Characterisation of the role of ING5

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ABSTRACT

The tumour suppressor protein p53 stops cell division by activating the expression of $p21^{WAF1/CIP1}$, whenever it senses that a cells DNA is damaged. Thus, giving the cell a chance to repair the damaged DNA before its errors are duplicated and passed onto daughter cells. When p53 is mutated, it loses its protective function. This allows mutations to accumulate in other genes and leads to more than 50% of all human cancers. $p21^{WAF1/CIP1}$ inhibits cyclin E/CDK2 complexes to prevent G1-S progression of cell cycle. Though the cyclin/CDK complexes are required for the orderly progression of the cell cycle, overexpression of cyclin E/CDK2 has been reported to lead to poor prognosis in breast cancer. The molecular mechanism underlying this is poorly understood.

The current investigation was undertaken to elucidate the role of ING5, in relation to cyclin E/CDK2 activity and p53 function. ING5, which was identified in a novel approach for screening the substrates of cyclin E/CDK2 is a member of the ING family of proteins that inhibit growth. The *in vitro* and *in vivo* interaction experiments and the reporter gene assays demonstrate that ING5 interacts with p53 to strongly activate p53-mediated transactivation of the promoters of the *p21, bax and mdm2* genes. The extensive mapping of their interaction domains showed that the PH domain of ING5 interacts with the DNA binding domain of p53 indicating that ING5 does not interfere with p53-MDM2 interaction. ING5 mediates apoptosis of breast cancer cells with an intact p53 pathway as demonstrated in MCF-7 cells. It was observed that overexpression of cyclin E/CDK2 negatively regulated ING5-mediated activation of p53 and apoptosis. This work for the first time also illustrates a member of the ING family to be interacting with p63 and p73. ING5 interacts with p63 and p73 both *in vitro* and *in vivo* to differentially modulate p63 and p73-mediated transactivation of *bax, p21* and *pig3* genes.

While one important function of p53 is to prevent S phase entry in response to cellular stress by activating the CKI p21, which interferes with the activities of the cyclin /CDKs, the identification of ING5 provides the first example of a reverse regulatory relationship between p53 and CDK activity. The findings suggest that the molecular network of ING5-p53-p21-cyclin E/CDK2 is an example of a positive feed back loop. Therefore ING5 could be useful in therapies for treating cancers with an intact p53 pathway.

ZUSAMMENFASSUNG

Der Tumor-Suppressor p53 kann, ausgelöst durch geschädigte DNA, die Expression von *p21*$^{WAF1/CIP1}$ aktivieren und dadurch die Zellteilung stoppen. Die Zelle erhält hierdurch die Möglichkeit die geschädigte DNA zu reparieren, bevor diese dupliziert und an die Tochterzellen weitergegeben wird. Mehr als 50% der menschlichen Krebserkrankungen resultieren aus Mutationen, die durch den Verlust der schützenden Eigenschaft von p53 angehäuft werden. p21$^{WAF1/CIP1}$ inhibiert den Cyclin E/CDK2 Komplex und verhindert dadurch den Übergang von der G1- in die S-Phase. Obwohl die Zyklin/CDK Komplexe für das ordnungsgemäße Fortschreiten des Zellzyklus benötigt werden, führt die Überexpression von Zyklin E/CDK2 nur mit geringer Wahrscheinlichkeit zu Brustkrebs. Über den zugrunde liegenden molekularen Mechanismus ist jedoch noch wenig bekannt.

Ziel der vorliegenden Arbeit war, die Rolle von ING5 in Bezug auf die Zyklin E/CDK2 Aktivität und die Funktion von p53 zu klären. ING5 ist Mitglied der ING-Protein Familie von Wachstumsinhibitoren und wurde in einem Screen für neue Zyklin E/CDK2 Substrate identifiziert. Interaktions-Experimente *in vitro* und *in vivo*, sowie Reportergen Assays konnten die Interaktion zwischen ING5 und p53 nachweisen, welche zu einer Verstärkung der durch p53 vermittelten Transaktivierung der Promotoren von *p21, bax* und *mdm2* führte. Durch ausführliche Untersuchung der Interaktions-Domänen konnte eine Bindung zwischen der PH-Domäne von ING5 mit der DNA-Bindungsdomäne von p53 gezeigt werden, was darauf hindeutet, dass ING5 die Interaktion zwischen p53 und MDM2 nicht beeinträchtigt. In MCF-7 Zellen konnte gezeigt werden, dass ING5 in Brustkrebszellen mit einem intakten p53-Signalweg, Apoptose vermitteln kann. Die durch ING5 vermittelte Aktivierung von p53 und des Zelltodes konnte durch überexprimiertes Zyklin E/CDK2 inhibiert werden. In dieser Arbeit wird zum ersten Mal die Interaktion eines Mitgliedes der ING-Familie mit p63 und p73 gezeigt. ING5 interagiert *in vitro* und *in vivo* mit p63 und p73 und moduliert die durch p63 und p73 vermittelte Transaktivierung von *bax, p21* und *pig3*.

Während die Fähigkeit von p53 den Eintritt in die S-Phase zu verhindern durch zellulären Stress ausgelöst wird und durch die Aktivierung des CKI p21, welcher wiederum die Aktivität der Zyklin/CDK Komplexe beeinflusst, vermittelt wird, liefert die Identifizierung von ING5 ein

erstes Beispiel für eine gegenläufige regulatorische Beziehung zwischen p53 und der Aktivität der CDK. Die Resultate dieser Arbeit deuten auf einen positiven “feed back loop” in dem molekularen Netzwerk zwischen ING5-p53-p21-Zyklin E/CDK2 hin. Wodurch ING5 eine wichtige rolle in der therapie von Krebsarten mit intaktem p53-Signalweg spielen könnte.

1. INTRODUCTION Chapter 1

The eukaryotic cell cycle is a co-ordinated series of events leading to replication of cells. The period between two mitotic divisions defines the **cell cycle**. The cellular processes required to successfully replicate and divide cells are driven by the sequential activation and inactivation of a family of cyclin dependent kinases (CDKs). Activation is driven predominantly by the periodic expression of the cyclin subunit and requires activating phosphorylation of the kinase subunit. Inactivation is controlled by inhibitory phosphorylation of the kinase subunit, ubiquitin mediated degradation of the cyclin subunit and by interaction of the cyclin dependent kinase complexes with small inhibitory molecules. Thus the timing of cell division is under strict constraint, involving a network of signals that work together to determine the time and frequency of an error free cell division. Defects in the regulation of cell cycle often leads to genomic instability and predispose the organism to cancer. Uncontrolled proliferation is a hallmark of cancer cells (Malumbres and Carnero 2003).

