On the function of the interferon-inducible p53 target gene TRIM22

Petersson, Jessica

2011

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Jessica Petersson

LUNDS UNIVERSITET

*`I should see the garden far better,' said Alice to herself, `if I could get to the top of that hill: and here's a path that leads straight to it - at least, no, it doesn't do that - '(after going a few yards along the path, and turning several sharp corners), `but I suppose it will at last. But how curiously it twists! It's more like a corkscrew than a path! Well, **this** turn goes to the hill, I suppose - no, it doesn't! This goes straight back to the house! Well then, I'll try it the other way.'*

Lewis Carroll, "Through the looking glass"

Contents

Selected abbreviations

4E-BP	4E-binding protein
aa	amino acid
bp	base pair
cDNA	complementary DNA
eIF	eukaryotic initiation factor
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorter
eGFP	enhanced green fluorescent protein
HIV-1	human immunodeficiency virus 1
IFN	interferon
IRES	internal ribosomal entry site
ISG15	interferon stimulated gene 15
IRF	interferon regulatory factor
kDa	kilodalton
LPS	lipopolysaccharide
Mdm2	murine double minute 2
mRNA	messenger RNA
NB	nuclear bodies
ORF	open reading frame
PCM	pericentriolar material
PBMC	peripheral blood mononuclear cell
PML	promyelocytic leukaemia protein
RING	really interesting new gene
Staf50	stimulated trans-acting factor of 50 kDa
TRIM	tripartite motif
UBL	ubiquitin like protein

UPR   unfolded protein response

UTR   untranslated region

List of papers

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

I.   *Petersson J.*, Lönnbro P., Herr A-M., Mörgelin M., Gullberg U. and Drott K. The human IFN-inducible p53 target gene TRIM22 colocalizes with the centrosome independently of cell cycle phase. *Exp Cell Res* 316, 568-579, 2010.

II.   *Petersson J.*, Ageberg M., Sandén C., Olofsson T., Gullberg U. and Drott K. The p53-target gene TRIM22 directly or indirectly interacts with the translation initiation factor eIF4E and inhibits the binding of eIF4E to eIF4G. *Pending revision, Biology of the Cell*.

III.   *Petersson J.*, Gullberg U. and Drott K. Antiproliferative and cell death inducing effects mediated by the IFN-inducible p53-target gene TRIM22. *Preliminary manuscript*.

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Introduction

Cancer

Cancer is a disease in which clonal expansion of cells invades, disturbs and erodes normal tissues. Mutations in genes that control and regulate growth and cell death are driving cancer development. The process of transformation is a multi-step process, where derived cell mutations accumulate and finally abolish the normal balance between proliferation, differentiation and cell death. Genomic instability is a fundamental hallmark of cancer and is associated with activated oncogenes (gain of function) and inactivated tumour suppressor genes (loss of function). Proto-oncogenes are responsible for the proliferation and survival of normal cells, but are in cancer cells often constitutively activated, creating oncogenes. Tumour suppressor genes have repressive effects on proliferation and the cell cycle, and induce apoptosis when all other measures fail. Thus, tumour suppressor genes are often inactivated in cancer cells. Since two alleles of every gene are present in the human genome, the loss of tumour suppressor genes most often requires inactivation of both copies [1].

The tumour suppressor p53

p53, often referred to as the "guardian of the genome", is probably one of the most important tumour suppressor genes, and it is positioned in the centre of a complex of signalling pathways that prevent proliferation and survival of potentially malignant cells. p53 was discovered in 1979, and was initially believed to be an oncogene, but data obtained 10 years after its discovery revealed it to be a tumour suppressor gene and transcription factor [2]. Today, we know p53 to be a tumour suppressor of paramount importance and numerous papers have been published regarding p53 and its functions. However, yet much remains to be discovered regarding the functions of p53. p53 or the pathways dependent of p53 are inactivated in almost all tumour types. Mutational analyses have shown that over 50% of

human tumours carry p53 mutations [3, 4]. The p53 protein has a broad range of biological functions, such as regulation of apoptosis, the cell cycle, senescence, DNA repair, differentiation, angiogenesis and translation (fig 1)[2].
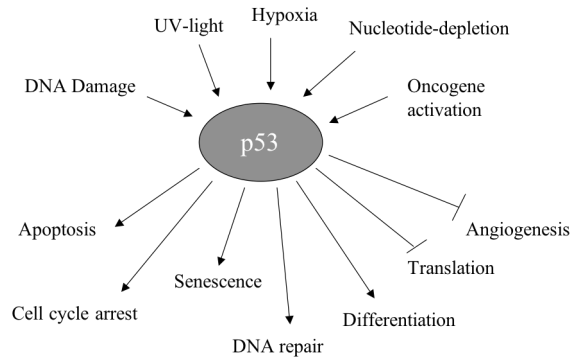
Fig 1: A schematic presentation of some activators of p53 and outcomes of p53 activation. p53 is activated upon DNA damage, UV-light exposure, hypoxia, nucleotide depletion and by oncogenes. Activation of p53 can result in induction of apoptosis, cell cycle arrest, senescence, DNA repair, differentiation or inhibition of angiogenesis and translation.

p53 is most often inactivated in cancer cells through missense mutations in the DNA binding domain [5]. Even though p53 is a tumour suppressor gene, haploinsufficiency (the loss of only one allele) of p53 can still cause transformation, suggesting the "dose" of p53 to play an important role in protection towards cancer [6]. Mutant forms of p53 often have a longer half-life than wild-type p53 and can have a dominant-negative effect on wild-type p53. Moreover, mutant forms of p53 can contribute to malignancy, e.g. through aberrant regulation of transcription [5]. In addition, p53 is also inactivated indirectly through alterations in genes of proteins that interacts with p53 or through binding of viral proteins. Indeed, p53 was initially discovered when co-precipitated with the SV40 large T antigen [7].

In normal, unstressed cells, p53 is expressed at low levels. The half-life of the p53 protein is short, due to feedback mechanisms leading to rapid degradation, and the amount of p53 is regulated on the degradational rather on a transcriptional or translational level. When a stressful stimulus activates p53, it is stabilised, accumulates in the nucleus and forms a homotetrameric complex. The activation of p53 is induced by several different factors, such as DNA damage, UV-light exposure, hypoxia, nucleotide-depletion and by oncogenes (fig 1). All these stimuli also lead to stabilisation of the p53 protein by inhibition of the degradation of p53. Thereafter, p53 mainly exerts its actions through sequence-specific

transcriptional activation and repression, even though p53 also can mediate cell cycle arrest and apoptosis also independently of transcriptional regulation [8, 9].

Activated p53 is regulated by posttranslational modifications and so far about 50 different patterns of posttranslational modifications have been identified, including phosphorylation, acetylation, mono- and di-methylation, glycosylation, ubiquitylation, neddylation, sumoylation and poly-ribosylation. The functional roles of the majority of these modifications are yet unknown [10]. However, recent accumulated studies have shown that p53 expressed under physiological conditions in cells not extremely stressed, also participates in milder adaptive processes such as modulation of metabolism, antioxidant defence and detoxification, rate of protein biosynthesis and supervision of the autophagy process [2].

p53 induces cell cycle arrest and apoptosis

p53 is capable of inducing different outcomes in the cell depending on cellular context, as well as severity of DNA damage. p53 functions as a "modular node" for converging signals, and receives, assess and integrate different signals and then induce cell death or cell survival. How this is performed is not yet fully understood. However, post-translational modifications, binding to different co-factors as well as protein levels of p53 is believed to be important in this process. For instance, p53 has been shown to bind to different genes with different affinity. Genes involved in cell cycle arrest bind to p53 with higher affinity than some genes involved in apoptosis and these apoptosis related genes are believed to be fully activated only when p53 abundance reaches a threshold [11].

p53 induces cell cycle arrest through regulation of both G1 and G2/M transition. Induction of G1 arrest is well studied. An important mediator of p53-induced cell cycle arrest is p21, which is a p53-target gene. Entry into the M phase can be prevented by inhibition of CDK1, which needs to bind to cyclin B1 for the cell cycle to proceed. Repression of cyclin B1 by p53 also arrests the cells in the G2 phase [12]. Other genes downstream of p53 that can contribute or mediate G2 arrest include 14-3-3-σ, GADD45 and Reprimo [13, 14].

p53 induces apoptosis by inducing transcription of pro-apoptotic genes and repressing antiapoptotic genes. NOXA, PUMA and p53AIP1 are p53-induced mitochondrial pro-apoptotic proteins, causing release of cytochrome c and thus apoptosis. PUMA, a member of the Bcl-2 family, has been shown to be a key mediator of the apoptotic pathway mediated by p53, since apoptosis induced by e.g. irradiation and cell stress was blocked in PUMA knock-out

mice [15]. Furthermore, p53 can also induce apoptosis independent of transcription, in the cytoplasm [16].

Regulation of p53 by its target genes

Due to the strong effects of p53 on the cell, aberrant expression of p53 would have deleterious consequences. Therefore, the levels of p53 must be tightly regulated. As already mentioned, the levels of p53 are mainly regulated by degradation. To date at least nineteen ubiquitin E3-ligases targeting p53 have been found [17]. The p53-target gene Mdm2 (murine double minute 2) is thought to be the major E3-ligase targeting p53 for degradation. However, while Mdm2 negatively regulates p53 protein levels, p53 positively regulates the transcription of Mdm2, thus forming a negative feedback loop aimed to maintain low levels of p53 in unstressed cells. Consequently, disruption of the Mdm2-p53 interaction through posttranslational modifications of p53 and/or Mdm2, results in rapid accumulation of p53 during stress. For instance, the tumour suppressor p14/19ARF can bind and prevent Mdm2 from targeting p53, thus inducing an increase in p53 protein levels [11]. The levels of p14/17ARF can be induced by oncogenes, e.g. E2F [18].

Furthermore, several similar feedback loops exists, making sure p53 levels are kept low. Recently, the ubiquitin E3-ligase Pirh2 (p53-induced RING-H2) has been shown to be more important than earlier thought [19]. Pirh2 and p53 constitute a feedback loop similar to Mdm2 and p53. Some of the other E3-ligases targeting p53 include COP1 (constitutively photomorphogenic 1), CHIP (chaperone associated ubiquitin ligase), topors (human topoisomerase I- and p53-binding protein) and ARF-BP1 (ARF-binding protein). The ubiquitylation of p53 can also be reversed by HAUSP (Herpes virus-associated ubiquitin-specific protease), which deubiquitylates p53 [19].

Interferons

Interferons (IFN) are pleiotropic cytokines with antiviral, antiproliferative, proapoptotic, and immunomodulatory functions. Although IFNs are utilised in the treatment of several malignancies, molecular pathways downstream of IFNs are not fully understood. IFNs are mainly categorised as type I and II, where IFNα/β belongs to the type I and IFNγ to type II. The production of type I IFNs is induced by viral infections, while type II is induced in response to mitogenic or antigenic stimuli. Most cells are capable of

producing type I, while type II is produced exclusively by immune cells, including natural killer cells, CD4+ T cells and CD8+ T cells [20]. Both type I and type II IFNs exerts their actions through receptor complexes present on the surface membranes. IFN receptor-mediated signalling results in activation of latent cytoplasmatic factors, STATs (signal transducers and activators of transcription) family of proteins. These STAT proteins are activated through the JAK (janus tyrosine kinase) family. Subsequently STATs together with IRFs (IFN regulatory factors), a family of transcription factors, which mediate many changes in gene expression in the cells responsible for the biological activities of IFNs. However, the IFNs also uses other pathways independently of JAKs or/and STATs [21].

IFNγ induces TRIM22 through a 5′ extended IFN-stimulating response element (5′ eISRE) in the TRIM22 nucleotide sequence. IRF-1 binds to the 5′ eISRE and probably induces the transcription of TRIM22 [22]. For transcription of TRIM22 to occur, the chromatin-remodelling enzyme BRG1 (Brahma regulated gene 1) is required [23]. Although TRIM22 is strongly induced by IFNα/β in most tissues, the mechanism for induction of TRIM22 in response to IFNα/β is yet not known.

