a mouse ortholog to TRIM22 [129]. This led us to examine if TRIM22 possessed the same capabilities (Figure 10). To that end, the human T lymphoblast cell line Jurkat was transfected with TRIM22 and endogenous CD25 expression was induced with CD28. However, in contrast to our expectations, TRIM22 expression did not repress CD25 expression, suggesting that TRIM22 does not act as its mouse ortholog, Rpt1, at least not in Jurkat cells.

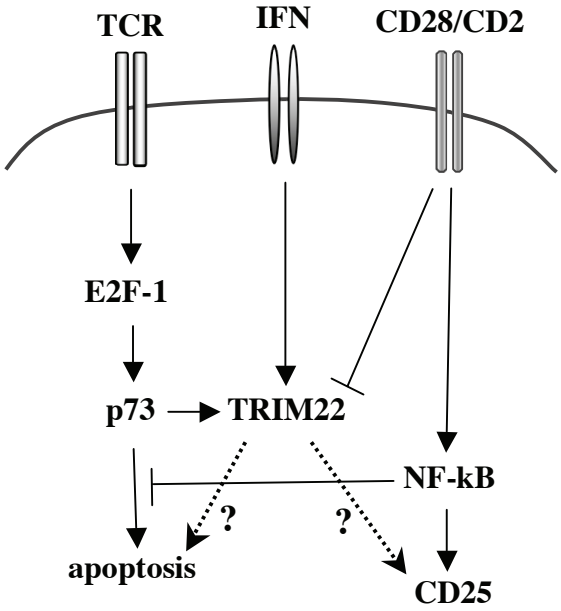


Figure 10. Hypothetical relationship between TRIM22, p73, NF-kB and CD25 during T-cell activation.
TRIM22 levels are regulated by IFN and CD2/CD28, and we speculate that TRIM22 could induce apoptosis or affect CD25 levels. The T cell receptor (TCR) induces apoptosis via p73 and E2F-1, which can be inhibited by NF-kB.

p73 induced cell death in T lymphocytes

Strong stimulation of the T cell receptor (TCR) on cycling peripheral T lymphocytes causes apoptosis, a process called TCR-activation induced cell death (TCR-AICD) [168-170]. T lymphocytes undergoing TCR-AICD show p73 expression. TCR-AICD is dependent on both E2F-1 and p73 activity (Figure 10) [171]. We have shown that TRIM22 is transcribed by p73 and that overexpression of TRIM22 mediates cell death (*Paper I and IV*). The cell death of TRIM22 will be discussed in detail below. The induced cell death by p73 can be inhibited by activation of NF-kB, which is essential for survival of mature T lymphocytes after activation (Figure 10) [172]. Gongora *et al* have briefly explored TRIM22 levels in T lymphocytes activated by CD2/CD28 and showed that TRIM22 expression was down regulated [94]. All these results combined made us hypothesize that the observed down regulation of TRIM22 mediated by CD2/CD28 could depend on that the cells should escape the observed cell death in *Paper IV*.

TRIM22 response depends on T lymphocyte activation

The observations made by Gongora prompted us to examine the TRIM22 expression during different stages of T lymphocyte activation [94]. The results obtained in *Paper III*, are partially divergent from the results by Gongora since down regulation of TRIM22 by CD2/CD28 was only seen in PBMCs and not in the isolated T lymphocyte populations, i.e. CD4+ and CD8+ T lymphocytes and NK cells. This could depend on the different cytokines produced by the PBMCs, suggesting a paracrine crosstalk between T lymphocyte populations. Furthermore, TRIM22 expression responded different to different stimuli; activation with IL2 and IL15 increased TRIM22 expression in PBMC, although only a modest increase in sorted populations. The data indicate that TRIM22 expression varies depending on the stage of T lymphocyte activation since we and others have shown that IL2 mediates partial T lymphocyte activation and CD2/CD28 complete. The terms partial and initial was based on the experiments with IL2, IL15 and CD2/CD28 on PBMCs with Celltiter test and CD25 expression (*Paper III*). CD25 is a T lymphocyte activation marker and showed highest expression levels in PBMCs cultured with CD2/CD28 and lowest with IL2, the same results were obtained with the Celltiter test which indicates cell proliferation.

Potential TRIM22 functions

Involvement of TRIM22 in proliferation arrest and cell death

TRIM22 was down regulated during T lymphocyte activation and it has been shown that many other genes involved in cell death and proliferation arrest are down regulated during T lymphocyte activation. Furthermore, apoptosis or cell cycle arrest are common functions for several p53 target genes [27,32]. We therefore examined involvement of TRIM22 in proliferation and survival of the cells. U937-4 wt cells, that lack endogenous p53, were electroporated with TRIM22, seeded on soft agar and colonies counted. As seen in *Paper I*, TRIM22 mediated reduced clonogenic growth, even more potent than $p21^{WAF1}$. The reduction in colonies suggested that proliferation arrest or apoptosis had occurred.

To discriminate between the two effects, cell cycle analysis was done by transfecting TRIM22 in the osteosarcoma cell lines U2OS (wt p53) and SAOS2 (p53 null). However, due to techninical difficulties, we had to abandon this technique.

Instead U2OS, SAOS2 and MAGI-CCR-5 cells were transfected with GFP-TRIM22 and viability of green cells was analysed with DAPI staining by FACS (*Paper IV*). Dead cells are permeable for DAPI staining, while live cells are not. SAOS2 (null p53), U2OS (wt p53) and MAGI-CCR-5 (wt p53) cells were

transfected and FACS analysis showed increased cell death in all cell lines in response to TRIM22, independently of p53 status. These results confirmed the results from the colony assay, which indicated proliferation arrest or apoptosis. However, the FACS analysis of DAPI does not discriminate between apoptosis and necrosis. Therefore, more experiments must be performed to distinguish between apoptosis and necrosis.

A broad spectrum caspase inhibitor (z.vad.fmk) was added to the DAPI experiments in order to see if the observed cell death was in fact apoptosis mediated by caspase cleavage. If so, there would be a decrease in cell death since the caspases would be inhibited and thereby unable to signal for apoptosis. Paradoxically, when measuring the cell death by DAPI stained green cells, an increased cell death was detected after treatment with z.vad.fmk. This phenomenon shows similarities with PML overexpression, which induces cell death in the absence of typical features of apoptosis, and when adding z.vad.fmk, apoptosis is rather enhanced than blocked. This suggests that at least when overexpressed, PML triggers apoptosis in a caspase independent fashion[173]. Maybe TRIM22 follows the same pattern for inducing cell death ?

However, at the same time morphology studies on TRIM22 was carried out revealing that TRIM22 had a tendency to form aggresomes. Aggresomes are defined as cytoplasmic accumulation of aggregation-prone misfolded proteins. The aggresome formation is a process that occurs when the capacity of the proteasome is exceeded by the misfolded proteins [174]. The cell usually deals with misfolded proteins by autophagic degradation. The tendency for aggresome formation has been shown for other TRIM proteins as well, TRIM19 and TRIM37 [175,176]. Therefore a plasmid titration was made in order to identify the lowest possible plasmid concentration mediating an effect but without formation of aggresomes.

According to our findings, the plasmid concentration could be a 100 times lower than recommended by the manufacturer of the transfection reagent, but still mediating a pronounced cell death. The aggresomes that arose could still be a problem. The strong cell death inducing effect in combination with the absence of aggresomes in experiments performed with very low amounts of transfected TRIM22, strongly suggest that the observed TRIM22 related effects depend on a specific function of TRIM22 protein, and not an unspecific effect of cellular deposits of misfolded protein.