1.1 Cancer

Although cancer has often been referred to as a disease of the cell cycle, it clearly does not mean that oncogenesis targets only the cell division machinery. It merely regards cell cycle deregulation as an important event in the multistep process of tumorigenesis.

Tumours are the outcome of a process of clonal selection in which multiple alterations, such as oncogene activation or the loss of tumour suppressors, are accumulated and give selective advantage to the cells that carry them. The end result is the generation of a cellular clone with aberrant growth and even metastatic properties in some cases (Hanahan and Weinberg 2000). Though cancer is being extensively researched, at the molecular level for over 25 years, it is not yet clear how many different pathways must be altered to generate tumour cells. The more recent research suggests that the alterations in the different pathways are not the same for all types of cancer cells. All cancer phenotypes are the manifestation of six essential physiological alterations of the cell shared by most types of tumours as suggested by Hanahan and Weinberg. The six alterations are as follows:

- Self-sufficiency in growth signals,
- Insensitivity to growth-inhibitory (antigrowth) signals
- Evasion of programmed cell death
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis

Each of these physiological changes acquired by the tumour cell during its development implies a successful breaching of an anticancer mechanism hardwired into cells and tissues. The functional importance and the strategies by which each of these capabilities is acquired is very briefly described below:

1.1.1 Self-sufficiency in growth signals

Normal cells cannot proliferate in the absence of mitogenic signals. The mitogenic signals are transmitted into the cells in part by transmembrane receptors that bind distinctive classes of signalling molecules such as diffusible growth factors, extracellular matrix components or molecules expressed. The signals conveyed stimulate the cells to go into an active state from a quiescent state. Oncogenes act by mimicking normal growth signals in one or the other way. This liberation from dependence on exogenously derived signals abolishes a critically important homeostatic mechanism that normally operates to ensure a proper behaviour of the various cell types within a tissue.

Many cancer cells acquire the ability to synthesise growth factors to which they are responsive thus creating a positive feed back loop which is termed ‘autocrine stimulation’ (Fedi et al., 1997). The production of PDGF (platelet-derived growth factor) and TGFα (tumour growth factor α) by glioblastomas and sarcomas, respectively, are two such examples (Fedi et al., 1997 see review (Hanahan and Weinberg 2000). On the other hand binding to specific moieties of the extracellular matrix (ECM) enables the integrin receptors to transduce signals into the cytoplasm that influence cell behaviour ranging from quiescence in normal cells to motility, resistance in apoptosis and entrance into the cell cycle. Failure of the integrins to mimic these extracellular links can impair cell motility, induce apoptosis or cause cell cycle arrest (Giancotti and Ruoslahti

1999). Thus acquisition of growth signalling autonomy by cancer cells proves to be an important tool in tumorigenesis.

1.1.2 Insensitivity to growth-inhibitory (antigrowth) signals

Antiproliferative signals received by the transmembrane cell surface receptors coupled to intracellular signalling circuits operate to maintain cellular quiescence and tissue homeostasis (Hanahan and Weinberg 2000). The antigrowth signals can block proliferation by two distinct mechanisms. Cells may be forced out of the active prolifertive cycle into the quiescent (G_0) state from which they may re-emerge on some future occasion when extracellular signals permit. Alternatively, the cells may be forced to permanently surrender their proliferative potential by being induced to enter into postmitotic states. The incipient cancer cells must obviate these anti-proliferative signals if they have to proliferate. The response of the normal cells to antigrowth signals is associated with the cell cycle block. During the G1-S transition the cells monitor their external environments and depending upon the signal received they decide to either proliferate or to be quiescent or to enter into a post mitotic state. At the molecular level, many of the anti-proliferative signals are channelled through the retinoblastoma protein, pRb, and its two relatives, p107 and p130. pRb in its hypophosphorylated state blocks proliferation by sequestering and altering the function of E2F transcription factors that control the expression of genes essential for progression from G1 to S phase (Weinberg 1995). The disruption of the pRb pathway liberates the E2Fs, thus allowing cell proliferation and rendering the cells insensitive to antigrowth factors such as $p15^{INK4B}$ and $^{p21WAF1/CIP1}$ that inhibit cyclin/CDK complexes. $p21^{WAF1/CIP1}$ inhibits the activity cyclin E/CDK2 complexes and thereby prevents the G1-S phase progression of the cell cycle. The expression of $^{p21WAF1/CIP1}$ is induced by p53 upon DNA damage in order to arrest the cell cycle and repair the damaged DNA. In the cells where $p21^{WAF1/CIP1}$ is downregulated the damaged DNA is replicated and segregated to daughter cells. Thus down regulation of $^{p21WAF1/CIP1}$ is one of the major ways in which cancerous cells gain unlimited replicative potential. The locus encoding $p15^{INK4B}$ may be deleted (Chin, Pomerantz et al. 1998) or alternatively its immediate downstream target, CDK4 may become unresponsive to the inhibitory action of p15INK4B due to mutations that create amino acid substitutions in its INK4A/B-interacting domain; the resulting cyclin D/CDK4 are then given a free hand to inactivate pRb by phosphorylation (Zuo, Weger et al. 1996). Finally functional pRb may be lost

through mutation of its gene or may be inactivated by some viral oncoproteins such as the E7 protein of human papillomavirus (HPV) as observed in cervical carcinomas (Dyson, Howley et al. 1989). The anti-growth circuit converging onto pRb and the disruption of the cell division cycle one way or the other defines and substantiates the purpose of loss of the tumour suppressor.