There are two main pathways for viral induction of IFNα/β production in cells. In the endosomal pathway a virus is taken up by the cell enclosed in an endosome and is then recognised by several TLRs (Toll-like receptors). This induces phosporylation and activation of the IRFs, which then induce the expression of IFNα/β. In the cytosolic pathway, viral RNA or DNA is sensed by several PRRs (pattern recognition receptors), including PKR (RNA-dependent protein kinase). Also this pathway activates IRFs and subsequently IFNα/β [24].

So far, over 300 genes have been shown to be induced upon treatment with IFNs. Several of these are involved in proliferation, apoptosis and angiogenesis [24, 25]. Different cell-lines display various degree of sensitivity towards the antiproliferative effects of IFNs. Furthermore, different types and subtypes of IFNs differ greatly in their proliferative effects [26, 27]. However, IFNs have been shown to target several proteins involved in cell cycle control, such as c-myc, pRb, cyclin D3, cdc25A and p21, and to induce a prolongation of the cell cycle [25, 28].

The involvement of apoptosis as an instrument for the cell to evade the spread of the viruses is also evident since several viruses have evolved mechanisms to inhibit apoptosis. Viruses such as herpesviruses, poxviruses and a number of adenoviruses encode proteins similar to the cellular anti-apoptotic Bcl-2 family proteins, thus inhibiting cell death. However, this is not true for all viruses, in contrary some viruses induce cell death, such as

HIV-1 which is associated with direct killing of infected peripheral blood mononuclear cell in AIDS patients [24].

There are several different pathways linking IFNs and cell death, but the mechanisms of their actions are so far not entirely understood. Mediators of apoptosis induced by IFNα include caspase 4, caspase 8, TRAIL (tumour necrosis-related apoptosis-inducing ligand), Fas/CD95, XIAP (the X-linked inhibitor of apoptosis), death-activating protein kinases, IRFs, dsRNA-activated protein and PML. These genes alone are probably not sufficient to induce apoptosis, but their cumulative effects probably result in apoptosis. The ability to induce apoptosis is at least partially independent of cell cycle arrest, presence of wild-type p53, or expression of the Bcl-2 family [20].

The connection between p53 and interferons

Interestingly, TRIM22 is an IFN-induced as well as a p53-target gene and may thus constitute a link between these two pathways. Connections between p53 and IFN-signalling have already been shown to exist. To that end, IFNα has been shown to enhance the transcription of p53-target genes and p53-dependent apoptosis, and IFNα/β can give rise to increased expression of p53 (fig 2)[29, 30]. Moreover, IFNγ cooperates with p53 in induction of differentiation of leukaemic cells [31], all in all suggesting that IFNs can potentiate certain aspects of the p53 response. Interestingly, p53 can also contribute to an increase in IFN-release from virally infected cells [30].

The connection between tumour suppressor pathways and immune response pathways makes sense since viral infections are one cause of tumour initiation. Several viruses have been shown to induce cancer; e.g. the lymhotropic viruses (Epstein-Barr virus, human herpersvirus 8 and human T-lymphotropic virus 1) as well as the human papilloma viruses. Also, chronic inflammation is clearly associated with increased frequency of development of cancer. Indeed, p53 has been suggested to work as a general inhibitor of inflammation, thereby suppressing cancer development [32]. Moreover, virus and pathogen infected cells need to induce the same response towards the infections as cancer cells in order to evade spread of the infections, including translational repression, decreased proliferation and apoptosis. Therefore, it seems logical to partially use the same pathways. Consequently, absence of p53 makes vesicular stomatitis-, encephalomyocarditis- and hepatitis C virus-infected cells more resistant to IFN-induced apoptosis [33, 34]. Also, p53 overexpressing mice are resistant while p53 null mouse embryonic fibroblasts are hypersensitive to viral infections [30][33]. Therefore, reactivation of p53 is proposed to help to

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control viral infections [35].
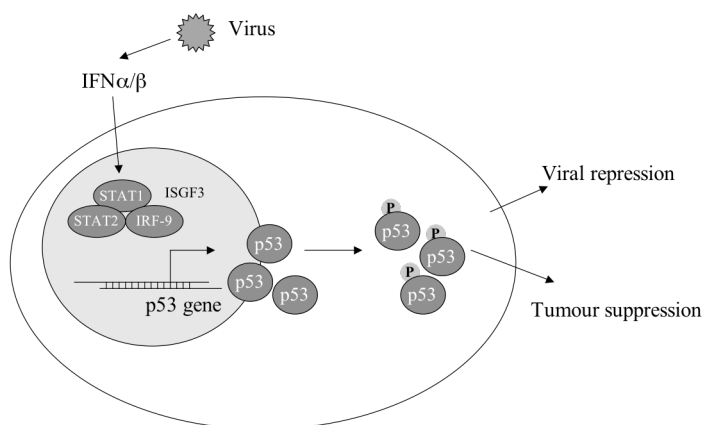
Fig 2: *A connection between viral infection, IFNs (α/β) and p53. Virally infected cells activates IFNα/β, leading to the formation of the heterotrimeric complex ISGF3, constituted by activated STAT1, STAT2 and IRF-9. ISGF-3 binds to two ISRE (IFN-stimulated response element) sites in the gene encoding p53, thus activating transcription and p53 protein synthesis. Viral infection also results in the phosphorylation and activation of p53, leading to antiviral defence and tumour suppression.*

Protein translation

Regulation of protein translation plays a critical role in many fundamental cellular processes, such as cell growth, development, and response to cell stress but also in viral replication.

Protein translation takes place in the cytoplasm, in the mitochondria and from ribosomes attached to the ER-membrane (on the cytosolic side). mRNA for cytosolic proteins are translated on free cytoplasmic ribosomes whereas mRNA for secretory and membrane proteins is translated on membrane-bound ribosomes. The membrane-bound ribosomes discharge polypeptide chains across the ER membrane [36].

After transcription, most cellular mRNAs are capped with a m^{7}GpppN cap in the 5´terminus and a poly-A-tail is added to the 3´terminus, after which the modified mRNA is transported to the cytoplasm where translation occurs. Capped translation is dependent on an array of eukaryotic translation initiation factors (eIFs), e.g. eIF4E and eIF2α. Both eIF4E and

eIF2α are extensively regulated in response to cell stress [37, 38]. eIF4E is active both in transport of the mRNA from nucleus into the cytoplasm as well as in initiation of translation by binding to the mRNA cap [38]. It belongs to a translation initiation complex called eIF4F. Except for eIF4E, eIF4F also consists of eIF4G (a large scaffold protein, responsible for the assembly of the eIF4F complex), and eIF4A (a RNA helicase, responsible for the unwinding of the mRNA). eIF2α is necessary for the assembly of the 43S translation initiation complex. Global translation is reduced in response to most, or perhaps all, types of cell stress. This results in saving of cellular energy as well as prevention of synthesis of unwanted proteins that might interfere with the cellular stress response. Furthermore, during stress a switch that favours translation of proteins required for cell survival occurs [37]. These proteins contain an internal ribosome entry segment (IRES), which allows them to be translated when cap-dependent translation is impaired. IRES is a RNA structure allowing translation without a cap structure, so called cap-independent translation. During cap-independent translation, the translational machinery is assembled at a position close to the initiation codon without the same composition of eIFs as cap-dependent translation. However, there is no common primary consensus sequence defining an IRES, but the function of an IRES is rather dependent on the three-dimensional structure, as judged from empirical studies [38]. Different IRES-structures require different composition of eIFs, some demand all the eIFs that are required for cap-dependent translation, where as others does not need eIFs at all but can bind directly to the ribosomes for translation [39]. Interestingly, viral replication often occurs through IRES-mediated translation and the IRES-structure was first discovered in picornavirus mRNA [37]. The IRES-structure allows the virus particles to be translated when cap-dependent translation is impaired (e.g., during stressful situations) as well as during mitosis.

The eIF4F complex is important for suppression of tumour progression, and consequently both eIF4E and eIF4G are up-regulated in many cancers. However, the regulation of the eIF4F complex in response to virus infections is a bit more complicated. Some viruses use the host cells complete translation machinery, including the entire eIF4F complex, for the replication of their genomic material. However, others rely on IRES-structures, and are thus not in need for as many translation initiation factors. Some of these viruses have evolved mechanisms to shut off the protein translation of the cell, to completely favour translation of their own genomic components. In these cases eIF4F is sometimes disturbed [40]. Therefore, in the case of viral infections, disruption of eIF4F inhibits or favours viral replication depending on the virus. For example, herpesvirus

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is translated from capped mRNA, and has evolved mechanisms to maintain an efficient cap-dependent translation dependent on the eIF4F complex [41]. In contrary, a synthetic inhibitor of the eIF4E and eIF4G binding was shown to repress coronavirus replication [42]. In conclusion, the targeting of eIF4F provides a new target of therapeutics toward both cancer cells as well as some virally infected cells, and compounds targeting eIF4F have recently been developed [43].

Both p53 and IFNs are involved in translational repression. IFNs interferes with global translation through induction of the IFN-inducible PKR, phosphorylating eIF2α. Phosphorylation of eIF2α leads to repressed protein translation [44, 45]. IFNs have also been shown to be able to inhibit IRES mediated translation [46]. p53 is able to inhibit protein synthesis through dephosporylation and accumulation of the translational inhibitor 4E-BP1 as well as by cleavage of eIF4G. The unphosphorylated form of 4E-BP1 binds and inhibits eIF4E resulting in inhibited translation [47, 48]. p53 also represses expression of eIF4E [49].

Cell cycle

The sequence of phases through which a cell passes between one division and the next is called the cell cycle. The cell cycle consist of four phases; G1, S, G2 and M. During the S phase the DNA is replicated, whereas the M phase includes mitosis and cytokinesis. Between the S and M phase are two gaps, G1 and G2, during which the cell is preparing for DNA synthesis (G1) and mitosis (G2). The length of the cell cycle varies between different cell-types. Cells that are not in the process of cell division enter a fifth phase; the inactive G0 phase. Cell cycle progression is a highly regulated and complex process controlled by multiple factors. The passage between the different phases is regulated by cyclins and cdks (cyclin-dependent kinases). The Cdks regulates the cell cycle by phosphorylation of target proteins, and are not fully activate without associated cyclins. The abundance of the cyclins varies throughout the cell cycle, hence their name. There are several checkpoints throughout the cell cycle in order to maintain the integrity of the genome. The checkpoints induce cell cycle arrest in response to e.g. damaged DNA, unreplicated DNA and misalignment on the mitotic spindle. Disruption of these checkpoints lead to mutations that may result in carcinogenesis [50]. As previous mentioned both p53 and IFNs affects several proteins involved in the control of the cell cycle.

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Cell death

Cell death is very broadly divided into necrosis and apoptosis. Apoptosis is a highly controlled process, and is associated with cell shrinkage and precise chromatin fragmentation, that results in a neat disposal of the cell. In contrary necrosis is a process where the cell spills out its contents into the surrounding tissue, thereby causing inflammation. Necrosis has long be thought of as a passive cell death, but is today thought of as an alternate form of cell death with possible important biological consequences, through induction of an inflammatory response [51]. However, cancer drugs that results in apoptosis of cells are preferable. Apoptosis is further divided into the extrinsic pathway and intrinsic pathway, initiated from membrane death-receptors and the mitochondria, respectively. The intrinsic pathway is associated with cytochrome C release from the mitochondria. Caspases (cystein-rich aspartate proteases), specific proteases that cleave intracellular proteins, are central for both pathways. They are synthesised in an inactive proform and are activated by cleavage. Upon activation caspases participates in a cascade of activation whereby one caspase can activate another caspase in a chain reaction. However, caspase-independent apoptosis also exists [52]. An alternative cell death pathway is provided by the mechanism of autophagy ("self-eating"). Autophagy is a more complex process in the sense that it sometimes rescues the cell from cell death and in other cases promotes cell death. Furthermore, autophagy does in some cases induce cell death in collaboration with apoptosis, and in other cases it can function as a back-up mechanism when apoptosis is defective [53]. Autophagy is also a mechanism for degradation of long-lived proteins and cytoplasmic organelles, such as mitochondria, parts of the ER and peroxisomes. Autophagocytotic vesicles fuse with lysosomes, resulting in degradation of the autophagocyted material [54].