But, if we observed aggresomes with TRIM22 transfection and do not believe in the results obtained with the highest concentration, how come we detected an effect in the colony assay in *Paper I* ? In the colony assay, $p21^{WAF1}$ was used as a positive control and behaved accordingly. This could depend on the fact that $p21^{WAF1}$ is not a TRIM protein and does not have a tendency to form aggresomes

like the TRIM proteins. Also a different technique was used to overexpress TRIM22, namely electroporation with lower transfection efficiency.

The partnership between PML and TRIM22

The similarity between TRIM22 and PML is remarkable. They are both interferon inducible and controlled by p53. Both have a p53 response element in intron 1 in their gene sequence, are involved in induction of cell death and viral defense and both are TRIM proteins. p53 and TRIM27 can be recruited to PML NBs and we therefore examined the possibility if TRIM22 could be directed to PML NBs [177,178].

The fibrosarcoma cell line HT1080 expressing endogenous PML was utilized in co-localisation experiments and transfected with TRIM22. As seen in *Paper IV*, TRIM22 was located at the same place as PML, however to ascertain that TRIM22 and PML were located in the NBs, further experiments must be done. NBs can be defined as nuclear structures that contain SUMOylated PML and increases in size and number after IFN treatment. A cell contains usually 10-30 NB per nucleus with diameters between 0.2-1.0 μM [177]. So far the criteria fulfilling the demands of size and amount of NBs match for PML and TRIM22. Nevertheless, the co-localization of PML and TRIM22 has to be confirmed in other cell lines. The co-localization of TRIM22 and PML will be studied in the presence and absence of activated p53. Co-immunoprecipitation experiments will be performed to see if PML and TRIM22 are able to bind to each other.

Furthermore, co-localisation experiments between TRIM22 and p53 was carried out, but TRIM22 was not found to co-localize with p53, neither endogenous nor exogenous. Nevertheless, when overexpressing TRIM22 in U2OS cells and performing a co-immunoprecipitation between endogenous p53 and over-expressed TRIM22, a small fraction of TRIM22 was found bound to endogenous p53. When repeating the experiment in SAOS2 with overexpressed p53 and TRIM22, they were not co-precipitated, suggesting that perhaps a small fraction of TRIM22 is available for p53 to be bound, maybe depending on the activation status of p53.

The UBL capabilities of TRIM22

As mentioned above, PML localizes in discrete nuclear speckles along with other proteins, structures that are called PODs (PML oncogenic domains), NB (nuclear body), Kremer bodies or ND10 [125]. So far more than 40 proteins involved in cellular processes such as apoptosis, DNA damage response, viral defense and transcriptional regulation have been found to co-localize with PML in the PML NBs [179,180]. However, modification of PML by the ubiquitin like protein (UBL)

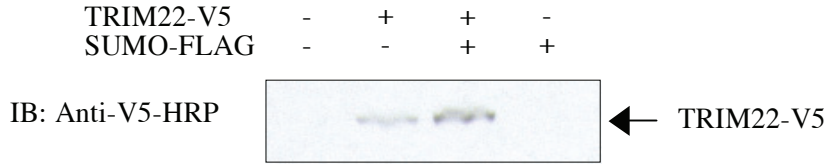


Figure 11. SUMO presence may stabilize TRIM22 expression. *293T/17 cells were transfected with TRIM22-V5 and SUMO-FLAG, and after 48 hours TRIM22 expression was analysed by Western blot using the V5-HRP conjugated antibody.*

SUMO is essential for proper formation of the PML-NBs [177]. PML contains a SUMO binding motif that is independent of its SUMOylation sites and is necessary for PML-NB formation [181].

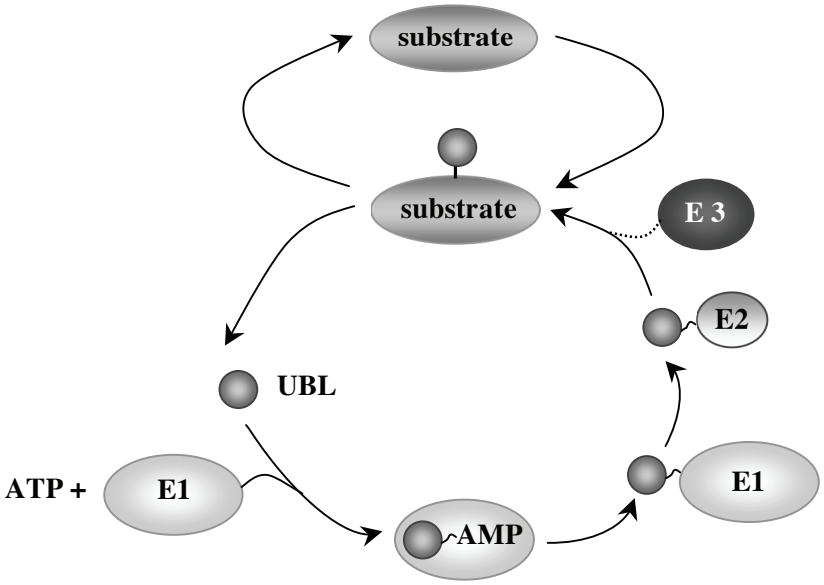
Furthermore, the PML RING domain is critical for PML-NB formation and PML SUMOylation [181]. A search for SUMOylation sites on TRIM22 protein using the SUMOplot™ Prediction tool available at http://www.abgent.com/doc/sumoplot reveals that TRIM22, like PML, possesses three sumoylation sites located in similar regions on both proteins (position of the conserved K: 65, 161, 489 for PML and 6, 153, 265 for TRIM22). This data lead us to imagine that, like PML, the SUMOylation of TRIM22 can alter its cellular distribution and regulation.

TRIM22 is probably not modified by SUMO

Lately, several RING finger containing proteins have been identified as enzymes transferring a growing class of ubiquitine-like proteins (UBLs), namely ISG15 (interferon stimulated gene of 15 kDa), ubiquitin and SUMO, to different target proteins [88].

To understand the potential involvement of TRIM22 in SUMOylation, TRIM22-V5 and SUMO-FLAG was co-transfected into the epithelial kidney cell line 293T/17. Cells were cultured for 48 hours and TRIM22 was analysed with Western blot. As shown in figure 11, there was no SUMOylation of TRIM22-V5 when co-expressing TRIM22-V5 and SUMO-FLAG. However, presence of SUMO stabilized TRIM22 levels, suggesting that SUMO could antagonize degradation of TRIM22. This is comparable with PML, which is SUMOylated and expression is stabilized with SUMO. The same experiments were done but adding 1000 U/ml IFNα, no changes in TRIM22 expression was however observed compared to without IFNα.

However, detection of SUMOylated proteins is complicated since detection of the SUMOylation bands with western are usually up to 10 times weaker than the



Modified from Schwartz and Hochstrasser, TRENDS in Biochem Sciences 2003

Figure 12. ***The UBL cycle.***
The UBL (ubiquitin-like proteins) is linked to an activating enzyme (E1), passed to an conjugating enzyme (E2), that alone or together with a ligase (E3) mediates transfer of the UBL to the substrate, a process which is reversible. The UBL could be ubiquitin, SUMO, Nedd8 or ISG15.

protein to be SUMOylated (TRIM22), suggesting that SUMOylation is transient. Therefore, it was impossible to conclude whether TRIM22 was SUMOylated or not.