1.1.3 Evasion of programmed cell death

The ability of tumour cell populations to expand in number is determined not only by the rate of cell proliferation but also by their ability of cell attrition. Programmed cell death or apoptosis represents a major source of this attrition. The studies in mouse models suggest that the increased resistance to apoptosis is a hallmark of cancer cells (Finlay, Hinds et al. 1989). The apoptotic machinery is in a latent form in all cell types but once triggered by a variety of signals, this program executes its command in a perfectly choreographed series of steps. Apoptosis is a well-defined series of morphological changes that involves chromatin compaction, cytoplasmic condensation and membrane blebbing. Ultimately the cells fragment to release small membrane bound apoptotic bodies that are generally phagocytosed by the neighbouring cells. Importantly the intracellular components are not released into the extracellular milieu where they might have deleterious effects on neighbouring cells (Wyllie, Kerr et al. 1980).

The apoptotic machinery can be broadly classified into sensors and effectors. Sensors are those components of the cell that are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the effectors of death. The abnormalities detected include DNA damage, signalling imbalance provoked by oncogene, survival factor insufficiency or hypoxia (Evan and Littlewood 1998).

A Classical example of such a sensor is the tumour suppressor p53 protein. Upon DNA damage, p53 is stabilised due to phosphorylation on ser15 by ATM/ATR. The p53 tumour suppressor protein can then elicit apoptosis by up regulating the expression of the proapoptotic genes in response to DNA damage, which then stimulate the release of cytochrome C. The cytochrome C, then stimulate the ultimate effectors of apoptosis, including the intracellular protease termed

caspase-9 (Thornberry and Lazebnik 1998). The caspase-9 then triggers the other effector caspases that selectively disrupt the other components of the cell to execute the death program.

Kerr, Wylie and Curie for the first time in 1972 described massive apoptosis in the rapidly growing, hormone-dependent tumours following hormone withdrawal (Kerr, Wyllie et al. 1972). In transgenic mice where the pRb tumour suppressor was functionally inactivated in the choroids complexes, slowly growing microscopic tumour arose, exhibiting high apoptotic rates; the additional inactivation of the p53 tumour suppressor protein, which is a component of the apoptotic machinery led to rapidly growing tumours containing low number of apoptotic cells (Symonds, Krall et al. 1994). Cancer cells through several strategies can acquire resistance to apoptosis but the most commonly occurring loss of a proapoptoic regulator through mutation involves the *p53* tumour suppressor gene. The inactivation of the p53 protein is observed in more than 50 % of human cancers and results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris 1996). Additionally the PI3 Kinase-Akt/PKB pathway is likely to be involved in mitigating apoptosis in a substantial fraction of human tumours (as reviewed in Hanahan and Weinberg 2000).

1.1.4 Limitless replicative potential

The cells in culture have a finite replicative potential (Hayflick 1997). Once such populations have passed through a certain number of doublings they stop to grow. This process is termed as "senescence". The senescence of cultured human cells could be circumvented by disabling their pRb and p53 tumour suppressors, enabling these cells to further multiply until they enter into a second state called "Crisis". This state is characterised by massive cell death, karyotypic disarray associated with end-to-end fusion of chromosomes, and the occasional emergence of variant (roughly 1 in 10^{7}) cell that have acquired an ability to multiply without limit, termed immortalization (Wright, Pereira-Smith et al. 1989). The tumour cells that are propagated in cell culture appear to be immortalized thus suggesting that this phenotype was acquired during the progression of tumourigenesis *in vivo* and was essential for the development of their malignant growth (Hayflick 1997).

The ends of chromosomes, telomeres, are composed of several thousand repeats of a short 6 bp sequence element. Due to the mechanism of DNA replication in eukaryotes, telomere DNA is

shortened by 50-100 bp at each round of replication leading to successive loss of telomeric DNA. The progressive loss of telomere sequences from the ends of the chromosomal DNA eventually causes them to lose their ability to protect the chromosomal DNA. These unprotected chromosomal ends are then susceptible to participate in end-to-end chromosomal fusions, yielding the karyotypic disarray associated with crisis and resulting in the death of the affected cell. Therefore for immortalisation it is essential to maintain telomeres.

Telomere maintenance is evident in almost all types of cancer cells (Shay and Bacchetti 1997) and is achieved in 85%-90% of them by upregulating the expression of the telomerase enzyme that adds hexanucleotide repeats onto the ends of telomeric DNA (Bryan and Cech 1999). Thus limitless replicative potential seems to be a characteristic feature of cancer cells.

1.1.5 Sustained Angiogenesis

The growth of new blood vessels – the process of angiogenesis is carefully regulated once a tissue has been formed. The angiogenesis initiating signals are demonstrated by the vascular endothelial growth factor (VEGF) and acidic and basic fibroblasts growth factors (FGF1/2). The integrin and adhesion molecules mediating cell-matrix and cell-cell association also play critical roles (Fedi et al., 1997) in angiogenic signalling. Tumours appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors (Hanahan and Folkman 1996).

1.1.6 Tissue invasion and Metastasis

Metastasis is the final step in tumorigenesis during which the cells form the primary tumour masses, move out, invade adjacent tissues and thence travel to different distant sites where they may succeed in founding new colonies. Tissue invasion is responsible for 90% of human cancer deaths (Sporn 1996). The genetic and biochemical determinants of invasion and metastasis remain incompletely understood (Hanahan and Weinberg 2000).

The proteins involved in the tethering of cells to their surroundings in a tissue are altered in cells possessing invasive or metastatic capabilities. The affected proteins include cell-cell adhesion molecules (CAMs), the members of the immunoglobulin and calcium dependent cadherin

families, which mediate cell-to-cell interactions and integrins, which link cells to extracellular matrix substrates.

Evidence suggests that all these hallmark traits are acquired directly or indirectly through changes in the genomes of cancer cells. Mutation of specific genes, however, is an inefficient process, reflecting the unceasing, fastidious maintenance of genomic integrity by a complex array of DNA monitoring and repair enzymes. Although there are several mechanisms to maintain the integrity of the cell, nevertheless mutations do occur, possibly predisposing the affected cells to cancer. Malfunction of specific components of these genomic caretaker systems explain this increased mutability (Lengauer, Kinzler et al. 1998). One such prominent member of this caretaker system maintaining the genomic integrity of the cell is the p53 tumour suppressor protein, which, in response to DNA damage, induces either cell cycle arrest to allow DNA repair to take place or apoptosis, if the damage is excessive. There are other tumour suppressors, whose loss has been envisioned to lead to genomic instability (Lengauer, Kinzler et al. 1998).