Therapies

p53 is due to its potent effects in cancer cells, very tempting to manipulate in development of cancer therapy. Since p53 is impaired in many cancers, reactivation of p53 is an obvious goal, and the idea has been supported by reactivation of wild-type p53 leading to successful repression of tumour development in animal models [55-57]. However, in humans, reactivation or introduction of genes is not an easy task. One way could be to use viral vectors. However, this technique of delivery probably still needs to be further assessed and improved in order for success. Problems so far have included insufficient spread of the virus from the injected site as well as

overcoming barriers at the tumour site, imposed by stroma and immune responses [58].

Furthermore, many synthetic small molecule drugs, stabilising and activating p53 have been developed. While most of them target the interaction between p53 and Mdm2, thus inhibiting the degradation of p53, some of them reactivates mutant p53, e.g. through change of p53 conformation into wild-type. Several of these drugs are currently in clinical trials [59].

IFNα became the first immune therapy to be approved as an anticancer drug when it received its approval in 1986. Since then, treatment with IFNα for almost all malignancies has been attempted. Unfortunately, IFNα is associated with a broad spectrum of dose-related toxicities. The patients experience dose-related fatigue and flue-like symptoms, which compromise planned treatment dosing and schedules. However, the treatment is better tolerated if administered late in the day and in combination with anti-inflammatory drugs [60]. IFNα is still in clinical use, often in combination with chemotherapy or monoclonal antibodies. In Sweden, Lund, IFNα is used to treat melanoma, follicular and diffuse large B-cell lymphomas, CLL, hairy cell leukaemia and sometimes renal cancer [165].

The TRIM protein family

Our group previously identified the IFN-inducible protein TRIM22 as a direct target gene to the tumour suppressor p53 [61]. TRIM22 belongs to the TRIM family of proteins, named based on a characteristic tripartite motif (TRIM), including a RING finger, one or two B-boxes and a Coiled-coil-domain. The tripartite motif is always present at the N-terminus of the TRIM proteins and the order of the domains is conserved [62] (fig 3).
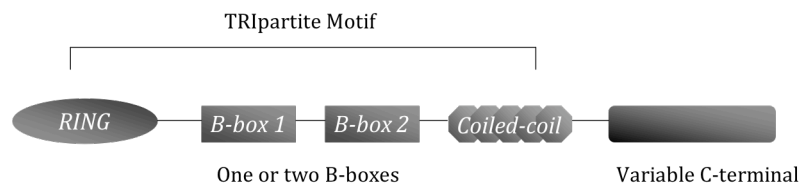
Fig 3: *The conserved TRIpartite Motif, containing a RING-domain, one or two B-boxes and a Coiled-coil-domain.*

Both RING fingers and B-boxes are cysteine-rich zinc-binding domains. The RING-domain is present in hundreds of proteins and is defined by a serie of

conserved cysteine and histidine residues that constitute zinc coordination sites. However, the TRIM family constitutes the largest RING-containing group [63]. The RING-domain is often associated with ubiquitin E3-ligase activity, resulting in ubiquitylation of target proteins targeting them for degradation or altered activity [64]. Many TRIM family members have been shown to exhibit E3-ligase activity, including TRIM22 [65-67]. Furthermore, almost all TRIM proteins interact with one or more E2-ligases, also suggesting these to function as E3-ligases [68]. The B-boxes are a defining domain of the TRIM family but the function is not yet known [63]. The Coiled-coil-domain is not unique for the TRIM family and is believed to mediate protein-protein interactions [69]. Consistently, the TRIM proteins have a high tendency of homo-dimerisation, but do also hetero-dimerise [69, 70]. The TRIM proteins contain a variable C-terminal-domain. The most common C-terminal-domain is the SPRY-domain. However, also the SPRY-domain is present in other proteins than the TRIM proteins [63]. The function of the SPRY-domain is not yet known but it has been suggested to be involved in protein binding, mediating specificity [63, 71]. Furthermore, in the context of TRIM5 the sequences of the SPRY-domain have been shown to be of paramount importance for the potency and specificity of the restriction of particular retroviruses [72, 73]. TRIM22 consists of a RING finger, a B-box (type 2), a Coiled-coil as well as a SPRY-domain (fig. 4).

Fig 4: TRIM22 consist of a RING finger, a B-box, a Coiled-coil and a SPRY-domain.

The function of the TRIM proteins

The TRIM family consists of more than 70 proteins, involved in many biological processes such as apoptosis, cell proliferation, viral defence and ubiquitylation [63, 65]. Some TRIM–NHL (NHL is one of the variable C-terminals of the TRIM proteins) proteins have also been suggested to be involved in miRNA-mediated gene silencing [74].

Furthermore, mutations in several TRIM proteins have been linked to human disease, e.g. mutations in TRIM18 is associated with X-linked Opitz syndrome, TRIM20 with familial mediterranean fever and TRIM54 with Muscle atrophy [65].

Several TRIM family members are also implicated in cancer. PML (TRIM19) forms an oncogenic fusion protein with the retinoic acid receptor α (RARα) in APL, whereas the Ret finger protein (TRIM27) forms an oncogenic fusion protein with the Ret proto-oncogene in human papillary thyroid carcinoma. Furthermore, estrogen responsive finger protein (TRIM25) and TRIM32 enhances proliferation and survival of breast tumour growth and squamous cancer cells, respectively [75-78].

Moreover, several TRIM family members are involved in apoptosis and proliferation; PML (TRIM19) is involved in apoptosis, senescence and inhibition of proliferation mediated by the tumour suppressor protein p53 [79, 80]. Furthermore RFP (TRIM27) induces apoptosis, TRIM17 induces neuronal apoptosis, TRIM36 delays cell cycle progression and TRIM32 represses apoptosis [78, 81-83]. Furthermore, TRIM16 acts as a tumour suppressor protein through binding to vimentin and E2F1, thus reducing cell motility and cell replication [84].

The fellowship of the RING

Several biological processes, e.g. transcription and translation of mRNAs, protein transport, protein modifications, protein turnover, intracellular concentration, localisation and activity of proteins must be controlled in order for cells to be able to function properly. One among eukaryotes highly conserved and important control mechanism is post-translational modification of proteins by ubiquitin [85]. Ubiquitin is a small protein, consisting of 76 amino acid (aa) residues. The poly-ubiquitylation of target proteins is most often associated with following degradation in the 26S-proteasome, but ubiquitylation can also result in other outcomes, such as DNA repair, kinase activation, transcriptional regulation and transport of membrane proteins [86]. A simplified rule is that poly-ubiquitylation results in degradation while mono- and multi- ubiquitylation results in other outcomes. Ubiquitylation is carried out by the ubiquitin system and is dependent on ATP and three classes of proteins; E1, E2 and E3 [87]. The specificity of ubiquitylation is mainly mediated by the E2- and E3-ligases. The RING-domain is present in hundreds of proteins and many proteins possessing this domain also function as ubiquitin E3-ligases mediating ubiquitylation of selected target proteins [64]. Many TRIM family members have been identified as ubiquitin E3-ligases, including TRIM22 [65-67]. Since the discovery of ubiquitin, several other ubiquitin like proteins (UBLs) have been identified, including SUMO (small ubiquitin-related modifier), Nedd8 (neural precursor cell expressed, developmentally down-regulated 8) and ISG15 (IFN-stimulated gene 15) [88]. In the context of

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TRIM22, ISG15 might be an interesting UBL since this as well as TRIM22 is induced by IFNs as well as by p53 [89, 90]. ISG15 requires E1-, E2- and E3-enzymes in the same manner as ubiquitin [91]. Interestingly, TRIM25 functions as both an ubiquitin E3-ligase as well as an ISG15 E3-ligase through its RING-domain, revealing the RING-domain to be able to perform both ubiquitylation as well as ISGylation, as well as providing proof that one protein could perform both [77, 92]. Furthermore, a subset of TRIM proteins, including PML and TRIM27, have been shown to be able to perform SUMOylation through theirs RING- and B-box domains. TRIM27 is able to perform both ubiquitylation and SUMOylation, demonstrating the possibility for one protein to perform both [93].

TRIM proteins and viral defence

Several TRIM family members are involved in viral defence, and about 20 TRIM family members have been shown to interfere with the retroviral life cycle [94]. Furthermore, TRIM proteins were shown to act at almost every stage of the viral replication cycle in a screen investigating a panel of TRIM proteins [94]. TRIM5α, the most thoroughly explored antiviral TRIM family member, is capable of repressing replication of HIV-1 as well as several other viruses [92]. Furthermore, the importance of TRIM proteins in viral defence is accentuated since viruses have evolved functions to disable TRIM proteins. For instance, enterovirus 71 induces degradation of TRIM38 and influenza A targets TRIM25 [95, 96]. However, intriguingly, gene silencing of TRIM25, TRIM31 and TRIM62 inhibited viral release, suggesting these proteins to aid viral release [94]. Accumulating evidence also suggests TRIM22 to be important in the defence toward viral infections through inhibition of viral replication [67, 97-103].

TRIM proteins and the immune system

Many TRIM proteins are involved in the immune system. Changed expression of specific TRIM proteins as well as autoantibodies towards TRIM proteins has been detected in a number of autoimmune diseases such as Sjögren´s syndrome and SLE (systemic lupus erythematosus). However, it is unclear whether autoantigens for specific TRIM family members display a role in the disease pathogenesis of these diseases or if they are only markers of the diseases. However, several TRIM proteins have also been shown to function as direct regulators of PRR (pattern recognition receptors) signalling and inflammasome activation (a multiprotein oligomer responsible for activation of inflammatory processes) [104-106].

TRIM22 has been shown to activate NF$_K$B and induce secretion of pro-inflammatory cytokines by the human macrophage cell-line U937 in an NF-κB-dependent manner [107].

TRIM proteins and p53

So far PML and TRIM22 are the only TRIM proteins that have been shown to be induced in response to p53. But, recently, several TRIM proteins have been shown to interact with and regulate p53, positively and negatively. TRIM13 (Ret finger protein 2) ubiquitylates and degrades Mdm2, thus causing stabilisation of p53. TRIM13 also induces apoptosis, suggesting a role as a tumour suppressor [108]. TRIM24 interacts with and ubiquitylates p53, resulting in reduced levels of p53 [109]. TRIM28 directly interacts with Mdm2 and cooperates with Mdm2 in the ubiquitylation and degradation of p53. TRIM28 in cooperation with Mdm2 also causes inhibition of the acetylation of p53 [110]. TRIM29 (ATDC) binds to p53 and counteracts p53-mediated functions through export of p53 from the nucleus to the cytoplasm, thus functioning as an oncogene [111]. Furthermore, overexpressed TRIM29 reduces acetylation of p53 at K120, through degradation by TIP60, which results in an enhancement of cell proliferation and transformation activity. Also UV-induced apoptosis is suppressed by TRIM29 [112]. All this taken together suggests TRIM proteins to be important regulators of p53 function.

Evolution of TRIM proteins

A genomic analysis of the TRIM family revealed human TRIM proteins to basically consist of two groups of genes with distinct evolutionary properties. Group one is composed of proteins with a RING-B1-B2-CC structure in combination with all the variants of C-terminal-domains present in TRIM proteins. Group two is composed of proteins which only hold the B-box 2-domain and in most cases the SPRY-domain. TRIM22 belongs to the second group. Group two is the smallest and most homogenous group. The members of the first group are also present in invertebrates, while group two is absent in invertebrates. Taken together this suggests the second group to be the youngest one. Analysis also suggests the second group to have evolved faster, compatible with roles in defence towards viruses [63]. Also, specific TRIM proteins not present in mammals are present in fish (finTRIMs; fish novel TRIM genes), probably involved in the fish immune response toward viruses [113]. Since group one is the most ancient it is most likely to contain basic functions, whilst the second group may have worked as a reservoir to develop new species-specific functions. However, the second group is not limited to restriction of

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viruses, but several of the members are involved in additional functions, such as apoptosis and differentiation [63].