TRIM22 is involved in ISGylation

Given that TRIM22 is IFN-inducible, ISG15 is a particularly interesting UBL since ISG15 is one of the most strongly induced genes after IFN treatment [182]. Generally UBLs are linked to different enzymes, E1, E2 and E3 (described previously). UBLs are linked to an activating enzyme called E1, passed to an E2 conjugating enzym, which alone or in combination with an E3 ligase mediates transfer of the UBL to a substrate (Figure 12) [114]. The attachment of the UBL is reversible by specific UBL proteases. Several other p53 target genes are both E3 ligases and contain RING finger domains (mdm2, COP1) [131,132]. TRIM22 fulfils both criteria and we therefore hypothesize that TRIM22 is an E3 ligase for UBLs, thus post translationally modifing protein targets, which regulate survival, differentiation and proliferation. A characteristic feature of UBL E3 ligases is the transfer of UBL to substrates but also to itself [88].

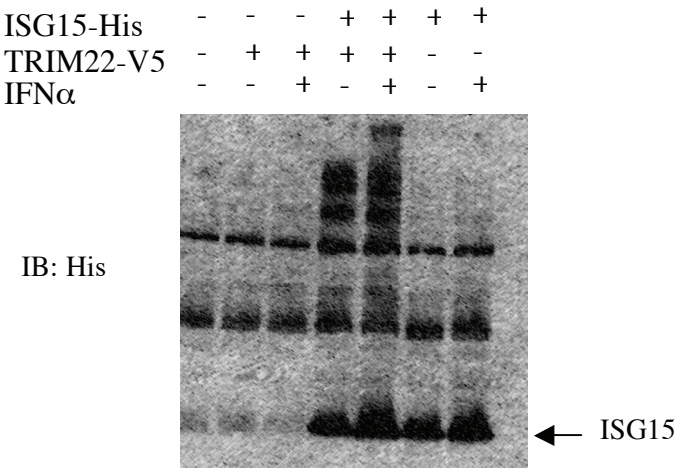
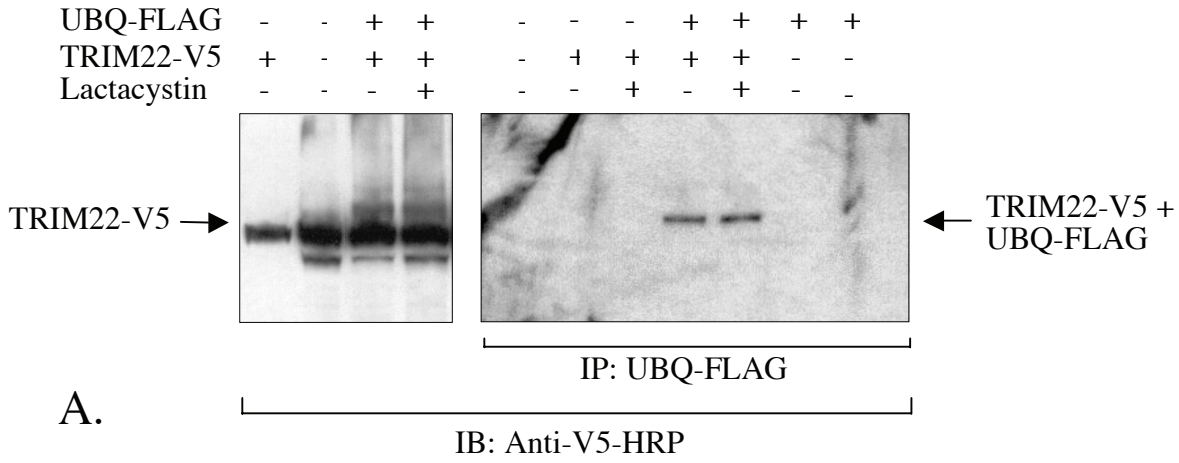


Figure 13. TRIM22 induces ISGylation in 293T/17 cells.
293T/17 cells were transiently transfected with ISG15-His and TRIM22-V5. After 48 hours ISGylation was analysed by immunoblotting with His-antibody. IFNα (1000 U/ml) was added to culture the last 24 hours.

293T/17 cells were transfected with TRIM22-V5 and ISG15-His and after 48 hours, ISGylation was estimated by direct Western blot with His-antibody. As shown in figure 13, ISG15 is detectable in ISG15 transfected cells, and cells transfected with ISG15 and TRIM22 showed an ISGylation ladder, indicating that TRIM22 indeed has the ability to induce ISGylation. We therefore hypothesize that TRIM22 might be an ISG15 ligase, post-transcriptionally modifying protein targets involved in immunomodulation or differentiation.

TRIM22 is ubiquitinated

Previous work has shown that RING-fingers are involved in ubiquitination [88]. To investigate this, V5-tagged TRIM22 was transfected into 293T/17 cells alone and together with FLAG-tagged ubiquitin and analyzed with Western blot. Cells were cultured for 48 hours and in the presence of the proteasome inhibitor lactacystine for 5 hours before harvest of cells, in order to prevent degradation of proteins. As shown in figure 14 (left), TRIM22 is mono-ubiquitinated, or possibly di-ubiquitinated in the presence of overexpressed ubiquitin. The mono-ubiquitination suggests that TRIM22 could be involved in histone regulation, endocytosis and the budding of retroviruses from the plasma membrane [115]. However, it cannot be ruled out that TRIM22 could be polyubiquitinated, since multi-ubiquitin chains may be degraded by the proteasome and therefore not visible [115].



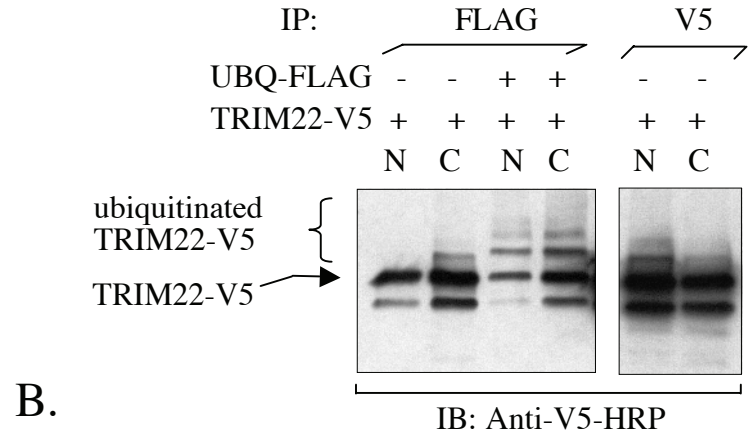


Figure 14. Ubiquitination of TRIM22.
293T/17 cells were transfected with tagged expression constructs of TRIM22 and ubiquitin (UBQ). ***A.)*** *After 48 hours TRIM22 expression was analysed by Western blot* ***(left panel)*** *or TRIM22 was immunoprecipitated with anti-FLAG and analysed by Western blot analysis (****right panel****). Both Western blots were performed with V5-HRP antibody. Cells were cultured in the presence of proteasomeinhibitor lactacystine (10 µmol/L) the last 5 hours of incubation.* ***B****.) After fractionation into nuclear (N) and cytosolic (C) fractions, immunoprecipitation and western blot analysis was performed.*

To verify that TRIM22 is ubiquitinated, we performed immunoprecipitation assays. 293T/17 cells were transiently transfected with TRIM22-V5 and ubiquitin-FLAG. Whole cell extracts of the transfectants were subjected to immunoprecipitation with anti-FLAG and immunoblotted with direct conjugated anti-V5-HRP in order to avoid the heavy chains of the antibody from the immunoprecipitation (50 kDa). As shown in figure 14A (right), the co-transfected immunoprecipitates contained TRIM22 and ubiquitin, while no

protein was detected in the immunoprecipitates transfected with TRIM22 or ubiquitin alone. This indicates that overexpressed TRIM22 indeed is ubiquitinated.