1.2 The Cell cycle

The cell cycle is divided into four phases, G1, S, G2, and M phases. The principle task of the cell division cycle is to replicate DNA without errors during S phase and to segregate the duplicated chromosomal DNA equally to two daughter cells during mitosis, that is the M phase after processing through the G2 phase. The M phase is divided into four stages, the prophase, during which the DNA condenses. During the metaphase, the two sister chromatids remain attached and become aligned in the centre of the cell. The sister chromatids then separate and move to the opposite ends of the mitotic apparatus, thus segregating one of the two sister chromatids to each daughter cell during the anaphase part of the cell cycle. The nuclear envelope that breaks down into vesicles in the early part of mitosis re-forms around the segregated chromosomes as they de-condense during the telophase, which is the last stage of mitosis. The physical division of the cytoplasm during cytokinesis yields two daughter cells. Following mitosis, the cycling cells enter the G1 phase, which is the phase before DNA synthesis starts taking place. G1 phase is the interval during which the cells respond to external cues that ultimately determine either to replicate DNA and divide or optionally to exit the cell cycle into a quiescent state (G_0) is made. Most cells are in this stage excluding some exceptions such as the progenitor cells. Once the

decision is made they are irreversibly committed to complete the cell cycle (Sherr 2000). The time, late in G1 phase when the cell makes this commitment is coined the "restriction point" (Pardee 1989). When cells are stimulated by growth factors to enter the cell cycle from G_0, they generally require continuous mitogenic stimulation to be driven to the restriction point after which mitogens can be withdrawn and cells will enter S phase and complete the cycle in their absence.

A family of kinases known as the cyclin dependent kinases regulates the progression of the cells through the cell cycle. The CDKs bind to cyclins and stimulate the progression through the cell cycle. Cyclins are synthesised and degraded in a highly coordinated manner. Thus the periodic appearance of the cyclins during the cell cycle is a fundamental level of control over the activity of the CDKs.

The first cyclin to be expressed upon stimulation of the resting (quiescent) cells to enter the cell cycle is Cyclin D. Mitogen induced signal transduction pathways activate cyclin D/CDK complexes Cyclin D assembles with cdk4 and cdk6. The complex enters the nucleus and phosphorylates pRb and the related pocket proteins, p107 and p130 (Sherr and Roberts 1999), thus activating the E2F transcription factors that are required to promote the transcription of genes required for G1 and S-phase. The two inhibitors of cyclin dependent kinases, $p21^{WAF1/CIP1cip1}$ and $p27^{kip1}$ bind to cyclin D/CDK4 without inhibiting its kinase activity and are required for the efficient assembly and nuclear import of cyclin D/CDK4 (Cheng, Olivier et al. 1999). Cyclin D complexes play a second non-catalytic role in G1 progression by sequestering $p21^{cip1}$ and $p27^{kip1}$, which are potent inhibitors of cyclin/CDK2. The cell cycle becomes independent of extracellular signals at the restriction point.

The activated cyclin E/CDK2 complexes initiate DNA replication and a number of target substrates responsible for duplicating and segregating centrosomes have been identified (Hinchcliffe and Sluder 2001); (Zhao, Dynlacht et al. 1998); (Hall, Nelson et al. 2001). Cyclin E/CDK2 also phosphorylates its negative regulator p27, which is subsequently recognised by an ubiquitin ligase and is targeted for destruction by the proteasome (Sheaff, Groudine et al. 1997). Cyclin E/CDK2 also autophosphorylates cyclin E, targeting the cyclin for degradation (Clurman,

Sheaff et al. 1996); (Won and Reed 1996). This feature of cyclin E/CDK2 renders it self-activating and self-limiting.

Cyclin A is expressed immediately after cyclin E at the G1-S boundary. Cyclin A/CDK2 complexes promote transcription of histones and other genes needed to accomplish replication (Obaya and Sedivy 2002). Cyclin E/CDK2 and cyclin A/CDK2 complexes are responsible for

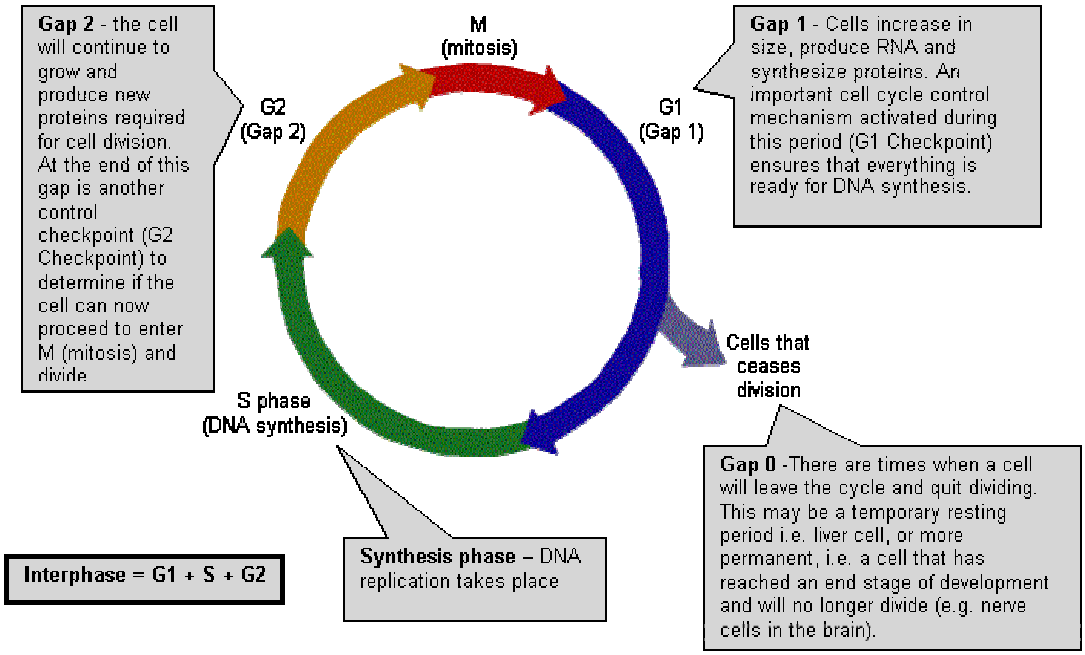

Figure 1.1: Schematic representation of the eukaryotic cell cycle. The different phases of the cell cycle and the events occurring in these phases are depicted here.

the initiation and completion of DNA replication, respectively (Pagano, Pepperkok et al. 1992; Girard, Strausfeld et al. 1991; Ohtsubo, Theodoras et al. 1995).