Furthermore, TRIM22 is positioned in a small cluster at 11p15.4 with three closely related TRIM proteins; TRIM5, TRIM6 and TRIM34. Interestingly, TRIM22 and TRIM5 have been suggested to evolve in a discordant manner. The cow genome has an expanded cluster of TRIM5 genes but no TRIM22 gene, while the dog genome contains TRIM22 but not TRIM5. Both these genes have also been suggested to evolve under strong positive selection. The whole cluster, including TRIM5 and TRIM22 as well as the cow orthologue LOC516599 was present in the last common ancestor of human, cow and dog. However, both TRIM5 and TRIM22 are present in humans. TRIM22 and TRIM5 have a similar domain-structure and are 58% identical in amino acid sequence. They are most dissimilar in their Coiled-coil- and SPRY-domains [63, 114, 115]. As earlier mentioned TRIM5 is known to restrict a plethora of different viruses, and is also recently suggested to work as a PRR (pattern recognition factor) activating innate immune signalling pathways [100, 116-118]. The functions of both TRIM6 and TRIM34 are so far not known, and the literature regarding these two genes is very limited. However, at least TRIM34 have been shown to possess antiviral capabilities [119]. Another, to TRIM22 (and TRIM5, TRIM6 and TRIM34), very closely related TRIM protein that deserves to be mentioned in this context is TRIM21 [63]. TRIM21/Ro52 was first discovered since autoantibodies against the protein are present in Sjögren´s syndrome and SLE (systemic lupus erythematosus). These autoantibodies are clinically used in the diagnosis of these diseases [120]. Also TRIM21 is included in the list of TRIM proteins that works as ubiquitin E3-ligases. Moreover, interestingly, overexpression of TRIM21 has been shown to repress proliferation and induce cell death in B cells [121]. TRIM21 also play roles in the immune system by regulation of IRF-3, IRF-8 and IRF-7 [122-124].

In conclusion, the evolutionary history of TRIM22 suggests a role in viral defence. However, roles in apoptosis, cell proliferation and tumour suppression are not unlikely since other TRIM members (also in the second group) also display these functions.

PML

PML (TRIM19) is the most well studied TRIM protein. It was originally identified as a part of the fusion protein PML-RARα t(15;17) characteristic of acute promyelocytic leukaemia [125]. PML is a versatile protein involved in many cellular responses. It is mainly localised in the nucleus in so called nuclear bodies (NB), which contain a large variety of proteins involved in

cell cycle regulation, tumour suppression and protein translation. Moreover, PML bodies are docking sites for several viruses. The function of PML is linked to interactions with other proteins. PML has been shown to induce cell cycle arrest, cell death, translation inhibition and viral restriction [79]. PML acts both upstream and downstream of p53. PML acts upstream of p53 to enhance transcription of p53-targets by recruiting p53 to nuclear bodies (NBs). PML is also induced by p53 and potentiate the antiproliferative effects downstream of p53. Interestingly, cells lacking PML show a reduced tendency to undergo senescence or apoptosis in response to p53 activation [80].

The translation regulatory activity of PML is mediated through interaction with the translation initiation factor eIF4E. PML binds to eIF4E through its RING-domain and represses mRNA transport from the nucleus to the cytoplasm as well as translation initiation in the cytoplasm [38, 126].

Interestingly, PML and TRIM22 are in some aspects remarkably similar. Both are TRIM proteins induced by both p53 and IFNs and contain a p53 response element in intron 1 in their gene sequence [61, 80, 127]. PML and TRIM22 are so far the only two TRIM proteins induced by both IFNs and p53. Furthermore, both are involved in viral defence. However, the amino acid sequence of PML is not very homologous to TRIM22, and the C-terminal of PML is the EXO III-domain unlike TRIM22s SPRY-domain. Furthermore they are evolutionary quite distantly related [63].

TRIM22

TRIM22 (Staf50) was originally recognised as an IFN-inducible gene in the human lymphoblastoid Daudi cell-line by Tissot and Mechti [102]. Our interest of TRIM22 started when we identified it as a novel target gene to the tumour suppressor protein p53, and found it to contain a p53 response element in intron 1 [61]. In the absence of IFN or p53 stimulation, TRIM22 is highly expressed in lymphoid tissues such as peripheral lymph nodes, thymus and spleen, as well as in peripheral blood leukocytes and in the ovary and lung [102]. In response to either IFNα, β, γ or p53 TRIM22 is strongly up regulated in most cells tested and emerges in screens of IFN-inducible as well as p53-target genes [127-130]. It is also up-regulated in response to LPS in monocyte-derived macrophages and progesterone in breast cancer cells [97, 131]. In contrast it is repressed during T cell activation by anti-CD28 and anti-CD2 monoclonal antibodies [132, 133], suggesting an anti-proliferative role of TRIM22. This is in concordance with data from our lab showing that overexpression of TRIM22 inhibits the

clonogenic growth of monoblastic U937-cells [61], as well as 293T/17- and U2OS-cells (Petersson et al, preliminary manuscript).

TRIM22 has been shown to restrict transcription of the HIV-1 promoter [102], as well as to repress HIV-1 replication [97-100, 103]. It has also been shown to repress encephalomyocarditis virus replication through ubiquitylation of the viral 3C protease ($3C^{PRO}$) [67], as well as to repress hepatitis C replication [101]. Taken together all these results suggest TRIM22 to play an important role in viral defence.

Furthermore, TRIM22 is able to undergo self-ubiquitylation through its RING-domain, and targets a viral component of encephalomyocarditis virus (a picorna virus) with ubiquitin for degradation; the 3C protease ($3C^{PRO}$). The $3C^{PRO}$ is a critical component both in the processing of picornaviral polyproteins and in the inhibition of the cellular defences towards the virus [67].

The clinical data regarding TRIM22 is so far very limited, but TRIM22 has been shown to be u-pregulated in HIV-1 positive individuals [103]. TRIM22 was also found to be up-regulated in an array analysis of human chronically hepatitis C infected liver biospecimens [134].

TRIM22 has also been shown to be up-regulated in some tumours and down-regulated in others [135-138]. However, it is not possible to elucidate the function of TRIM22 from these studies, since we cannot know whether the induction/repression of TRIM22 is specific or is an unspecific response toward p53/IFN-signalling. Neither is it possible to say whether it is regulated to drive or to oppose transformation.

The present investigation

Aims

The general objective of this thesis is to understand the function of the IFN-inducible p53-target gene TRIM22.

Specific aims:

I)   To study the subcellular localisation pattern of TRIM22 in cell-lines as well as in primary cells *(paper I)*

II)   To study the role of TRIM22 in protein translation *(paper II)*

III)   To study the role of TRIM22 in proliferation and cell death *(paper III)*

Experimental considerations

The advantages and disadvantages of the methods used in this thesis are briefly discussed. For a more detailed discussion of the methods utilised in relation to obtained results, please see *papers I-III*.

Cell-lines versus primary cells

Cell-lines are utilised as model systems of normal and malignant cells because of their pronounced experimental advantages. Cell-lines are defined as a population of immortalised cells. They are maintained in cultures over extended periods of time and have an unlimited culture lifespan. To become immortalised they have usually undergone a spontaneous process of transformation, thus expressing a changed genetic repertoire as compared to normal cells. This is a disadvantage if to be used as models for normal cells. However, cell-lines are easy to work with and are easy to transfect, as compared to primary cells, where enough transfected cells are hard to achieve. We have used only human cell-lines to obtain our results. However, when considered relevant, the results have

been reproduced in normal cells, as we have done in human peripheral mononuclear cells in *paper I.*

Cell-line models

The human osteosarcoma cell-line U2OS was used in *paper I*. U2OS-cells are advantageous because they express high levels of endogenous TRIM22 also in the absence of p53 or IFNs. It is also a suitable cell-line for immunoflourescence studies since the cells are large, making it easy to produce comprehensible immunoflourescence pictures. The human kidney cell-line 293T/17 was used in *paper II*, because of its robust proliferation and viability, facilitating reproducible results. In this cell-line the expression of TRIM22 is very low, but can be induced in response to either p53 or IFNs. In *paper III* both U2OS- and 293T/17-cells were utilised. We wanted to continue to use 293T/17-cells since they produced a robust effect in *paper II*. 293T/17-cells do not have active p53 protein, since they express the SV40 large T-antigen, which binds to and inactivates wild-type p53. Therefore, we also used U2OS-, which harbours wild-type p53, in order to compare a wild-type p53 cell-line to a p53 null cell-line.

Transient and stable protein overexpression studies

Transient or stable overexpression of a protein is a common way to study its function. In both cases, the cDNA for the protein of interest is placed behind a strong promoter in a vector construct, which is introduced into cells by transfection-reagents or electroporation. In transient transfections the protein is expressed during a limited period of time, whereas during stable transfection the protein is constantly overproduced. Therefore, these two methods of overexpression may give different pictures of the function of a protein. To get a picture of the immediate effects of overexpression of TRIM22, in *paper II & III* we mainly utilised transient transfections, utilising eGFP-tagged TRIM22 as described in the section below. By these means, we demonstrated that TRIM22 has cell death-inducing and anti-proliferative properties. These properties may explain why U2OS-cells do not tolerate stable overexpression of TRIM22 in *paper III*. In stable transfection, the protein of interest is placed in a vector that might also contain an additional gene conferring resistance to a certain antibiotic. In this manner positive cells can be selected using antibiotics, creating a stable overexpressing cell-line. It is, however, important to interpret the data with caution since overexpression of a protein often results in higher levels of the protein than in an endogenous setting. Furthermore, since the overexpressed protein is

not under the control of its own promoter, it cannot be regulated by feedback mechanisms, such as transcriptional up- or down-regulation, as its endogenous counterpart would be subject to.

Tagged TRIM22

In *paper II and III*, we are in some cases using eGFP (enhanced Green Fluorescent Protein)-tagged TRIM22. Tagging a protein with eGFP makes it easy to sort cells expressing the protein of interest. A fluorescence activated cell sorter (FACS) can be utilised to sort eGFP-expressing cells. FACS is a method where individual cells in a liquid suspension are studied. Different proportions of the cells are determined through the size, granularity, fluorescence (e.g. in the case of fluorescent proteins) as well as fluorescent labelling of different proteins with antibodies. Sorting of cells provide the opportunity to obtain a pure population of cells with certain properties. Drawbacks when sorting cells include a higher risk of infections as well as possible toxicity from the FACS flow. Also, long-lasting sorting sessions may result in compromised viability of the cells.

Since TRIM22 is an antiproliferative or/and cell death-inducing protein the phenotype is easy to neglect when studying an unsorted bulk of transiently transfected cells. Here, cells not successfully transfected, rapidly take over the culture masking the effects of TRIM22. Indeed, even when we sorted eGFP-positive cells, the phenotype is to some extent disappearing over time since cells losing the transient expression take over the culture. However, repeated FACS analysis of cells expressing eGFP-tagged TRIM22 allowed us to capture the effects of TRIM22 in *paper III.* Then again, eGFP is a large protein and might change a proteins localisation as well as function. Furthermore, GFP has in some cases been shown to induce cell death [139, 140]. Therefore, we have limited the use of TRIM22-eGFP to the experiments where it has been required to sort the cells, and in *paper III* we reproduce our results without the eGFP-tag using a stable cell-clone-formation-assay, based on antibiotic resistance.