The same experimental setup was also made with another proteasome inhibitor, MG132 (*data not shown*), and the results obtained were the same as in figure 14A with lactacystin, i.e. TRIM22 is ubiquitinated in the presence of overexpressed ubiquitin. The fact that TRIM22 is ubiquitinated itself is consistent with our hypothesis that TRIM22 could be an ubiquitin E3 ligase.

To identify where in the cell this process takes place, tagged TRIM22 and ubiquitin were co-transfected into 293T/17 cells and the lysate was divided into nucleus and cytoplasm fractions, immunoprecipitated and analyzed by Western blot. As shown in figure 14B, most ubiquitinated TRIM22 is present in the cytoplasm fraction. Since the RING domain is the domain that has been shown to be responsible for the ubiquitination effect in other E3 ligases, it would be interesting to delete the RING domain of the TRIM22 sequence to see if it still could be ubiquitinated.

As TRIM22 appeared to be involved in ubiquitination, p53 seemed to be a potential substrate for TRIM22 to ubiquitinate. My results suggest, however, that this was not the case. Since many E3 UBL ligases are known to either degrade or stabilize the protein levels of their substrates, p53 and TRIM22 were co-transfected in 293T/17 cells to examine p53 protein levels in the presence of TRIM22. Western blot analysis showed no effect on p53 protein levels during these experimental conditions.

All these results described in this section have helped in the understanding of the molecular function of TRIM22 and its putative role in differentiation, proliferation and apoptosis. Nevertheless, many questions are still unanswered; Does TRIM22 have to be downregulated in order for normal erythroid differentiation to proceed ? Is TRIM22 localized in the PML NBs ? Similarities to PML ? Does TRIM22 have to be recruited to the NBs in order to mediate cell death ? What are the molecular mechanisms by which TRIM22 induces cell death ? The observed cell death by TRIM22, is it apoptosis or necrosis ?

CONCLUSION

- The interferon inducible TRIM22/Staf50 is a novel p53 target gene
- Overexpression of TRIM22 mediates reduced clonogenic growth of the leukemic cell line U-937-4
- Overexpression of TRIM22 in SAOS2 induces cell death independently of p53
- Endogenous TRIM22 is upregulated in response to ATRA-mediated differentiation of the promyelocytic cell lines NB4 and HL60
- During normal hematopoiesis TRIM22 expression is down regulated during maturation of most lineages, most pronounced in the erythroid lineage.
- In activated T lymphocyte populations TRIM22 could pertain activation-stage specific roles connected to paracrine crosstalk
- Contrary to the mouse ortholog of TRIM22, i.e. Rpt1, TRIM22 does not affect levels of CD25 (IL-2Rα) in Jurkat cells
- Overexpressed TRIM22 co-localizes with endogenous TRIM19/PML, but not with endogenous p53
- TRIM22 can be conjugated with ubiquitin or ISG15

Populärvetenskaplig sammanfattning på svenska

(summary in Swedish)

Skulle p53-målgenen TRIM22 kunna skydda mot cancer uppkomst ?

Frisk blodbildning

En frisk vuxen människa har mellan fyra och sex liter blod, beroende på individens kroppsstorlek. Blodet produceras i benmärgen som finns i våra lårben och höftben. Varje sekund bildas normalt i benmärgen tre till fyra miljoner blodkroppar från våra stamceller. Blodkropparna är specialiserade celler som har olika funktion. De röda blodkropparna (*erytrocyterna*) sköter syretransporten, medan blodplättarna (*trombocyterna*) förhindrar blödning. Det finns flera olika typer av vita blodkroppar (*leukocyter*); granulocyter, monocyter, makrofager och lymfocyter. De har alla olika funktion, storlek och form, men alla är en del av kroppens immunförsvar, som försvarar oss bland annat mot virus och bakterier. Halten av olika typer av vita blodkroppar kan mätas i ett blodprov för att t.ex. se om tecken på en inflammatorisk process finns. Vanligen har man en relativt konstant fördelning av de olika blodkropparna.

Leukemi (blodcancer)

Leukemi är samlingsnamnet för en rad olika cancertyper i blodet som innebär att blodkropparna förändrats och förökar sig okontrollerat och ansamlas i benmärgen och blodet. Det är framförallt brist på normalt fungerande blodkroppar som ger de symptom (trötthet, infektionsbenägenhet och blödningar) som förknippas med de olika typerna av leukemi. Orsakerna till leukemi är inte helt kända men faktorer som strålning eller tidigare cytostatika behandling kan påverka.

På senare år har utsikterna till bot ökat kraftigt och en utav behandlingsformerna som ibland används är interferon. Interferon produceras naturligt i kroppen som ett försvar mot infektioner. Cancerceller är olika känsliga för interferon och genom att behandla med interferon (vanligtvis i kombination med andra läkemedel) kan goda resultat uppnås. Tyvärr, så som med många andra behandlingar av cancer, är biverkningar vanliga med bland annat influensaliknade symptom, t.ex. feber, muskelvärk, men även depressioner.

Interferon-inducibla gener

Interferon-inducibla geners uttryck regleras av interferon nivåerna. Generna är mallar för proteiner, vilkas funktion är att utföra olika uppgifter i cellen och de interferon-inducibla generna ger på så sätt upphov till interferon-inducibla proteiner. Dessa proteiner kan ha till uppgift att förhindra celldelning eller ge programmerad celldöd.

I TRIM familjen finns en grupp proteiner som induceras av interferon. Deras uppgift kan vara att t.ex. orsaka programmerad celldöd eller förhindra celldelning. Till denna familj hör bl.a. TRIM22.

Cancerskyddande gener

Cancerskyddande gener (tumörsuppressor gener) är viktiga för att förhindra uppkomsten av cancer. Om de cancerskyddande generna förstörs eller förloras så ökar risken för olika cancerformer, inklusive leukemi. De cancerskyddande generna och proteinerna de kodar för har till uppgift att förhindra cancerutvecklingen, vilket kan ske genom att förhindra cancercellen att dela sig eller genom att skicka signaler till cellen att begå programmerad celldöd (*apoptos*). Möjligen skulle även vissa cancerskyddande proteiner kunna vara involverade i specialiseringen av celler (*differentiering*). p53 är en tumörsuppressor gen som skyddar mot uppkomsten av cancer och den gen som oftast är defekt i tumörceller, t.ex. vid leukemi. p53 skickar ut order till specifika målgener som utför ovan nämnda uppgifter. I dagsläget känner man inte till alla p53s målgener.

Syftet med avhandlingen

Målet med avhandlingen har varit att försöka att identifiera nya målgener till p53 och försöka kartlägga cellulära och molekylära mekanismer för dessa. Avsikten är att bidra till ökad kunskap om cancer celler, hur dessa kan styras och göras ofarliga.

Resultat

Jag har identifierat en tidigare okänd målgen till p53 som kallas TRIM22 (även kallad Staf50). I artikel I beskrivs identifieringsprocessen och fastställandet att TRIM22 står under direkt kontroll av p53. Tillsammans med resultaten från artikel IV har det visat sig att TRIM22 ger ökad celldöd, oberoende om p53 finns närvarande eller inte.

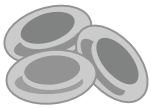
TRIM22 uttrycket i olika blodceller kartlades i artikel II, som visade att TRIM22 uttrycket överlag var lågt i de olika cellerna. I förstadierna till röda

blodkroppar var TRIM22 uttrycket inte ens detekterbart, vilket skulle kunna innebära att TRIM22 uttrycket i dessa celler måste förbli lågt för att cellerna skall kunna överleva. I artikel III fortsatte kartläggningen av TRIM22 uttrycket i blodets celler, men nu i lymfocyter, vilka ingår i vårt immunförsvar. Här visade sig TRIM22 ha en koppling till lymfocyternas aktivering, vilket innebär att TRIM22 skulle kunna ha en roll i immunförsvaret.