The next task of the cell is to divide itself into two – the process termed mitosis. The dramatic morphological changes are under the control of CDK1 (Cdc2) in association with cyclin A and B. The expression of cyclin B follows that of cyclin A, rising late in S-phase and remaining high throughout G2 and mitosis. Cyclin B/CDK1 is largely maintained in the inactive phosphorylated state. Dephosphorylation and activation of cyclin B/CDK1 complex is closely related with the morphological changes that accompany mitosis. The nuclear lamins, nucleolar proteins centrosomal proteins and Eg5 (a kinesin-related motor) have all been described as cyclin B-CDK1 substrates (Obaya and Sedivy 2002).

The multi copy organelles such as mitochondria are equally divided in the cytoplasm so that both the daughter cells acquire a copy of the organelle by chance, whereas the single copy organelles such as golgi apparatus are actively divided. CDK1 in association with cyclin B2, which localizes predominantly to the endoplasmic reticulum, may play a role in dispersing the golgi apparatus to ensure that cytokinesis will provide the two daughter cells with sufficient components to rebuild the secretory apparatus (Draviam, Orrechia et al. 2001). The Anaphase Promoting Complex (APC) mediates the destruction of the mitotic cyclins thus ensuring that the cells return to interphase before initiating the next round of DNA replication.

1.3 Inhibitors of cyclin/CDK complexes

Interaction of the cyclin/CDK complexes with their inhibitors is an important mode of their

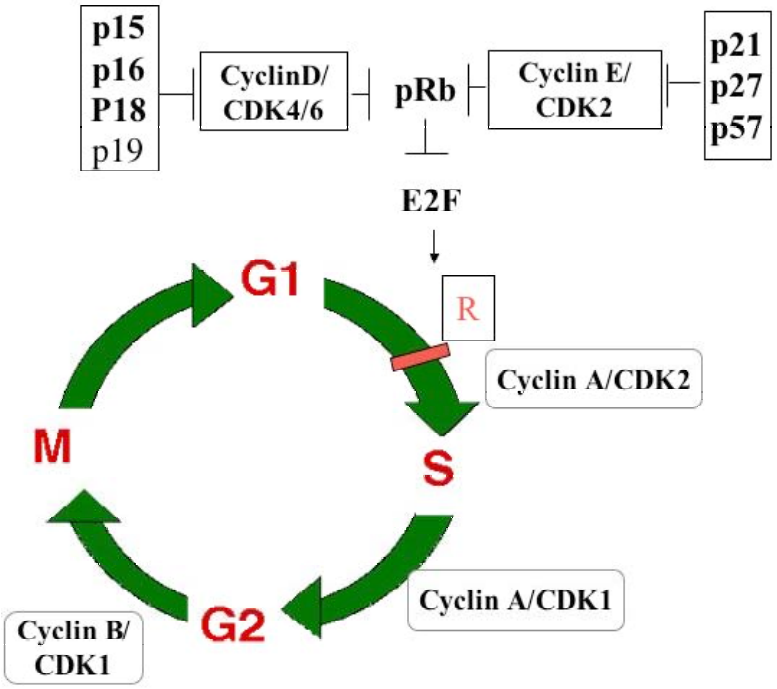

Figure 1.2: The cell cycle progresses through the G1, S, G2 and M phases. The different cyclins, CDK inhibitors involved are depicted here. (R, Restriction point). The details of the cell cycle are described in section 1.3.

negative regulation. The two distinct families of CDK inhibitors are the INK4 family and the CKI family. The INK4 family (p15, p16, p18 and p19) named for the ability to inhibit CDK4 bind to monomeric CDK4 and CDK6 on the same region as cyclin D, thus inhibiting the

formation of active cyclin D/CDK4/CDK6 complexes. Normally cyclin D/CDK4 complexes bind to p27 and prevent their binding to cyclin E/CDK2. In case when INK4 binds to cyclin D the pool of p27 available to interact and inhibit cyclin E/CDK2 is high. Therefore INK4 inhibits cyclin D/CDK4 complexes directly and cyclin E/CDK2 complexes indirectly. The second family of CDK inhibitors (CKIs), the Cip/Kip family ($p21^{WAF1/cip1}$, p27kip1 and p57kip2), strongly inhibit CDK2 containing complexes (Sherr and Roberts 1999). This family of inhibitors binds to preformed cyclin/CDK complexes, thus blocking substrate access. As mentioned earlier, $p21^{WAF1/CIP1}$ is upregulated by p53 in response to DNA damage.

1.3.1 Checkpoint Signalling

The checkpoints are active processes that control the timing and order of critical events in the cell cycle. A problem in any of the pathways of the cell cycle can generate a signal that stops progression in all the others, though they are not physically dependent. If the genome is damaged, cell cycle progression is delayed in G1 or G2 until the damage is repaired. Delay in G1 allows time for repair and hence prevents replicating damaged DNA. Arrest in G2 allows repair through homologous recombination, which requires two complete copies of the genome. Checkpoint mechanisms that act through phosphorylation or degradation of key regulators are expected to operate very rapidly in response to DNA damage whereas transcriptional induction of cell cycle inhibitor proteins is not so rapid. Such mechanisms are critical in maintaining long-term arrest if repair is not easily completed. Ex: of one such mechanism is activation of p53 by ATM and ATR, which has already been discussed (Bunz, Dutriaux et al. 1998).

The high degree of conservation of cell cycle and checkpoint control mechanisms accentuate the significance of these pathways for individual cells and whole organisms. There is increasing evidence that failures in checkpoint signalling increase genomic instability and predispose the organism to cancer.