Deletion mutants of TRIM22

To map the functionally important domains of TRIM22 the RING-, Coiled-coil- and SPRY-domain of TRIM22 were deleted, producing proteins of 50 kDa, 43.8 kDa and 40.3 kDa, respectively. For information regarding the localisation of the different domains within the TRIM22 sequence, the gene-information provided at NCBI was used. The RING-domain was defined as aa´s 14-63, the B-box as aa´s 92-133, the Coiled-coil-domain as aa´s 132-

248 and the SPRY-domain as aa´s 352-496 [166]. The deletion-mutants were sequenced and in vitro-translated in order to ensure the correct sequence and molecular size. In the case of TRIM22-delRING, also the short 13 aa´s residue sequence in front of the RING-domain was deleted. However, surprisingly, the TRIM22-delRING migrated on a gel at the same rate as full-length TRIM22, despite a difference in size of approximately 7 kDa. Nevertheless, *in vitro* translated TRIM22-delRING migrated as expected according to its size. We cannot explain the discrepancy in SDS-PAGE migration between *in vitro* and *in vivo* translated TRIM22-delRING. However, a larger size of *in vivo* translated than *in vitro* translated TRIM22-delRING suggests the *in vivo* translated TRIM22-delRING to be post-trancriptionally modified *in vivo.* However, full-length TRIM22 is suggested not to be modified in the same manner since the *in vivo* produced two proteins migrated in the same manner on the gel. An explanation could be that ubiquitin or another of the ubiquitin-like proteins is transiently attached to TRIM22, and subsequently moved to a target protein through the E3-ligase RING-domain. A TRIM22 protein lacking the RING-domain would thus be stuck with the attached UBL since it lacks the E3-ligase activity. The difference of 7 kDa would approximately match an ubiquitin molecule (9 kDa).

Specificity of the TRIM22 antibodies used

We have used two commercially available TRIM22 antibodies from Atlas antibodies (rabbit, polyclonal) and Abnova (mouse, polyclonal) as well as one produced by our collaborator Anna-Maria Herr (rabbit, polyclonal) [141]. Of course specificity is vital when using antibodies, and we have evaluated them all. Monoclonal and polyclonal antibodies differ in specificity; monoclonal antibodies consist of only one antibody subtype and only detect one epitope on the antigen whereas polyclonal antibodies consist of a heterogeneous mix of antibodies of different affinities and therefore detect several epitopes of the antigen. Therefore, polyclonal antibodies are more prone to give background signals, and specificity has to be carefully tested. Furthermore, specificity is especially important when studying localisation of proteins by immunoflourescence, as done in *paper I*. Here, the TRIM22 antibodies from Atlas and Herr were utilised. The specificity of the antibody obtained from Herr has been evaluated in her paper [141]. Since the unspecificity linked to polyclonal antibodies could indicate false positive results, and also because we did not posses the preimmune rabbit serum as a control, in addition to the controls provided in *Paper I,* an additional experiment was performed. The Atlas antibody is

made by rabbit immunisation against a peptide of the Coiled-coil-domain of TRIM22. Therefore, this antiserum should not react to TRIM22 with a deletion of the Coiled-coil-domain. As demonstrated in the Western blots in figure 5, this is exactly the case. Here, the antibody by Atlas detects full-length TRIM22 but not TRIM22-delCoiled-coil (panel B). An anti-his antibody detects both his-tagged full-length TRIM22 as well as his-tagged TRIM22-delCoiled-coil (fig 5A). As expected, also bands for endogenous TRIM22 are detected utilising the Atlas anti-TRIM22 antibody (fig 5B).
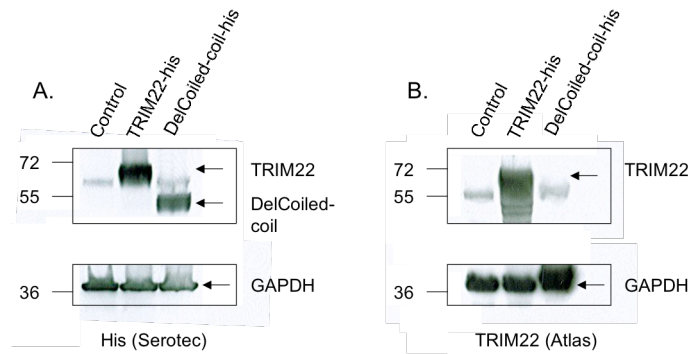
Fig 5: *A. The mouse monoclonal anti-His antibody (Serotec) recognises both his-tagged full-length TRIM22 as well as TRIM22-delCoiled-coil. B. The rabbit polyclonal anti-TRIM22 antibody (Atlas antibodies) detects full-length TRIM22 but not TRIM22-delCoiled-coil. As expected also endogenous TRIM22 is recognised by the anti-TRIM22 antibody.*

Results from immunoflourescence were similar; full-length TRIM22 is detected by anti-TRIM22 from Atlas antibodies but not TRIM22-delCoiled-coil. These results strongly suggest that the Atlas antibody indeed is specific for TRIM22. However, when we needed to blot for the TRIM22-delCoiled-coil construct, we used the Abnova antibody, a mouse polyclonal antibody made by immunisation of the whole TRIM22 protein.

Luciferase reporter assay

In order to test the effect of TRIM22 on protein translation, luciferase reporter experiments with multiple promoters were performed (*paper II*). The luciferase reporter system is most commonly used to study transcriptional regulation, but in combination with measurements of mRNA levels, estimation of the amount of luciferase protein by luminescence

measurement can also be used to study translation. Initially, we utilised the dual luciferase system, consisting of a plasmid with a promoter of interest conjugated to a firefly (Photinus pyralis) luciferase reporter gene, and a second plasmid with a control promoter conjugated to a renilla (Renilla reniformis) luciferase reporter gene. The renilla luciferase activity is used as an internal control to compensate for inter-experimental variability, such as cell viability and transfection efficiency. But, since TRIM22 suppressed luminescence from all construct used, also renilla luminescence was suppressed, making it impossible to use as an internal control. However, numerous experiments with the p21-, Bax-, NF$_K$B-, cyclin E- and SV40-promoters resulted in reproduction of the effect, supporting the reliability of the technique even without the internal renilla control.

Polysome fractionation

Polysome fractionation was performed in *paper II* in order to further characterise TRIM22 as a regulator of translational initiation. Polysome fractionation is a method for investigating the association of mRNAs with the translation machinery under varying conditions. When an mRNA is actively translated, it is associated with at least one ribosome. The number of ribosomes attached determines how effectively the mRNA is translated. An mRNA with attached ribosomes is termed polysome. Centrifugation of RNA through a sucrose gradient enables separation of mRNA attached to a different number of ribosomes as well as free, inactive ribosomal 40S, 60S and 80S subunits. In this manner one can determine how active the translation machinery is. Repression of translation initiation increases the amount of free 40S, 60S and 80S subunits and decreases the amount of polysomes, since polysomes cannot be formed. In contrast, repression of translation elongation increases the fraction of polysomes, since the ribosomes are stalled upon the mRNA. However, when we performed polysome fractionation upon TRIM22 transfected cells we observed an increase in the inactive subunits, and especially free 80S subunits, but no decrease in the polysome fraction. This suggests repression of translation initiation, thus in line with our other results, but we do not entirely understand why we lack the decrease in the polysome fraction. One possible source of error is that while performing polysome fractionation it was not possible to FACS-sort transfected TRIM22-expressing cells as performed in some of the other experiments. Running polysome fractionation requires a minimum of 10-15 millions of cells. To avoid unspecific polysome run-off, the cells need to be pre-treated with cycloheximide to stall the polysomes. However, too long exposure to

cycloheximide is toxic for the cells, making it impossible for the cells to survive during a FACS-sorting procedure. Evaluation of eGFP positive cells revealed a transfection efficiency of approximately 20-30 %. Therefore, since only 20-30% of the studied cell population expresses TRIM22 in the polysome fractionation experiments, it is not surprising that the TRIM22-related effects are modest.

Cell-titer test

The cell-titer96®AQ$_{ueous}$ one solution cell proliferation assay (here after referred to as cell-titer test) from Promega was used to measure proliferation in *paper III*. For cells growing in suspension, counting of cells in a light microscope is a usual way to measure proliferation, and inclusion of trypan blue also provides a measurement of cell death. However, since we used adherent cells this method would require removal of the cells from the bottom of the wells, by trypsination. This yields an uncertainty whether all the cells have been successfully removed from the bottom of the wells or not, and thus an uncertainty in the method. However, the use of the cell-titer technique for measurement of proliferation is widely spread. Cell-titer is a colorimetric assay, and it determines the amount of viable cells in the wells, thus providing a tool for measuring proliferation or cell death. It contains a MTS tetrazolium compound that is bioreduced by the viable cells into a soluble coloured formazan product, which is measured by absorbance at 490 nm. The conversion of MTS tetrazolium is thought of as being accomplished by NAPDH or NADH produced by dehydrogenase enzymes in metabolically active cells, but the mechanism is yet not entirely known. According to the manufacturer the absorbance at 490 nm is directly proportional to the number of living cells in the culture. However, because levels of NAPDH and NADH may sometimes reflect the metabolic state of a cell, the test probably does not exclude the possibility of an effect on the cell metabolism rather than on proliferation. Therefore, ideally, complementary methods should be used in order to ascertain a pure anti-proliferative effect. For this purpose, methods such as [3H]thymidine-incorporation and cell cycle analyses could be considered.

General discussion

TRIM22 was originally discovered in 1995, during a screening of IFN-inducible genes in the human lymphoblastoid Daudi cell-line [102]. It was introduced into our research group when Susanna Obad found it to be a p53-target gene [61]. We find TRIM22 a protein of major interest since it links tumour-suppressing pathways with pathways of the immune response, due to its dual properties as a p53-target gene as well as an IFN-inducible gene. When the work for this thesis was started, the functions and mechanisms of TRIM22 were poorly elucidated. TRIM22 was known to repress transcription of the long terminal repeat promoter region of HIV-1, suggesting an antiviral effect [97]. During our work we have studied the subcellular localisation and function of TRIM22. Our main hypothesis was centred on the possibility that TRIM22 might be a novel tumour suppressor. However, the possibility of the contrary, that TRIM22 might repress p53-function and induce malignancy, was never ruled out.

TRIM22 localises to both nucleus and cytoplasm

In *paper I* we found endogenous TRIM22 to be localised both to the nucleus and cytoplasm in the human osteosarcoma cell-line U2OS as well as human peripheral mononuclear cells (PBMCs). We found TRIM22 to stain the cytoplasm in a diffuse manner, and the nucleus in a speckled manner. Thus, TRIM22 probably has functions both in the nucleus as well as in the cytoplasm. This is in agreement with Sivaramakrishnan et al [131], who also found TRIM22 to be localised both to the nucleus and cytoplasm. Furthermore, consistent with our findings, they found TRIM22 to form nuclear bodies in the nucleus, similar to the nuclear bodies formed by PML [142]. However, we have evaluated the expression of TRIM22 compared to the expression of PML, but have not found any co-localisation, suggesting TRIM22 not to co-localise to the nuclear bodies harbouring PML. Nevertheless, the data in this thesis are mainly focused on the function of TRIM22 in the cytoplasm.

TRIM22 localises to the centrosomes and ER

Our immunoflourescence data also revealed TRIM22 to co-localise with the centrosomes and the endoplasmatic reticulum (ER) in the cytoplasm. Furthermore, the centrosomal localisation was preserved throughout the cell cycle. The centrosomes are structures in the cell, essential for mitosis,

and also for the organisation of the microtubule network. The centrosomes consist of two centrioles, the surrounding pericentriolar material (PCM) and an additional number of proteins, such as 14-3-3σ, cyclin E, cyclin A and Cdk2 [143]. Interestingly, the PCM is also a localisation for proteasomes and chaperones, and is a site where degradation of proteins occurs. When proteins are misfolded in the cytoplasm, they can form aggregates which are transported on the microtubules to the centrosome where they are degraded [144]. By these means they create a scenario where the role of TRIM22 as a ubiquitin E3-ligase might fit in. Centrosomal TRIM22 might target these proteins with ubiquitin to induce their degradation in the proteasomes. However, when the degradational capacity of the proteasomal machinery is exceeded, aggregates accumulate and form aggresomes close to the centrosomes. Vimentin filaments surround these aggresomes [145].