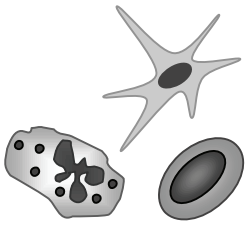
I det sista arbetet gavs det även bevis på att TRIM22 befinner sig på samma plats i cellen som en annan medlem från samma familj som TRIM22 tillhör, TRIM19 (PML). Detta ger ledtrådar till de uppgifter TRIM22 har, vilket är en viktig fråga då dess funktion till stor del är okänd.

Betydelse

Projektet bidrar med ny kunskap om de molekylära mekanismer genom vilka leukemicellerna uppvisar blockerad utmognad och ökad överlevnad. Det har visat sig att denna samlade kunskapen genom åren har lett till utvecklingen av flertalet nya läkemedel mot leukemi och cancer.



Röda Blodkroppar



Vita Blodkroppar

Trombocyter

Acknowledgements

I would like to thank all past and present people at the Department of Hematology and Transfusion Medicine of Laboratory Medicine in Lund. I wish to extend my gratitude to all those people who have given me their support and made my time as a PhD-student inspiring and adventurous.
In particular, I would like to express my sincere gratitude and appreciation to:

Urban Gullberg, my head supervisor and excellent guide in the world of science. Thank you for giving me the opportunity to be your PhD-student, and giving me the chance to learn so many different things. Your words of wisdom and advice have been greatly appreciated.

Kristina Drott, my co-supervisor, thank you for your never-ending enthusiasm and your great source of knowledge that always had the answers to my questions.

Tor Olofsson, for introducing me to the world of FACS and cell sorting and for all the help you have given me with sorted cells and differentiation markers. Last but not least, I will miss your wonderful sense of humor

May-Louise Andersson, for helping with administrative matters in such an amicable way and for always being so nice and friendly.

Karina, for being the best room colleague one can wish for and for sharing my view for sense of order.

André, for sharing this last period of headaches and cell culturing with me and for helping me with my computer when I lost control over it.

Louise E. for all support and encouragement during the last part of my thesis and for our lunches and snacks, which gave me a much needed break from work.

Anna, Kristina & Pia, thank you for your friendship throughout these years and the dinners where we could discuss our fascination and frustration with science.

Malin, my dear old friend. Thank you for your friendship that I always can count on. For all the fun we have had and have and for all our phone calls throughout the years.

Louise B. for all our "fika"-times, e-mail chats and all the great moments we have had throughout the years (including Plump and Poker). I could not wish for a better friend or neighbour.

Mamma & Pappa, ni har varit mitt största stöd genom livet och alltid uppmuntrat mig till att fortsätta med studierna och att aldrig ge upp. Tack för allt ni gett mig …….

Richard ♥, my best friend and greatest love. Thank you for always being by my side and for giving me endless support and help.

All my other friends. Thank you for being there for me and enriching my social life.

This work was supported by grants from the Swedish Cancer Society, the Swedish Research Council (No 11546), the Swedish Childhood Cancer Foundation, the Georg Danielsson Foundation, the Gunnar Nilsson Cancer Foundation, the John and Augusta Persson Foundation, the Lundberg Foundation, the Tornspiran Foundation, the Greta and Johan Kock Foundation, funding from Lund University Hospital and the Alfred Österlund Foundation.

REFERENCES

1. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood. 1993;81:2844-2853.
2. Pappenheim A. Prizipen der neuren morphologischen haematozytologie nach zytogenetischer grundlage. Folia Haematol. 1917;21:91-101.
3. Huntly BJ, Gilliland DG. Cancer biology: summing up cancer stem cells. Nature. 2005;435:1169-1170.
4. Passegue E, Jamieson CH, Ailles LE, Weissman IL. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci U S A. 2003;100 Suppl 1:11842-11849.
5. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57-70.
6. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414:105-111.
7. Rosenbauer F, Koschmieder S, Steidl U, Tenen DG. Effect of transcription-factor concentrations on leukemic stem cells. Blood. 2005;106:1519-1524.
8. Sawyers C. Targeted cancer therapy. Nature. 2004;432:294-297.
9. Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin. 2005;55:178-194.
10. Takaoka A, Hayakawa S, Yanai H, et al. Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature. 2003;424:516-523.
11. Attardi LD, Jacks T. The role of p53 in tumour suppression: lessons from mouse models. Cell Mol Life Sci. 1999;55:48-63.
12. Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev. 1996;10:1054-1072.
13. Preudhomme C, Fenaux P. The clinical significance of mutations of the P53 tumour suppressor gene in haematological malignancies. Br J Haematol. 1997;98:502-511.
14. Krug U, Ganser A, Koeffler HP. Tumor suppressor genes in normal and malignant hematopoiesis. Oncogene. 2002;21:3475-3495.
15. Harris CC. p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. Carcinogenesis. 1996;17:1187-1198.
16. Prokocimer M, Rotter V. Structure and function of p53 in normal cells and their aberrations in cancer cells: projection on the hematologic cell lineages. Blood. 1994;84:2391-2411.
17. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature. 1991;351:453-456.
18. Gronbaek K, de Nully Brown P, Moller MB, et al. Concurrent disruption of p16INK4a and the ARF-p53 pathway predicts poor prognosis in aggressive non-Hodgkin's lymphoma. Leukemia. 2000;14:1727-1735.
19. Eischen CM, Weber JD, Roussel MF, Sherr CJ, Cleveland JL. Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Dev. 1999;13:2658-2669.

20. Moller MB, Ino Y, Gerdes AM, Skjodt K, Louis DN, Pedersen NT. Aberrations of the p53 pathway components p53, MDM2 and CDKN2A appear independent in diffuse large B cell lymphoma. Leukemia. 1999;13:453-459.

21. Sherr CJ, Weber JD. The ARF/p53 pathway. Curr Opin Genet Dev. 2000;10:94-99.

22. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. Oncogene. 2005;24:2899-2908.

23. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000;408:307-310.

24. Lavin MF, Gueven N. The complexity of p53 stabilization and activation. Cell Death Differ. 2006;13:941-950.

25. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell. 1993;75:817-825.

26. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature. 1995;377:552-557.

27. Bates S, Ryan KM, Phillips AC, Vousden KH. Cell cycle arrest and DNA endoreduplication following p21Waf1/Cip1 expression. Oncogene. 1998;17:1691-1703.

28. Hermeking H, Lengauer C, Polyak K, et al. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell. 1997;1:3-11.

29. Waterman MJ, Stavridi ES, Waterman JL, Halazonetis TD. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. Nat Genet. 1998;19:175-178.

30. Wang XW, Zhan Q, Coursen JD, et al. GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci U S A. 1999;96:3706-3711.

31. Ohki R, Nemoto J, Murasawa H, et al. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. J Biol Chem. 2000;275:22627-22630.

32. Miyashita T, Krajewski S, Krajewska M, et al. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene. 1994;9:1799-1805.

33. Oda E, Ohki R, Murasawa H, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science. 2000;288:1053-1058.

34. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell. 2001;7:683-694.

35. Bossy-Wetzel E, Green DR. Apoptosis: checkpoint at the mitochondrial frontier. Mutat Res. 1999;434:243-251.

36. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. Nature. 1997;389:300-305.