1.3.2 Other cell cycle related diseases

Cell cycle deregulation leading to cancer has already been described in the earlier part of this section. The other diseases related to cell cycle include cardiovascular and neurodegenerative diseases. Cardiovascular diseases are the leading cause of death in industrialised countries. Most

of the cardiovascular diseases arise from complications of atherosclerosis, which is a chronic and progressive inflammatory condition characterised by excessive cellular proliferation of vascular smooth muscle, endothelial and inflammatory cells leading to occlusive vascular disease, myocardial infarction and stroke. Recent studies have revealed the important role of the cyclins, CDKs and the CKIs (CDK inhibitors) in vascular and cardiac tissue injury (as reviewed by (Boehm and Nabel 2003). The mechanism by which neurons die in human degenerative diseases remains an enigma until today. Terminally differentiated neurons of normal brain are incapable of cell division. However, evidence suggests that aberrant activation of cell cycle in certain degenerative diseases lead to their demise. In Alzheimer's disease, regulators spanning every phase of the cell cycle are upregulated in affected neurons, leading to successful DNA replication, but unsuccessful mitosis. The end point of this non-productive cycle of division is death (as reviewed by Vincent, Pae et al. 2003).

Thus the orderly execution of the different events of the cell cycle proves to be a key role of a normal cell. Any sort of aberrations in these events that go unchecked, predispose the cells to various diseases.

1.3.3 Significance of Cyclin E/CDK2

As outlined earlier, the cyclin E/CDK2 complexes participate in the regulation of the retinoblastoma protein inactivation, establishment of the pre-replication complex (pre-RC) and initiation of S phase. The cyclin E/CDK2 complexes phosphorylate nucleophosmin (NPM/B23; (Okuda, Horn et al. 2000); (Tokuyama, Horn et al. 2001), mouse Mps1 (Fisk and Winey 2001) and CP110 (Chen, Indjeian et al. 2002) to positively regulate centrosome duplication. Cyclin E/CDK2 kinase is activated in response to several oncoproteins, including c-Myc and the adenoviral E1A protein, thus supporting a role for this kinase in tumorigenesis (Amati, Alevizopoulos et al. 1998)**;** (Luscher 2001). Cyclin E/CDK2 phosphorylate NPAT, which is involved in cell cycle dependent transcriptional regulation of histone *H2B, H4 and H3* (Zhao, Kennedy et al. 2000; Ma, Yuan et al. 2000). The other known substrates are the tumour suppressor pRb and PRC1, a regulator of cytokinesis (Jiang, Jimenez et al. 1998). Overexpression of cyclin E has been linked to poor prognosis in breast cancer (Keyomarsi, Tucker et al. 2002). Thus cyclin E/CDK2 proves to be an important cyclin/CDK complex, whose

targets play a key role in various cellular processes. Although cyclin E/CDK2 is known to be active during a very crucial period of the cell cycle, the knowledge of its substrates is very limited (except for the few described above). A further knowledge of the substrates for cyclin E/CDK2 would help a better understanding of the cell cycle.

1.4 The tumour suppressor p53

1.4.1 p53 protein

The human p53 protein consists of 393 amino acids and contains five major functional domains, the transactivation domain, proline rich domain, the DNA binding domain, the oligomerization domain and the regulatory domain. p53 has been conserved during evolution (Soussi, Caron de Fromentel et al. 1987). The cross-species comparison of the amino acid sequences showed five highly conserved regions within the amino acid residues 13-23, 117-142, 171-181, 234-250 and 270-286 (Soussi and May 1996). These regions are termed box I to V. (Fig 1.3).

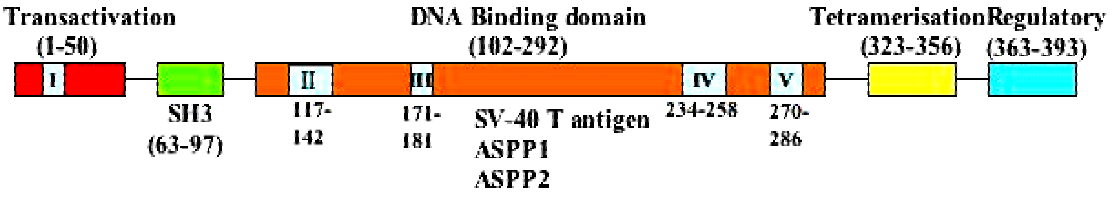

Figure 1.3: Schematic representation of the p53 protein. The different functional domains of the p53 protein are represented. SH3, proline rich region. Marked in small boxes- I, II, III, IV and V are the evolutionarily conserved regions of p53. ASPP1, ASPP2, SV-40 T antigen are the proteins binding to the DNA binding domain of p53

1.4.1.1 The transcativation domain of p53

The amino acids 1-50 on the N-terminal region represent the transactivation domain of p53 (Fields and Jang 1990); (Unger, Nau et al. 1992). The acidic N-terminal domain recruits the basal transcriptional machinery including the TATA binding proteins (TBP) and TBP associated factors, TAFs, components of TFIID (Lu and Levine 1995); (Thut, Chen et al. 1995). This region also contains one of the five conserved regions thus emphasizing its importance.

1.4.1.2 The proline rich domain

The amino acids 63-97 represent the proline rich domain of p53, with striking similarity to SH3-binding proteins. It contains five repeats of the SH3 binding motif PXXP, thus suggesting that it might be involved in interaction with the components of the signal-transduction pathways. This region is required for p53-mediated apoptosis in some experimental systems (Sakamuro, Sabbatini et al. 1997), for suppressing tumour cell growth (Walker and Levine 1996) and for human papilloma virus (HPV) E6 to target for degradation (Li and Coffino 1996). p53 is polymorphic at amino acid 72, which results in either a proline (p53pro) or an arginine (p53arg) at this position. The replacement of proline with arginine disrupts one of the five PXXP motifs. It has also been reported that the p53 arg is significantly more susceptible than p53 pro to E6 mediated degratdation to HPV-induced tumours (Storey, Thomas et al. 1998).