After protein translation secretory and transmembrane proteins are transported through the ER, where they are folded correctly, and subsequently sorted in the Golgi apparatus before being transported to the outside of the cell. In the ER proteins go through a "quality control" mechanism, and if incorrectly folded they are retrotranslocated back to the cytosol and transported to the proteasomes for degradation [144]. This constitutes a connection between the ER and the centrosome, compatible with the localisation of TRIM22 to both these compartments. Also in this scenario TRIM22 would fit in as a ubiquitin E3-ligase (fig 6).
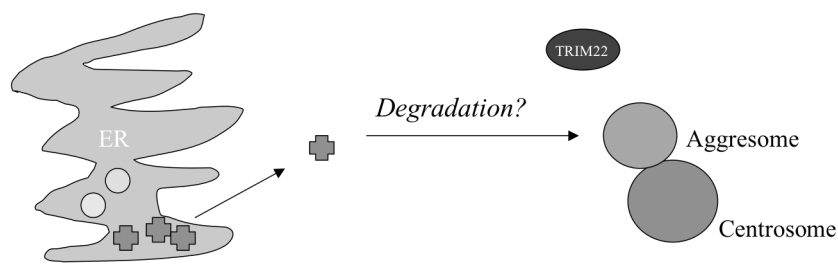
Fig 6: A hypothetical explanation of the localisation of TRIM22 to the centrosome and ER. Incorrectly folded proteins in the ER are transported to the cytoplasm, where they form aggregates and are transported to the centrosome area for degradation by the proteasomal machinery. The unfolded proteins are targeted by ubiquitin E3-ligases, such as TRIM22?

TRIM22 and the UPR

When the ER is overloaded with unfolded and/or misfolded proteins, the unfolded protein response (UPR) is triggered. The UPR pathway induces increased transcription of chaperones increasing protein folding, decreases protein translation to diminish the protein overload, and increased protein degradation. If all these measures fail, apoptosis is induced. Interestingly, many tumour cells rely on the ER folding machinery to correctly fold key signalling pathway proteins. Furthermore, the UPR has been shown to be important for cancer cells in order to survive the unfriendly tumour microenvironment. GRP78 (BiP) is a marker of activated UPR and increased GRP78 levels correlates with higher pathological grade, recurrent state and poor survival in several cancer types. Therefore, several UPR targeting cancer drugs are in development and in clinical trials [146].

As shown in *paper I* a subset of the U2OS-cells displayed distinct accumulations of TRIM22 in the ER. When we starved the cells by lowering the amount of serum in the culture medium, the amount of cells with distinct TRIM22 accumulations in the ER increased. These TRIM22 accumulations co-localised with vimentin filaments, thus resembling vimentin-enclosed aggresomes. Vimentin is an intermediate filament (IF) protein, which along with tubulin-based microtubules and actin-based microfilaments constitutes the cytoskeleton. Interestingly, serum starvation is one of the triggers of the UPR pathway [147]. However, we do not entirely understand the ER associated aggregates of TRIM22, but speculate that TRIM22 might be involved in the UPR. However, the possibility that TRIM22 is simply one of many proteins degraded in the UPR exists.

When the aggregation of unfolded proteins exceeds the degradational capacity of the aggresomes autophagy is induced. Autophagy is a more efficient way to get rid of unfolded proteins as well as to degrade large cellular structures such as mitochondria and peroxisomes. The components meant for degradation are engulfed by autophagosomes that fuses with lysosomes [144]. The connection between the UPR and autophagosomes leads our thoughts to this mechanism. Therefore we immunostained cells with TRIM22 and the autophagosomal marker LC3, as well as the 20S proteasome, to see whether TRIM22 was involved. However, no co-localisation could be shown with neither of the markers. In conclusion, TRIM22 could be involved in the UPR, but no proof of this has yet been obtained.

The centrosomes as viral assembly factories

An additional hypothesis regarding the localisation of TRIM22 to the centrosomes and ER adresses the role of TRIM22 as a repressor of viral replication. Virally infected cells harbour similar structures to pericentrolar aggresomes, which are thought to function as viral replication and assembly factories. Similar to aggresomes and to the TRIM22 aggregates formed in the ER of starved cells, these pericentriolar aggresomes are also interspersed with vimentin. Furthermore, many viruses have been suggested to use the aggresome pathway by transporting their proteins along the microtubules to the virus assembly factories. Disruption of the microtubules does in the case of some viruses, e.g. herpes viruses and retroviruses disrupt the virus factories. It is, however, not yet clear whether the viruses actually use the aggresomal pathway to facilitate their replication and assembly or if the aggresomes are part of an innate cellular response recognising viral components targeting them for storage and degradation [147, 148]. Moreover, some viruses are capable of hijacking the centrosomes and impair cell cycle progression. In the case of HIV-1, infected cells have been shown to accumulate in the G2 phase in vitro [149]. Consequently, the role of TRIM22 as a viral repressor and its localisation to the centrosome makes it plausible that TRIM22 targets viral replication at a centrosomal localisation.

Interestingly, HIV-1 Gag has been shown to transiently accumulate at the centrosome. The Gag protein is necessary for assembly of the HIV-1 virus to occur, for release of HIV-1 virions from the host-cell, as well as for other steps during viral replication [150]. It is thought that the assembly of the HIV-1 Gag protein with viral genomic RNA (gRNA) takes place at the centrosome [151]. This is interesting in the aspect of TRIM22 since TRIM22 has been suggested to bind to HIV-1 Gag and inhibit the transport of the virus to the cell membrane, where the release of a mature HIV-1 particle takes place. However, pulse chase experiments performed by Barr et al [98] revealed HIV-1 Gag not to be degraded by TRIM22, suggesting the inhibitory mechanism not to be due to degradation. Interestingly, Gag needs to be ubiquitylated in order for assembly and release of HIV-1 virions to occur. Furthermore, siRNA mediated knockdown of the ubiquitin like protein ISG15 have been shown to disrupt the IFN-mediated inhibition of HIV-1 replication and ISG15 has been suggested to inhibit the ubiquitylation of the Gag protein, thus inhibiting HIV-1 replication [91, 152, 153]. Also this is interesting in regard to TRIM22. We speculate that TRIM22 could be an ISG15 E3-ligase responsible for ISGylation of target proteins. Both TRIM22 and ISG15 are up-regulated in response to both p53- and IFN-induction [89, 90]. Both genes are involved in antiviral suppression. Furthermore, TRIM22 is a ubiquitin E3-ligase, and TRIM25 has proven a protein to be able to be both a ubiquitin and ISG15 E3-ligase

simultaneously [77, 92]. All taken together, makes it tempting to believe that TRIM22 might repress the replication of HIV-1 by ISGylation of Gag (fig 7). However, future experiments will relieve whether this is true.

In any case, the role of TRIM22 as an antiviral repressor is supported by increasing amount of evidence [67, 97-102], and therefore repression of viral assembly at a centrosomal localisation would not be surprising.
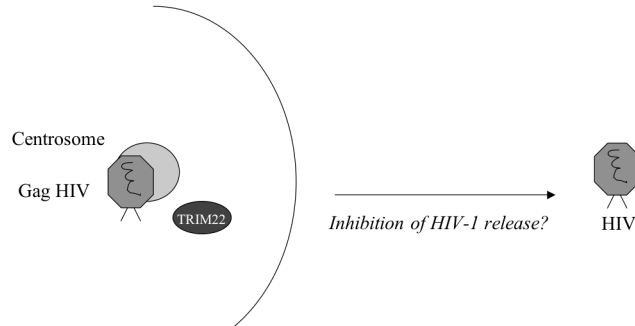
Fig 7: A hypothetical explanation of the role of TRIM22 at the centrosome; TRIM22 might interfere with the release of HIV-1 at a centrosomal localisation, through ISGylation of HIV-1 Gag.

TRIM22 represses protein translation

In *paper II* we reveal TRIM22 to be a repressor of protein translation. Translation is a very expensive mechanism in terms of ATP consumption, and the ability of the cell to control translation provides a rapid way to respond to environmental cues such as lack of nutrients and oxygen, as well as to avoid production of unwanted proteins. Consequently the initiation of translation is highly controlled [37].

From our data, as examined by production of luciferase protein and global protein synthesis experiments, we observe a TRIM22-mediated inhibitory effect on protein translation. As previously described in *paper I,* we suspected a correlation of TRIM22 to the UPR. One of the consequences in response to activation of the UPR pathway is a decrease in protein translation, effected by down regulation of the translation initiation factor eIF2α. Thus, we have performed experiments to evaluate if TRIM22 affects either eIF2α levels or phosporylation status, but we could detect neither. Protein translation is most commonly regulated at the initiation of translation. The two most highly regulated translation initiation factors are eIF2α and eIF4E. Since the TRIM protein PML is known to repress translation by binding to eIF4E, our next move was to investigate whether

also TRIM22 interacts with eIF4E. Indeed, reciprocal co-immunoprecipitation experiments revealed TRIM22 to interact with eIF4E. Furthermore, when eIF4E was pulled down with a synthetic cap-analogue, also endogenous TRIM22 was pulled down, further confirming the interaction with eIF4E. However, our experiments do not reveal whether this interaction consists of a direct binding of TRIM22 to eIF4E or whether the binding is indirect. Since eIF4E functions in a complex associated with other proteins and RNA, the interaction could also be due to binding to either an associated protein or associated RNA. Even though further experiments are warranted to elucidate this matter, the interaction of TRIM22 with eIF4E still is an in our opinion important observation.

eIF4E belongs to the eIF4F complex, which in addition to eIF4E consists of the scaffold protein eIF4G and the mRNA unwinding protein eIF4A. In order for translation to occur, eIF4E binds to the cap of the mRNA, whereupon the rest of the eIF4F complex is assembled. The binding between eIF4E and eIF4G is an essential step for the formation of the eIF4F complex. Therefore, we next examined whether eIF4G was pulled down with cap-analogue-precipitated eIF4E in the presence of TRIM22. Indeed we did find significantly less eIF4G when TRIM22 was overexpressed, suggesting TRIM22 to repress the binding of eIF4E to eIF4G (fig 8).

Protein translation occurs from free ribosomes in the cytoplasm as well as from membrane-bound ribosomes at the cytoplasmatic side of the ER. Thus, the localisation of TRIM22 in the cytoplasm (as shown in *paper I*) is in agreement with a role of TRIM22 in translation. Furthermore, we also found TRIM22 to co-localise with the ER. Thus TRIM22 could also be associated with ribosomes at an ER-localisation.

In conclusion, the translational repression mediated by TRIM22 is probably uncoupled from a possible involvement of TRIM22 in the UPR, but rather dependent on the assembly of the eIF4F complex.

The translational repression is independent of the RING-domain

Interestingly, in *paper II* the translational repression mediated by TRIM22 was shown to be independent of the RING-domain. Also the localisation of TRIM22 to the centrosome shown in *paper I* was independent of the RING-domain. This indicates the translation repression meditated by TRIM22 not to be dependent on the E3-ligase activity of TRIM22, since the RING-domain is required for the E3-ligase activity of TRIM22 [66, 67].

Translation initiation:
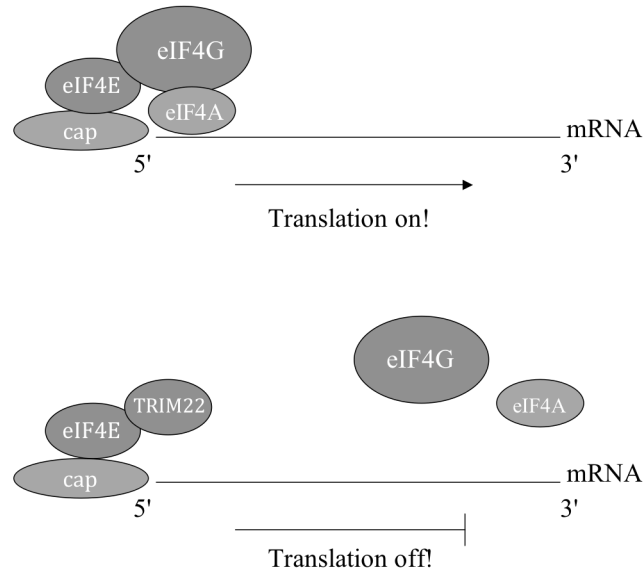
Fig 8: hypothetical role of TRIM22 in translation initiation repression; TRIM22 binds to eIF4E, thus inhibiting the binding of eIF4G by sterical obtrusion.