37. Li PF, Dietz R, von Harsdorf R. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. Embo J. 1999;18:6027-6036.

38. Bachelder RE, Ribick MJ, Marchetti A, et al. p53 inhibits alpha 6 beta 4 integrin survival signaling by promoting the caspase 3-dependent cleavage of AKT/PKB. J Cell Biol. 1999;147:1063-1072.

39. Owen-Schaub LB, Zhang W, Cusack JC, et al. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol. 1995;15:3032-3040.

40. Wu GS, Burns TF, McDonald ER, 3rd, et al. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet. 1997;17:141-143.

41. Takimoto R, El-Deiry WS. Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. Oncogene. 2000;19:1735-1743.

42. Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science. 1998;282:290-293.

43. Lin Y, Ma W, Benchimol S. Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. Nat Genet. 2000;26:122-127.

44. Mack DH, Vartikar J, Pipas JM, Laimins LA. Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. Nature. 1993;363:281-283.

45. Zhang CC, Yang JM, White E, Murphy M, Levine A, Hait WN. The role of MAP4 expression in the sensitivity to paclitaxel and resistance to vinca alkaloids in p53 mutant cells. Oncogene. 1998;16:1617-1624.

46. Zhang CC, Yang JM, Bash-Babula J, et al. DNA damage increases sensitivity to vinca alkaloids and decreases sensitivity to taxanes through p53-dependent repression of microtubule-associated protein 4. Cancer Res. 1999;59:3663-3670.

47. Murphy M, Ahn J, Walker KK, et al. Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes Dev. 1999;13:2490-2501.

48. Koumenis C, Alarcon R, Hammond E, et al. Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. Mol Cell Biol. 2001;21:1297-1310.

49. Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature. 1994;370:220-223.

50. Haupt Y, Rowan S, Shaulian E, Vousden KH, Oren M. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev. 1995;9:2170-2183.

51. Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992;356:215-221.

52. Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. A subset of p53-deficient embryos exhibit exencephaly. Nat Genet. 1995;10:175-180.

53. Armstrong JF, Kaufman MH, Harrison DJ, Clarke AR. High-frequency developmental abnormalities in p53-deficient mice. Curr Biol. 1995;5:931-936.

54. Almog N, Rotter V. Involvement of p53 in cell differentiation and development. Biochim Biophys Acta. 1997;1333:F1-27.

55. Soddu S, Blandino G, Scardigli R, et al. Interference with p53 protein inhibits hematopoietic and muscle differentiation. J Cell Biol. 1996;134:193-204.

56. Lang D, Miknyoczki SJ, Huang L, Ruggeri BA. Stable reintroduction of wild-type P53 (MTmp53ts) causes the induction of apoptosis and neuroendocrine-like

differentiation in human ductal pancreatic carcinoma cells. Oncogene. 1998;16:1593-1602.

57. Soddu S, Blandino G, Citro G, et al. Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells. Blood. 1994;83:2230-2237.

58. Banerjee D, Lenz HJ, Schnieders B, et al. Transfection of wild-type but not mutant p53 induces early monocytic differentiation in HL60 cells and increases their sensitivity to stress. Cell Growth Differ. 1995;6:1405-1413.

59. Feinstein E, Gale RP, Reed J, Canaani E. Expression of the normal p53 gene induces differentiation of K562 cells. Oncogene. 1992;7:1853-1857.

60. Chylicki K, Ehinger M, Svedberg H, Bergh G, Olsson I, Gullberg U. p53-mediated differentiation of the erythroleukemia cell line K562. Cell Growth Differ. 2000;11:315-324.

61. Chylicki K, Ehinger M, Svedberg H, Gullberg U. Characterization of the molecular mechanisms for p53-mediated differentiation. Cell Growth Differ. 2000;11:561-571.

62. Aloni-Grinstein R, Zan-Bar I, Alboum I, Goldfinger N, Rotter V. Wild type p53 functions as a control protein in the differentiation pathway of the B-cell lineage. Oncogene. 1993;8:3297-3305.

63. Weintraub H, Hauschka S, Tapscott SJ. The MCK enhancer contains a p53 responsive element. Proc Natl Acad Sci U S A. 1991;88:4570-4571.

64. Saifudeen Z, Dipp S, El-Dahr SS. A role for p53 in terminal epithelial cell differentiation. J Clin Invest. 2002;109:1021-1030.

65. Lengner CJ, Steinman HA, Gagnon J, et al. Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. J Cell Biol. 2006;172:909-921.

66. Wang X, Kua HY, Hu Y, et al. p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. J Cell Biol. 2006;172:115-125.

67. Kaghad M, Bonnet H, Yang A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell. 1997;90:809-819.

68. Yang A, Schweitzer R, Sun D, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature. 1999;398:714-718.

69. Schmale H, Bamberger C. A novel protein with strong homology to the tumor suppressor p53. Oncogene. 1997;15:1363-1367.

70. Trink B, Okami K, Wu L, Sriuranpong V, Jen J, Sidransky D. A new human p53 homologue. Nat Med. 1998;4:747-748.

71. Senoo M, Seki N, Ohira M, et al. A second p53-related protein, p73L, with high homology to p73. Biochem Biophys Res Commun. 1998;248:603-607.

72. Zeng X, Zhu Y, Lu H. NBP is the p53 homolog p63. Carcinogenesis. 2001;22:215-219.

73. Yang A, Kaghad M, Wang Y, et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell. 1998;2:305-316.

74. Jost CA, Marin MC, Kaelin WG, Jr. p73 is a simian [correction of human] p53-related protein that can induce apoptosis. Nature. 1997;389:191-194.

75. Yang A, Walker N, Bronson R, et al. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature. 2000;404:99-103.

76. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem. 1998;67:227-264.

77. Pfeffer LM, Dinarello CA, Herberman RB, et al. Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. Cancer Res. 1998;58:2489-2499.

78. Barber GN. Host defense, viruses and apoptosis. Cell Death Differ. 2001;8:113-126.

79. Higuchi T, Hannigan GE, Malkin D, Yeger H, Williams BR. Enhancement by retinoic acid and dibutyryl cyclic adenosine 3':5'-monophosphate of the differentiation and gene expression of human neuroblastoma cells induced by interferon. Cancer Res. 1991;51:3958-3964.

80. Nason-Burchenal K, Gandini D, Botto M, et al. Interferon augments PML and PML/RAR alpha expression in normal myeloid and acute promyelocytic cells and cooperates with all-trans retinoic acid to induce maturation of a retinoid-resistant promyelocytic cell line. Blood. 1996;88:3926-3936.

81. Porta C, Hadj-Slimane R, Nejmeddine M, et al. Interferons alpha and gamma induce p53-dependent and p53-independent apoptosis, respectively. Oncogene. 2005;24:605-615.

82. Munoz-Fontela C, Angel Garcia M, Garcia-Cao I, et al. Resistance to viral infection of super p53 mice. Oncogene. 2005.

83. Teodoro JG, Branton PE. Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. J Virol. 1997;71:3620-3627.

84. Roulston A, Marcellus RC, Branton PE. Viruses and apoptosis. Annu Rev Microbiol. 1999;53:577-628.

85. Reymond A, Meroni G, Fantozzi A, et al. The tripartite motif family identifies cell compartments. Embo J. 2001;20:2140-2151.

86. Nisole S, Stoye JP, Saib A. TRIM family proteins: retroviral restriction and antiviral defence. Nat Rev Microbiol. 2005;3:799-808.

87. Borden KL. RING domains: master builders of molecular scaffolds? J Mol Biol. 2000;295:1103-1112.

88. Joazeiro CA, Weissman AM. RING finger proteins: mediators of ubiquitin ligase activity. Cell. 2000;102:549-552.

89. Meroni G, Diez-Roux G. TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases. Bioessays. 2005;27:1147-1157.

90. Short KM, Cox TC. Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. J Biol Chem. 2006;281:8970-8980.

91. Rhodes DA, de Bono B, Trowsdale J. Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence? Immunology. 2005;116:411-417.