1.4.1.3 The central DNA binding domain

The amino acids 102-292 represent the central DNA binding domain. This region contains four of the five evolutionarily conserved regions. This region is responsible for sequence specific DNA binding (el-Deiry, Kern et al. 1992). The crystal structure of the core domain binding to its cognate sites revealed that the three conserved boxes (box II-box IV) form three loops. The core domain consists of a large ß sandwich that acts as a scaffold for three loop-based elements. The loop L1 (box II) binds to the major groove whereas L2 (box III) binds to the minor grove of the target DNA. L3 (box IV) packs against L1 thus stabilising it (Cho, Gorina et al. 1994). The loops, L2 and L3, are connected by a zinc atom tetrahedrally co-ordinated on amino acids cys176, his179, cys238 and cys242). The zinc atom stabilises the structure of the loops (Hainaut and Milner 1993; Hainaut and Milner 1993)**.**

1.4.1.4 The oligomerisation domain

It is well established that p53 forms tetramers via its oligomerisation domain (Kraiss, Quaiser et al. 1988). X-ray crystallography shows that p53 forms a highly symmetrical tetramer. Each monomer is composed of a turn (asp324-Gly325), a β strand (glu326-arg333), a second turn (gly 334) and an α helix (arg 335- gly ^{356}). Two monomers form a dimer and two dimers interact to form a tetramer. The tetramerization domain is required for efficient transactivation *in vivo* and for p53-mediated suppression of growth of carcinoma cell lines (Pietenpol, Tokino et al. 1994).

There are three nuclear localisation sequences (NLS), NLS1 (316-325), NLS2 (369-375) and NLS3 (379-384) as well in the C-terminus. Mutation in the most N-terminal NLS leads to a complete cytoplasmic protein whereas mutations in the other two NLS lead to both nuclear and cytoplasmic localisation (Dang and Lee 1989); (Shaulsky, Goldfinger et al. 1990).

1.4.1.5 The regulatory domain

The regulatory domain is a basic region referred to as an apoptotic domain (Wang, Vermeulen et al. 1996), a regulatory domain (Wang and Prives 1995) or a DNA damage recognition domain (Lee and La Thangue 1999). This region seems act as a negative regulator of p53 sequence specific DNA binding.

1.4.2 p53 function

In general the tumour suppressor p53 functions as an inhibitor of cell growth by inducing cell cycle arrest or apoptotic cell death. Normally in cells, the p53 protein is kept at low concentration. Upon upstream stress signals, the p53 protein is activated and stabilized to induce the expression of a series of genes that guard the integrity of the cell. The various upstream stress signals include DNA damage (double strand breaks) induced by γ radiation, the presence of DNA repair intermediates after ultraviolet irradiation or mutagens, hypoxia, nucleotide depletion, hyperoxia, activated oncogenes. The level of p53 increases rapidly and is proportional to the intensity of DNA damage. The cellular proteins that recognise the DNA damage communicate with p53. For example, ATM protein which activate DNA damage and which contains a protein kinase domain signals p53 in this fashion (Kastan, Zhan et al. 1992). Following DNA damage due to ionizing radiation, p53 induces specifically the expression of GADD45, which has been found to associate with proliferating cell nuclear antigen, a protein involved in both DNA replication and repair (Kastan, Zhan et al. 1992); (Zhan, Bae et al. 1994). Upon DNA damage p53 also induces the expression of the $p21^{WAF1/CIP1}$ gene to arrest the cells in the G1 phase of the cell cycle.

p53 is also a component of the spindle checkpoint (Cross, Sanchez et al. 1995). The G2/M block following DNA damage is also p53-dependent (Agarwal, Agarwal et al. 1995); (Stewart, Hicks et al. 1995). p53 induces the expression of *14-3-3σ* upon exposure to ionizing radiation to arrest

the cells at G2 phase of the cell cycle. 14-3-3σ is also known as stratifin/SFN due to its high expression in stratifying keratinocytes where it was identified. Its removal has been observed to lead to inability in maintaining stable G2/M arrest after DNA damage (Hermeking, Lengauer et al. 1997).

In certain cell types activation of the p53 pathway leads to apoptosis. The same stress signals that induce cell cycle arrest also induce apoptosis. Analysis of p53 null mice has shown that functional p53 is necessary for DNA damage-induced apoptosis in many cell types including cortical thymocytes (Clarke, Purdie et al. 1993), myeloid progenitor cells (Lotem and Sachs 1993) marrow preB cells, quiescent peripheral B and T lymphocytes (Strasser, Harris et al. 1994), keratinocytes (Ziegler, Jonason et al. 1994) and small intestine cells (Clarke, Gledhill et al. 1994). p53 induces both transcriptionally dependent and independent (Chipuk, Kuwana et al. 2004) apoptosis. p53 induces the expression of Fas/APO1 (Owen-Schaub, Zhang et al. 1995) member of the death receptor family, BAX (Miyashita, Krajewski et al. 1994; Selvakumaran, Lin et al. 1994; Miyashita and Reed 1995; Han, Sabbatini et al. 1996), Noxa (Oda, Ohki et al. 2000) and p53AIP1 (Oda, Arakawa et al. 2000). p53 also up regulates the expression of the p53 responsive gene *PUMA*, which binds to Bcl-2, localises to the mitochondria to induce cytochrome c release and activate rapid induction of programmed cell death (Nakano and Vousden 2001).

1.4.3 p53 as a tumour suppressor

A tumour suppressor is a gene that when restored to activity can reverse the tumourigenic property of the cell, a requirement that has been rarely met experimentally. The properties of a tumour suppressor gene are:

- They are recessive and undergo biallellic inactivation in tumours
- Inheritance of a single mutant allele accelerates the susceptibility to form tumours
- The same gene is frequently inactivated in sporadic cancers

p53 is not included among the classical tumour suppressors as the mutant form of p53 has a dominant negative effect. The wild type p53 can suppress the transformation by mutant *p53* gene and *ras* (Finlay, Hinds et al. 1989; Eliyahu, Michalovitz et al. 1989). *p53* has shown to be lost or contain mutations that inactivate its activity in more than 50% of cancer arising from a wide

spectrum of tissues (Baker, Fearon et al. 1989; Caron de Fromentel and Soussi 1992; Hollstein, Rice et al. 1994; Nigro, Baker et al. 1989) and holds the fame of most frequently altered gene in tumours. Overexpression of the wild type p53 gene in the human osteosarcoma cells lacking endogenous p53 abrogates the neoplasticity of the cells (Chen et al., 1990). Mutations in the p53 gene has also been linked to the Li Fraumeni syndrome, an inherited disorder in which the affected individuals are at increased risk of developing a variety of cancers, including soft tissue sarcoma and cancers of the bone, breast, brain and genito-urinary tract (Malkin, Li et al. 1990). Thymic lymphoma is the most common tumour that arises in p53 nullizygous mice. In addition p53 is also inactivated by nuclear exclusion of the protein (Moll, Ostermeyer et al. 1996).