However, since the independency of the RING-domain for TRIM proteins is somewhat controversial we did also compare our TRIM22-delRING-construct, to TRIM22-delRING-constructs used in other publications. Two conserved cysteine residues (C15/C18) in the RING-domain were included in the deletions of the RING-domain in all publications we have found. These two residues have been shown to inactivate the ubiquitin E3-ligase activity in other TRIM proteins [154-156]. In some publications constructs specifically mapping C15/C18 are utilised, and show that substitution of these residues are enough to remove the capability of TRIM22 to function as a ubiquitin ligase (exchange of C15 to A15) [66], up-regulate NF_KB (exchange of C15 to A15) [107], restrict hepatitis B (exchange of C15 to A15) [101] (all these three publications uses the same construct) and restrict HIV-1 particle production (exchange of C15 and C18 to A15 and A18)[98]. Eldin et al [67] deleted the entire RING-domain and abolished TRIM22 ability to function as a ubiquitin E3-ligase. In contrast, Kajaste et al [99] revealed the RING-domain to be dispensible for inhibiting HIV-1 transcription using the same construct as Eldin et al [67] as well as a specific C15/C18 to A15/A18 construct. In conclusion, our TRIM22-

delRING-construct includes deletion of the crucial C15/C18 residues, thus suggesting our effects to be truly independent of the RING-domain and E3-ligase activity. Furthermore, the viral restriction ability of the closely related gene TRIM5α seems to depend on the RING-domain depending on a combination of host and virus [157]. As well, TRIM21 interacts and activates IRF-3 independently of its RING-domain [122]. Taken together this suggests these proteins to have abilities that are independent of the RING-domain.

Importantly, TRIM22-delCoiled-coil and TRIM22-delSPRY did not repress translation to the same extent as full-length TRIM22, as shown by luciferase experiments, thus confirming specificity of the effect. The Coiled-coil-domain is believed to mediate protein-protein interactions [69], suggesting that the translation inhibitory effect of TRIM22 may depend on disturbed interaction of TRIM22 with one or more of its interaction-partners. As can be observed from our data in *paper II*, in the case of TRIM22, the deletion of the Coiled-coil-domain suggests the TRIM22 protein to be more instable since less protein is visible on a Western blot compared to full-length TRIM22. Hence, it cannot be excluded that a mere reduction in levels of TRIM22 explains the loss of translation inhibition mediated by TRIM22-delCoiled-coil.

Interestingly, consistent with our data, the SPRY-domain has been shown to be of particular importance for some TRIM proteins. Regarding TRIM22, Herr et al [141] found the SPRY-domain in rhesus versus human TRIM22 to alter the localisation of TRIM22 in a species-specific manner, suggesting the SPRY-domain to be of importance for the subcellular localisation of TRIM22. For TRIM5α the SPRY-domain has been shown to play a major importance for the potency and specificity of restriction of particular retroviruses. Actually, the substitution of one aa is enough to abolish the potent HIV-1 restriction activity of rhesus TRIM5α [72, 73]. Furthermore, the SPRY-domain has been suggested to be involved in protein-protein interactions. The SPRY-domain has also been subject to positive selection of the TRIM proteins, giving rise to different restriction patterns towards different viruses [63]. Different primate species harbouring TRIM5α, has shown that there are some parts of the SPRY-domain that has shown to be hotspots for mutational changes [158]. In the context of TRIM22 and its anti-translational activity, it would be interesting to investigate whether mutations in these regions would be important for the effect.

TRIM22 probably mainly represses translation of "weak" mRNAs

One of the functions of the eIF4F complex is to unwind the secondary structure of the mRNA, allowing the translation machinery to "reach" the mRNA. Consequently, dependency of eIF4E and the eIF4F complex is higher for some mRNAs than others, depending upon the secondary structure of the mRNA. Since the eIF4F complex unwinds the secondary structure of the mRNA, mRNAs with a more complex secondary structure are more highly dependent on eIF4F. Typically oncogenes, such as cyclin D, cyclin E, c-myc and HIF-1α, are translated from mRNAs with a more complex structure and long 5′ and 3′ UTRs, and are thus more dependent upon eIF4F. These mRNAs are often referred to as "weak" mRNAs. Housekeeping genes, such as GAPDH and actin, are translated from mRNAs with a simple structure and short 5′ and 3′ UTRs and are easily translated even when low levels of eIF4F complex are present in the cell.

Thus, this constitutes a control mechanism in the cell, where the cell can control the production of proteins involved in proliferation without affecting the crucial housekeeping genes. Moreover, different genes are controlled differently depending on cellular and tissue context. Since TRIM22 affects the binding between eIF4E and eIF4G and thus the formation of the eIF4F complex, we hypothesise that TRIM22 primarily affects the translation of certain mRNAs, which are especially dependent on eIF4F for their translation. This is indeed true for other proteins affecting the binding between eIF4E and eIF4G. For example 4E-BP1 inhibits this interaction and affects translation of a subset of mRNAs, particularly dependent on eIF4F for their translation [159]. In addition, induction of eIF4E levels or activity, does not primarily lead to an increase in global translation but to an increase in translation from a subset of mRNAs. These observations further strengthen our assumption that the TRIM22 mediated inhibition of the eIF4F complex leads to inhibited translation of a subset of mRNAs. Finding these certain mRNAs is however not an easy task, since the mRNAs affected are tissue specific. However, we have investigated some of the proteins usually regulated in this manner, but did not find any effect on cyclin E, c-myc, Pim-1 or Survivin. However, IRF-7C was strongly repressed in the presence of TRIM22, thus supporting our theory.

Further clues regarding the translation repression effect of TRIM22 might be obtained by further studies on the target mRNAs of the translational repression of TRIM22. Proteinarray techniques or mRNA screening techniques on polysome fractions may reveal a signature of the translational repression of TRIM22. Hopefully, this will contribute to

increased understanding of the complex interplay between inflammation and cancer, and perhaps also the evolution of new therapies within these fields.

TRIM22 represses IRF-7C protein levels

Since IRF-7 (interferon regulatory factor 7) mRNA is known to be highly dependent upon eIF4F for its translation, we tested whether TRIM22 did decrease IRF-7 protein levels. To that end, TRIM22 was overexpressed in 293T/17-cells and IRF-7 protein levels were evaluated with Western blot. Also mRNA levels were measured in order to exclude the possibility of TRIM22 to repress transcription of IRF-7. IRF-7 is primarily expressed in spleen, thymus and peripheral blood leukocytes [160]. Normally, four different isoforms of IRF-7, of varying size, are encoded by alternative splicing. 293T/17-cells did express IRF-7 but only the smallest isoform; IRF-7C. However, indeed TRIM22 did significantly repress the IRF-7C protein levels. Interestingly, this isoform of IRF-7 has been shown to block the expression of IFNα/β, otherwise induced by IRF-7 A, B and D. Moreover, IRF-7C has oncogenic properties [161]. This leads us to the speculation that TRIM22 could stimulate IFNα/β production through repression of IRF-7C, and thereby block the oncogenic potential of IRF-7C (fig 9A). However, since the 293T/17-cells we performed the experiments in did not express the other IRF-7 splicing isoforms, we do not know if TRIM22 also suppresses these. Another, also very interesting, scenario would be that TRIM22 represses not only IRF-7C, but all IRF-7 isoforms, thus establishing a negative feedback loop towards IFN production and innate immune signalling (fig 9B).

Tight control of IFN production is necessary to avoid harmful consequences in the cells as well as autoimmunity. The closely related protein TRIM21, does indeed repress IRF-7 levels, and forms a negative feeback loop towards IFN production. However, the mechanism is different from the one executed by TRIM22; TRIM21 targets IRF-7 for degradation [124].

Taken together, our results show TRIM22 to repress IRF-7 but further studies are warranted to understand the role of TRIM22 in regulation of IRF-7 and IFNs.

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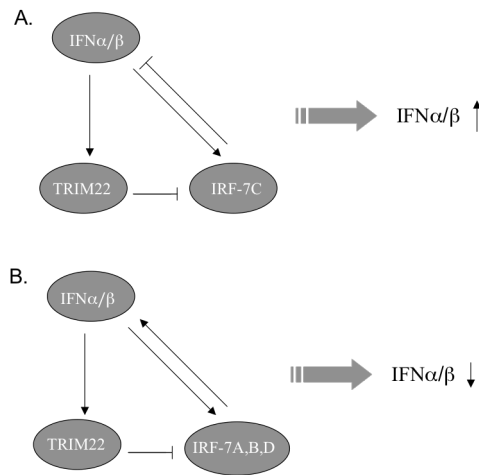
Fig 9: *Hypothetical illustration of the effect of TRIM22 on IFN production. A. IRF-7C and TRIM22 is induced by IFN. Since IRF-7C inhibits IFN production, TRIM22 would in this context stimulate excretion of IFNs. B. IRF-7 isoforms A-D and TRIM22 is induced by IFN. Since IRF-7A, IRF-7B and IRF-7D induces IFN, hypothetical TRIM22-mediated inhibition of these isoforms would in this context inhibit IFNs, thus mediating a negative feedback loop towards IFNs.*

TRIM22 represses proliferation and induces cell death depending on cellular context

In *paper III* we show TRIM22 to repress proliferation in 293T/17-cells, and to induce cell death in U2OS-cells. One feature distinguishing these two cell-lines is their p53-status. In 293T/17-cells, p53 is inactivated through the SV40 large T-antigen, whereas U2OS-cells express wild-type p53. Therefore it could be speculated that the p53-background is necessary to induce cell death in response to TRIM22. However, this comparison is unfortunately very limited since two completely different cell-types from different tissues have been used, and therefore a mere comparison of p53-status does not cover their diverging cellular contexts. To further characterise the importance of p53 with regard to these phenotypes, better models need to be used, e.g. U2OS (wt p53) versus SAOS (null p53), a common model for p53-studies [162]. However, also this model has limitations since SAOS and U2OS, although being the same cell-type are different cell-lines, probably harbouring different mutations resulting in their transformation. A better choice would be to use the same cell-line with an inducible p53 protein. Thus, more experiments and a better p53-model need to be utilised in order to establish the role of TRIM22 in the presence/absence of p53. In particular experiments regarding post-translational modifications of p53 in the presence of p53 would perhaps elucidate the role of TRIM22 with

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regard to p53. We have already performed Western blot experiments using a pan-p53 antibody in 293T/17- and U2OS-cells overexpressing TRIM22, but we have not observed any effect either in p53-stability nor in its size. Post-translational modifications in terms of addition of proteins such as ubiquitin or SUMO probably should have been visible in these experiments. However, yet again SUMOylation is very transient, and might be lost during a simple Western blot. Furthermore, we have not yet tried antibodies targeting phospo-specific forms of p53. Since p53 executes different outcomes in cells depending on phosporylation status, this would be a very interesting future experiment.

Interestingly, others have established cell-lines stably overexpressing TRIM22, when using retroviral or lentiviral transient transfections, demonstrating that it is possible to overexpress TRIM22 in the cell-lines U-937, A3.01 [99], HOS-CD4/CXCR4 [98] and HeLa [70]. However, we speculate that these cells have acquired resistance towards the proliferative-inhibiting and cell death-inducing features of TRIM22. In fact, we have also established a cell-line with an inducible vector of TRIM22 (GeneSwitch, Invitrogen) in 293-cells. However, unfortunately, the vector is allowing expression of TRIM22 also when not induced, by this means creating a cell-line stably overexpressing TRIM22. As a consequence, no phenotype regarding proliferation is observed when further inducing TRIM22, leading us to the speculation that these cells have acquired resistance toward TRIM22.

Interestingly, similar to the translation repression effect of TRIM22, the TRIM22 deletion mutant lacking the SPRY-domain did not suppress colony establishment in U2OS-cells to the same extent as full-length TRIM22. As discussed regarding the translation repression effect, the SPRY-domain has been shown to be important for the subcellular localisation of TRIM22. Interestingly, the lack of the SPRY-domain has been shown to exclude TRIM22 from entering the nucleus [141, 163]. This raises the possibility of that TRIM22 exerts its actions regarding cell death of U20S-cells in the nucleus. Interestingly, activated p53 primarily executes its actions in the nucleus, through its role as a transcription factor.