92. Bradley JR, Pober JS. Tumor necrosis factor receptor-associated factors (TRAFs). Oncogene. 2001;20:6482-6491.

93. Lavau C, Marchio A, Fagioli M, et al. The acute promyelocytic leukaemia-associated PML gene is induced by interferon. Oncogene. 1995;11:871-876.

94. Gongora C, Tissot C, Cerdan C, Mechti N. The interferon-inducible Staf50 gene is downregulated during T cell costimulation by CD2 and CD28. J Interferon Cytokine Res. 2000;20:955-961.

95. Asaoka K, Ikeda K, Hishinuma T, Horie-Inoue K, Takeda S, Inoue S. A retrovirus restriction factor TRIM5alpha is transcriptionally regulated by interferons. Biochem Biophys Res Commun. 2005;338:1950-1956.

96. Yap MW, Nisole S, Lynch C, Stoye JP. Trim5alpha protein restricts both HIV-1 and murine leukemia virus. Proc Natl Acad Sci U S A. 2004;101:10786-10791.

97. Fridell RA, Harding LS, Bogerd HP, Cullen BR. Identification of a novel human zinc finger protein that specifically interacts with the activation domain of lentiviral Tat proteins. Virology. 1995;209:347-357.

98. Chelbi-Alix MK, Quignon F, Pelicano L, Koken MH, de The H. Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. J Virol. 1998;72:1043-1051.

99. Regad T, Saib A, Lallemand-Breitenbach V, Pandolfi PP, de The H, Chelbi-Alix MK. PML mediates the interferon-induced antiviral state against a complex retrovirus via its association with the viral transactivator. Embo J. 2001;20:3495-3505.

100. Stoye JP. Fv1, the mouse retrovirus resistance gene. Rev Sci Tech. 1998;17:269-277.

101. Bouazzaoui A, Kreutz M, Eisert V, et al. Stimulated trans-acting factor of 50 kDa (Staf50) inhibits HIV-1 replication in human monocyte-derived macrophages. Virology. 2006.

102. Tissot C, Mechti N. Molecular cloning of a new interferon-induced factor that represses human immunodeficiency virus type 1 long terminal repeat expression. J Biol Chem. 1995;270:14891-14898.

103. Harada H, Harada Y, O'Brien DP, Rice DS, Naeve CW, Downing JR. HERF1, a novel hematopoiesis-specific RING finger protein, is required for terminal differentiation of erythroid cells. Mol Cell Biol. 1999;19:3808-3815.

104. Hiroi H, Momoeda M, Inoue S, et al. Stage-specific expression of estrogen receptor subtypes and estrogen responsive finger protein in preimplantational mouse embryos. Endocr J. 1999;46:153-158.

105. Inoue S, Urano T, Ogawa S, et al. Molecular cloning of rat efp: expression and regulation in primary osteoblasts. Biochem Biophys Res Commun. 1999;261:412-418.

106. Obad S, Olofsson T, Mechti N, Gullberg U and Drott K. Expression of the IFN-inducible p53-target gene TRIM22 is down-regulated during erythroid differentiation of human bone marrow. Leukemia Research. 2007;Ahead of print.

107. Hirose S, Nishizumi H, Sakano H. Pub, a novel PU.1 binding protein, regulates the transcriptional activity of PU.1. Biochem Biophys Res Commun. 2003;311:351-360.

108. Krutzfeldt M, Ellis M, Weekes DB, et al. Selective ablation of retinoblastoma protein function by the RET finger protein. Mol Cell. 2005;18:213-224.

109. Yergeau DA, Cornell CN, Parker SK, Zhou Y, Detrich HW, 3rd. bloodthirsty, an RBCC/TRIM gene required for erythropoiesis in zebrafish. Dev Biol. 2005;283:97-112.

110. Kudryashova E, Kudryashov D, Kramerova I, Spencer MJ. Trim32 is a ubiquitin ligase mutated in limb girdle muscular dystrophy type 2H that binds to skeletal muscle myosin and ubiquitinates actin. J Mol Biol. 2005;354:413-424.

111. Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem. 2001;70:503-533.

112. Yang Y, Yu X. Regulation of apoptosis: the ubiquitous way. Faseb J. 2003;17:790-799.

113. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev. 2002;82:373-428.

114. Schwartz DC, Hochstrasser M. A superfamily of protein tags: ubiquitin, SUMO and related modifiers. Trends Biochem Sci. 2003;28:321-328.

115. Hicke L. Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol. 2001;2:195-201.

116. Urano T, Saito T, Tsukui T, et al. Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth. Nature. 2002;417:871-875.

117. Dupont S, Zacchigna L, Cordenonsi M, et al. Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell. 2005;121:87-99.

118. Orimo A, Inoue S, Ikeda K, Noji S, Muramatsu M. Molecular cloning, structure, and expression of mouse estrogen-responsive finger protein Efp. Co-localization with estrogen receptor mRNA in target organs. J Biol Chem. 1995;270:24406-24413.

119. Ikeda K, Orimo A, Higashi Y, Muramatsu M, Inoue S. Efp as a primary estrogen-responsive gene in human breast cancer. FEBS Lett. 2000;472:9-13.

120. Horn EJ, Albor A, Liu Y, et al. RING protein Trim32 associated with skin carcinogenesis has anti-apoptotic and E3-ubiquitin ligase properties. Carcinogenesis. 2004;25:157-167.

121. de The H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A. The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell. 1991;66:675-684.

122. Takahashi M, Inaguma Y, Hiai H, Hirose F. Developmentally regulated expression of a human "finger"-containing gene encoded by the 5' half of the ret transforming gene. Mol Cell Biol. 1988;8:1853-1856.

123. Klugbauer S, Rabes HM. The transcription coactivator HTIF1 and a related protein are fused to the RET receptor tyrosine kinase in childhood papillary thyroid carcinomas. Oncogene. 1999;18:4388-4393.

124. Le Douarin B, Zechel C, Garnier JM, et al. The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. Embo J. 1995;14:2020-2033.

125. Koken MH, Puvion-Dutilleul F, Guillemin MC, et al. The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. Embo J. 1994;13:1073-1083.

126. Cao T, Duprez E, Borden KL, Freemont PS, Etkin LD. Ret finger protein is a normal component of PML nuclear bodies and interacts directly with PML. J Cell Sci. 1998;111 (Pt 10):1319-1329.

127. Obad S, Brunnstrom H, Vallon-Christersson J, Borg A, Drott K, Gullberg U. Staf50 is a novel p53 target gene conferring reduced clonogenic growth of leukemic U-937 cells. Oncogene. 2004;23:4050-4059.

128. Wei CL, Wu Q, Vega VB, et al. A global map of p53 transcription-factor binding sites in the human genome. Cell. 2006;124:207-219.

129. Patarca R, Freeman GJ, Schwartz J, et al. rpt-1, an intracellular protein from helper/inducer T cells that regulates gene expression of interleukin 2 receptor and human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. 1988;85:2733-2737.

130. Leng RP, Lin Y, Ma W, et al. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell. 2003;112:779-791.

131. Dornan D, Wertz I, Shimizu H, et al. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature. 2004;429:86-92.

132. Honda R, Yasuda H. Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. Oncogene. 2000;19:1473-1476.

133. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell. 1992;69:1237-1245.

134. Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature. 1993;362:857-860.