A common mechanism to affect cell death in U2OS- and decreased proliferation in 293T/17-cells?

Could TRIM22 affect the proliferation and cell death in *paper III* by a common mechanism? Hypothetically, the indication that TRIM22 inhibits translation of certain mRNAs (*paper II*), with are more dependent on eIF4F for their translation, could explain both TRIM22 induced proliferation arrest and cell death. It is possible that inhibition of proliferation related proteins (e.g. cyclins) dominates in the first scenario, while inhibition of

survival-related proteins (e.g. bcl-2, bcl-xl) dominates in the latter. The mRNAs most severely affected by disrupted eIF4F formation differ between different cell types [159]. Furthermore, the observation that the SPRY-domain, but not the RING-domain, is essential both for intact TRIM22 mediated translation inhibition and repression of cell clone-formation connects the two TRIM22-mediated effects. Translational repression as a way to repress proliferation is also a common way to regulate proliferation for the cells. This is proven by eIF4E, which is often found overexpressed in malignant cells, and induces higher proliferation of the cells due to overexpression of certain oncogenic proteins [164]. Thus it would not be surprising if TRIM22 did execute its proliferation repression and cell death effects through a translational repression mechanism. However, further studies are needed to elucidate these matters.

Conclusions

The main conclusions from this thesis can be summarised as follows:

- Endogenously expressed TRIM22 is localised to the nucleus in a speckled pattern and to the cytoplasm in a diffuse pattern
- Endogenous TRIM22 co-localises with the centrosome irrespective of cell cycle phase and with the ER
- TRIM22 interferes with the binding between the translation initiation factors eIF4E and eIF4G
- TRIM22 modestly represses total protein translation
- TRIM22 represses IRF-7C protein levels, and may affect translation of mRNAs that are particularly dependent on eIF4F for their translation
- TRIM22 induces cell death and/or represses proliferation depending on cellular context

Concluding remarks and future perspectives

In conclusion, we have provided new data, which have shed further light on the function of TRIM22. We have shown TRIM22 to localise to the centrosome and ER, to repress protein translation through the disruption of the eIF4E and eIF4G interaction, to induce decreased proliferation and to induce cell death; compatible with a role as a novel tumour suppressor. However, according to the nature of research, many new questions have

been raised and more studies are required to understand all these aspects of the function of TRIM22.

Although the data in this thesis have been primarily focused on the role of TRIM22 in the cytoplasm, TRIM22 are also present in the nucleus, and thus probably have functions also at this localisation. Our results regarding translational repression are targeted to the cytoplasm since translation takes place in the cytoplasm. However, the cell death inducing effect of TRIM22 in a p53-background might take place in the nucleus, since activated p53 primarily executes its actions in the nucleus.

The position of TRIM22 at the crossroads between p53- and IFN-signalling, and thus tumour suppressor pathways and immunological pathways, makes it a protein of great potential. More knowledge of TRIM22 might contribute to new therapies, both towards cancer and viral infections. It is possible that the bad side-effects of IFNs could be limited by use of smaller doses of IFN in combination with p53-activating small peptides, thus resulting in an elevated expression of TRIM22 and hopefully tumour suppression.

Populärvetenskaplig sammanfattning

Cancer är en sjukdom som beror på att kroppsegna celler invaderar, stör och förstör normala vävnader. Cancerceller drivs av *mutationer* (ändringar) i cellens *gener* (arvsmassa) som styr produktionen av proteiner som reglerar celltillväxt och celldöd. För att en cell ska omvandlas till en cancercell krävs oftast mutationer i flera gener. Mutationer som kan ge cancerutveckling sker främst i så kallade *tumörsuppressorgener* och *onkogener*. Tumörsuppressor-gener är gener som i vanliga fall skyddar cellen från cancerutveckling genom att hämma celltillväxt samt beordra potentiella cancerceller att begå självmord. I cancerceller är således dessa ofta inaktiverade. Onkogener är gener som främjar celltillväxt. I cancerceller är dessa därför ofta över-aktiverade.

p53 är förmodligen cellens viktigaste tumörsuppressorgen. Den styr de flesta signaleringsvägar, som behövs för att hindra överdriven celltillväxt och cancerutveckling. p53 och/eller olika signaleringsvägar som är beroende av p53 är inaktiverade i nästan alla cancertyper. Denna gen och dess signaleringsvägar är därför väldigt intressanta när man studerar cancer, och flera olika cancerbehandlingar där man återaktiverar p53 är på väg.

Interferon tillhör kroppens immunförsvar och skyddar cellerna dels från virusinfektioner men också från cancerutveckling. Interferon gör att det tillverkas en mängd av proteiner som är aktiva i tumörsuppression. Interferon används idag som cancerterapi för flera olika cancertyper, ofta i kombination med cellgifter. Tyvärr ger interferon ofta dosberoende biverkningar hos patienterna, med influensaliknande symptom.

Jag har studerat funktionen av *TRIM22*, ett protein som produceras i cellen som svar på både p53 och interferon. Det speciella och intressanta med TRIM22 är att det induceras av både p53 och interferon och därmed kopplar ihop försvaret mot cancer med immunförsvaret. TRIM22 tillhör även den så kallade TRIM-familjen. Proteinerna i TRIM-familjen är delvis lika genom att de har en liknande genstruktur med evolutionärt bevarade *domäner* ("genbitar"). Många TRIM-proteiner är dessutom uppreglerade av

interferon, hämmar virusinfektion och använder den så kallade RING-domänen för att märka in specifika proteiner för destruktion. Proteiner är cellens byggstenar och på detta sätt kan TRIM-proteinerna styra ett händelseförlopp i cellen. De olika TRIM-proteinerna har dock olika funktioner och syften i cellen.

Vad som hitintills är känt om TRIM22 är att det verkar motverka flera olika typer av virusinfektioner, t.ex. HIV. Man vet också att det har en RING-domän som kan märka in proteiner och göra att de bryts ner. Under mina studier av TRIM22 har jag hittat nya funktioner som utförs som svar på TRIM22. Utifrån mina resultat verkar det som att TRIM22 också kan motverka cancer-uppkomst.

I *artikel I* visar vi var i cellen TRIM22 finns. TRIM22 finns i både cellens *cytoplasma* och *cellkärna*. Det betyder att TRIM22 förmodligen har funktioner på båda dessa ställen. Jag visar också att TRIM22 lokaliserar till cellstrukturer som kallas *centrosomer* och det *endoplasmatiska nätverket*. Centrosomer är viktiga cellulära strukturer som bland annat är nödvändiga för celldelning. Det endoplasmatiska nätverket är en struktur som bland annat är viktig då proteiner ska utsöndras från cellerna. Vi vet ännu inte vad TRIM22 har för funktion vid dessa cellstrukturer men föreslår att denna lokalisation kan ha att göra med TRIM22s roll i virusförsvar.

I *artikel II* visar vi att TRIM22 hämmar *translation* (produktion) av nya proteiner. Att hämma protein-translation är ett effektivt och snabbt sätt för cellen att svara på omgivningens signaler. Att stänga av protein-translation kan t.ex. ge som konsekvens att cellväxten minskar, och det kan därför vara ett effektivt sätt att hämma cancertillväxt på.

I *artikel III* visar vi att TRIM22 gör att vissa celler växer långsammare och att vissa celler dör. Båda dessa cellsvar är förstås mycket önskvärda i cancer-celler.

För att sammanfatta våra resultat så har vi visat att TRIM22 gör att cellerna tillväxer långsammare och i vissa celler inducerar celldöd. Vi vet ännu inte exakt hur detta går till men har hittat en mekanism där TRIM22 hämmar protein-translation och vi föreslår att denna mekanism kan vara ansvarig för dessa effekter. Alla dessa egenskaper stämmer väl in på ett protein som skyddar mot canceruppkomst.

Vår forskning har betydelse eftersom man för att kunna utveckla nya och bättre cancerterapier, bättre behöver förstå hur cellerna fungerar. Eftersom TRIM22 också hämmar virusinfektion kan våra resultat även vara till nytta för framställningen av nya och bättre läkemedel mot virusinfektioner.

Acknowledgments

Jag vill tacka alla som på ett eller annat sätt gjort denna avhandling möjlig! Inledningsvis börjar jag med att tacka alla mina kollegor på B13 för att ni alla bidrar till att göra avdelningen till en så bra plats! Tack för alla utflykter, fester, fikapauser och diskussioner.

Vissa personer har bidragit lite extra till denna avhandling och därför vill jag särskilt tacka:

- min huvudhandledare, **Kristina Drott**, för din fantastiska entusiasm och optimism! Tack för att du valde mig som din doktorand och för att du har delat med dig av din stora kunskap. Tack också för alla råd och diskussioner, och för den stora självständigheten du har gett mig;

- min biträdande handledare **Urban Gullberg**. Tack för att jag alltid har varit välkommen att diskutera mina, ibland motsägelsefulla, resultat med dig. Tack också för att du har inspirerat genom din genuina fascination för naturvetenskapen, och för alla råd om allt från den stora bilden till de små tekniska detaljerna;

- **Karina Vidovic** för uppmuntrande ord och råd när försöken inte blev som de borde, för alla skratt, och även för peppandet i skrylle;

- **Carl Sandén** för trevligt sällskap på labbet och även för de fantastiska resorna till Denver och Orlando;

- **Malin Ageberg** för alla dina utmanande frågor och såklart även för trevligt sällskap på labbet;

- **Tove Ullmark** för att det har varit så trevligt att ha dig som rumskompis;

- **Bodil Rosberg och Ann-Maj Persson** för att ni alltid tålamodigt har svarat på diverse frågor;

- **Tor Olofsson** för all hjälp med att sortera celler, för allt du har lärt mig om FACS:en och även för din speciella humor;

- *Per Lönnbro* för att du har hjälpt mig genom att producera så imponerande immunoflourescence-bilder och för att du även lärt mig så mycket om denna vackra teknik;

- *Sara Heras* for welcoming me to the lab at the human genetics unit, for helping me with the polysome fractionation and for all the other fun things during my two weeks in Edinburgh;

- *Susanna Obad* för att du fann TRIM22 och därmed överlämnade ett sådant spännande projekt till mig. Tack också för dina mycket noggranna och färgkoordinerade pärmar som gjorde det lätt för mig att se vilka experiment som du redan hade provat;

- *Christina Annerstedt* och *May-Louise Andersson* för all fantastisk hjälp med de där ekonomiska papperna;

- *Anna* för studierna ihop innan du valde den gröna vägen, för fikorna och festerna under och efter studentlivet, och för bussfärderna och vandringarna i Bolivia och på Island;

- *Maria* för studierna ihop, för sommaren i Örebro, för alla skratt och för alla diskussioner om allt och ingenting;

- *Tina* för att du är en så inspirerande eldsjäl! Tack för att du alltid förstår, för katterna, och för allt det roliga ihop;

- *Susanna* och *Allie* för massor av kul Malmö-häng;

- min barndomsbästis, *Hanna*, för mängder av fantastiska barndoms-minnen ihop, och för att jag är glad över att vi båda hamnade i Malmö så vi kunde börja umgås igen;

- klättergänget, *Hanna*, *Niclas*, *Florian, Håkan, Henrik* m.fl. för hänget på klubben, ute på klippan och inte minst för de fantastiska taco-luncherna. Jag ser fram emot alla 2012s klätteräventyr;

- *alla mina vänner* som berikar mitt liv så mycket;

- *Peluza* och *Phiaf* som med sitt spinnande alltid får mig lugn och harmonisk. Tack också till *Olli* såklart;

- *Farmor* och *Mormor*, för så mycket omtanke;

51

- min bror, **Jimmy**, för att vi är och förhoppningsvis alltid kommer att förbli så bra vänner! Tack även för att du alltid har trott på mig och försökt peppa mig att följa mina drömmar; och

- sist men absolut inte minst, **Mamma och Pappa**, som jag älskar så mycket och som har gjort så mycket för mig. Tack för att ni alltid har trott på mig och för allt mentalt stöd ni ger mig.

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