135. Honda R, Yasuda H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. Embo J. 1999;18:22-27.

136. Li Y, Wu D, Chen B, et al. ATM activity contributes to the tumor-suppressing functions of p14ARF. Oncogene. 2004;23:7355-7365.

137. Ehinger M, Bergh G, Olofsson T, Baldetorp B, Olsson I, Gullberg U. Expression of the p53 tumor suppressor gene induces differentiation and promotes induction of differentiation by 1,25-dihydroxycholecalciferol in leukemic U-937 cells. Blood. 1996;87:1064-1074.

138. Michalovitz D, Halevy O, Oren M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell. 1990;62:671-680.

139. Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M, Levine AJ. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol Cell Biol. 1988;8:531-539.

140. Sabbatini P, Lin J, Levine AJ, White E. Essential role for p53-mediated transcription in E1A-induced apoptosis. Genes Dev. 1995;9:2184-2192.

141. Park YB, Park MJ, Kimura K, Shimizu K, Lee SH, Yokota J. Alterations in the INK4a/ARF locus and their effects on the growth of human osteosarcoma cell lines. Cancer Genet Cytogenet. 2002;133:105-111.

142. Zhu JJ, Li FB, Zhu XF, Liao WM. The p33ING1b tumor suppressor cooperates with p53 to induce apoptosis in response to etoposide in human osteosarcoma cells. Life Sci. 2006;78:1469-1477.

143. Applied Biosystems Essentials of Real Time PCR, PN 105622.

144. Applied Biosystems Users Bullitin #2: Relative Quantitation of Gene Expression,PN 4303859.

145. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol. 2002;30:503-512.

146. Carey MS, S. Transcriptional Regulation in Eukaryotes. Cold Spring Harbor Laboratory Press, New York. 1999.

147. Bronstein I, Fortin J, Stanley PE, Stewart GS, Kricka LJ. Chemiluminescent and bioluminescent reporter gene assays. Anal Biochem. 1994;219:169-181.

148. Thavathiru E, Das GM. Activation of pRL-TK by 12S E1A oncoprotein: drawbacks of using an internal reference reporter in transcription assays. Biotechniques. 2001;31:528-530, 532.

149. Munsch D, Watanabe-Fukunaga R, Bourdon JC, et al. Human and mouse Fas (APO-1/CD95) death receptor genes each contain a p53-responsive element that is activated by p53 mutants unable to induce apoptosis. J Biol Chem. 2000;275:3867-3872.

150. Momand J, Zambetti GP. Mdm-2: "big brother" of p53. J Cell Biochem. 1997;64:343-352.

151. Wu GS, Burns TF, McDonald ER, 3rd, et al. Induction of the TRAIL receptor KILLER/DR5 in p53-dependent apoptosis but not growth arrest. Oncogene. 1999;18:6411-6418.

152. Andrews NC. The NF-E2 transcription factor. Int J Biochem Cell Biol. 1998;30:429-432.

153. Breitman TR, Selonick SE, Collins SJ. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci U S A. 1980;77:2936-2940.

154. Olsson IL, Breitman TR. Induction of differentiation of the human histiocytic lymphoma cell line U-937 by retinoic acid and cyclic adenosine 3':5'-monophosphate-inducing agents. Cancer Res. 1982;42:3924-3927.

155. Olsson I, Gullberg U, Ivhed I, Nilsson K. Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1 alpha,25-dihydroxycholecalciferol. Cancer Res. 1983;43:5862-5867.

156. Sutherland JA, Turner AR, Mannoni P, McGann LE, Turc JM. Differentiation of K562 leukemia cells along erythroid, macrophage, and megakaryocyte lineages. J Biol Response Mod. 1986;5:250-262.

157. Dao CT, Luo JK, Zhang DE. Retinoic acid-induced protein ISGylation is dependent on interferon signal transduction. Blood Cells Mol Dis. 2006;36:406-413.

158. Takano Y, Adachi S, Okuno M, et al. The RING finger protein, RNF8, interacts with retinoid X receptor alpha and enhances its transcription-stimulating activity. J Biol Chem. 2004;279:18926-18934.

159. Takeshita K, Benz EJ. Gene expression during erythropoiesis. Int J Cell Cloning. 1991;9:109-122.

160. Ozaki ME, Webb SR. Controlling mature CD4+ T cell responses. Immunol Res. 2000;21:345-355.

161. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu Rev Immunol. 1996;14:207-232.

162. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 2002;20:323-370.

163. Harris NL, Ronchese F. The role of B7 costimulation in T-cell immunity. Immunol Cell Biol. 1999;77:304-311.

164. Gross JA, Callas E, Allison JP. Identification and distribution of the costimulatory receptor CD28 in the mouse. J Immunol. 1992;149:380-388.

165. O'Garra A, Murphy K. Role of cytokines in determining T-lymphocyte function. Curr Opin Immunol. 1994;6:458-466.

166. Mosmann TR, Li L, Sad S. Functions of CD8 T-cell subsets secreting different cytokine patterns. Semin Immunol. 1997;9:87-92.

167. Civil A, Verweij CL. Regulation of IL2 gene transcription via the T-cell accessory molecule CD28. Res Immunol. 1995;146:158-164.

168. Jones LA, Chin LT, Longo DL, Kruisbeek AM. Peripheral clonal elimination of functional T cells. Science. 1990;250:1726-1729.

169. Rocha B, von Boehmer H. Peripheral selection of the T cell repertoire. Science. 1991;251:1225-1228.

170. Kabelitz D, Pohl T, Pechhold K. Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. Immunol Today. 1993;14:338-339.

171. Lissy NA, Davis PK, Irwin M, Kaelin WG, Dowdy SF. A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. Nature. 2000;407:642-645.

172. Senftleben U, Li ZW, Baud V, Karin M. IKKbeta is essential for protecting T cells from TNFalpha-induced apoptosis. Immunity. 2001;14:217-230.

173. Quignon F, De Bels F, Koken M, Feunteun J, Ameisen JC, de The H. PML induces a novel caspase-independent death process. Nat Genet. 1998;20:259-265.

174. Johnston JA, Ward CL, Kopito RR. Aggresomes: a cellular response to misfolded proteins. J Cell Biol. 1998;143:1883-1898.

175. Kallijarvi J, Lahtinen U, Hamalainen R, Lipsanen-Nyman M, Palvimo JJ, Lehesjoki AE. TRIM37 defective in mulibrey nanism is a novel RING finger ubiquitin E3 ligase. Exp Cell Res. 2005;308:146-155.

176. Boe SO, Haave M, Jul-Larsen A, Grudic A, Bjerkvig R, Lonning PE. Promyelocytic leukemia nuclear bodies are predetermined processing sites for damaged DNA. J Cell Sci. 2006;119:3284-3295.

177. Zhong S, Salomoni P, Pandolfi PP. The transcriptional role of PML and the nuclear body. Nat Cell Biol. 2000;2:E85-90.

178. Morris-Desbois C, Bochard V, Reynaud C, Jalinot P. Interaction between the Ret finger protein and the Int-6 gene product and co-localisation into nuclear bodies. J Cell Sci. 1999;112 (Pt 19):3331-3342.

179. Hofmann TG, Will H. Body language: the function of PML nuclear bodies in apoptosis regulation. Cell Death Differ. 2003;10:1290-1299.

180. Jensen K, Shiels C, Freemont PS. PML protein isoforms and the RBCC/TRIM motif. Oncogene. 2001;20:7223-7233.

181. Shen TH, Lin HK, Scaglioni PP, Yung TM, Pandolfi PP. The mechanisms of PML-nuclear body formation. Mol Cell. 2006;24:331-339.

182. Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang DE. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J Biol Chem. 2002;277:9976-9981.