1.4.4 p53 protein regulation

The various post-translational modifications of p53 that positively and negatively regulate p53 activity are phosphorylation, acetylation, deacetylation glycosylation, sumoylation, ubiquitination and methylation. The most frequently occurring modifications are phosphorylation and acetylation. As already discussed phosphorylation of p53 at ser15 leads to stabilisation of the protein. Acetylation of p53 at lysines 373 and 382 and to a lesser extent on lysines 372 and 381 by p300/CBP and on lysine 320 by P/CAF increases the transactivational capacity of p53 by accentuating its sequence specific DNA binding (Avantaggiati, Ogryzko et al. 1997; Gu and Roeder 1997; Lill, Grossman et al. 1997; Luo, Li et al. 2004) or leads to co-activator recruitment (Barlev, Liu et al. 2001). Further MDM2 recruits HDAC1 to deacetylate p53 (Ito, Kawaguchi et al. 2002) and overexpression of SIRT1 deacetylates p53 and reduces p53-mediated transactivation (Langley, Pearson et al. 2002). Further Set9-mediated methylation of p53 on a single lysine residue (K 372) on the carboxyl-terminus positively affects its stability (Chuikov, Kurash et al. 2004).

The interaction of p53 with MDM2 negatively regulates it. In normal unstressed cells the expression of p53 is very low. Tight regulation of the abundance of the p53 protein is essential for normal growth and development. In the unstressed cells, mouse double minute 2 (MDM2), an E3 ligase binds to the N-terminal transactivation domain of p53 to inhibit p53 mediated transcription of the target genes and transfers ubiquitin moieties onto several sites on the C-terminus of p53. The ubiquitinated p53 is subsequently exported from the nucleus and degraded

by the proteasome (Kubbutat, Jones et al. 1997). The expression of the oncoprotein, MDM2 is under the control of p53, thus falling into a negative feed back loop, which provides a mechanism of keeping the p53 protein low in the unstressed cells and also in the stressed cells where the damage has already been taken care of (Jones, Roe et al. 1995). Upon stress induction, p53 is stablised. One such example is the phosphorylation of p53 at ser15 by ATM/ATR kinase, which results in an increase in p53 stability. Alternatively, they initiate a phosphorylation cascade where ATM/ATR target the checkpoint kinases, Chk1 and Chk2 (Lukas and Bartek 2004), which subsequently phosphorylate p53 resulting in the release of MDM2 from p53. Thus the interaction between p53 and MDM2 is disrutpted.

In addition to stability p53 is regulated by its subcellular localisation. Since p53 functions as a transcription factor its localisation to nucleus plays a key role in regulating its activity. There are two ways how p53 could be recruited to the nucleus. Firstly p53 can be transported to the nucleus by dynein and microtubule network, which requires the N-terminus of p53. Secondly, there are NLS in the C-terminal region of p53, which are recognised by the nuclear import factors (Vousden 2002). Due to the apoptotic and the cell cycle inhibiting properties of p53, the levels of p53 need to be checked for the normal progression of the cell cycle. One way of achieving this is the cytoplasmic export of the nuclear p53 by MDM2 where it is subsequently degraded by the proteasomal machinery. There are two nuclear export sequences in p53, one in the N-terminal MDM2 binding region (Stommel, Marchenko et al. 1999) and one in the C-terminal oligomerisation domain (Zhang and Xiong 2001), that help in the nuclear export of p53.

1.4.5 The p53 Family

The p53 family comprises of three members, p53, p63 and p73. Similar to p53, both p63 and p73 can form homo-oligomers, bind DNA and activate transcription of the different p53-responsive genes and induce apoptosis. In contrast to p53, p63 and p73 give rise to multiple functionally distinct protein isoforms some of which lack the amino-terminal transactivation domain and can function as “dominant negative proteins”, which block the function of the corresponding full-length proteins. In addition, p63 and p73 are only rarely mutated in the large number of tumours examined and are thus unlikely to be classical tumour suppressor genes. On the contrary the p63

and p73 null mice suffer severe developmental defects indicating a tissue specific role for p63 and p73 during development (as reviewed in (Irwin and Kaelin 2001).

Like p53, the p63 and p73 proteins are divided into different functional domains, the N-terminal transactivation domain, the proline rich domain, the central DNA binding domain and the oligomerisation domain. The SAM domain is unique to p63 and p73. The central core domain of p63 and p73 are 63% and 60% identical to that of p53. Six different isoforms of p73 have been identified (α, β, γ, δ, ε, ζ; (Kaghad, Bonnet et al. 1997; Casciano, Ponzoni et al. 1999; De Laurenzi, Costanzo et al. 1998; De Laurenzi, Catani et al. 1999; Kong, Valentine et al. 1999). In addition to these C-terminal splice isoforms, two additional isoforms, ΔNp73α and ΔNp73β, result from the use of alternative promoters located in intron 3 (Yang, Kaghad et al. 1998). The p63 gene also encodes atleast six different proteins that share common open reading frames (Yang, Kaghad et al. 1998), TA-p63α, TA-p63β and TA-p63γ are transcribed from a 5' promoter, and ΔN-p63α, ΔN-p63β and ΔN-p63γ are transcribed from a promoter located in intron 3. Various p63 and p73 isoforms vary in their expression pattern and also in their function. The degree of transactivation for certain genes varies between different p73 and p63 COOH-terminal isoforms, suggesting that COOH terminal sequences affect function (Zhu, Jiang et al. 1998); (Shimada, Kato et al. 1999) for example p73β is a more potent transcriptional activator than p73α (De Laurenzi, Costanzo et al. 1998); (Lee and La Thangue 1999). Likewise, p73 and p63β isoforms are more potent inducers of apoptosis than their α isoforms (Yang, Kaghad et al. 1998).

p63 and p73 can activate certain p53-responsive promoters leading to p53 like effects such as apoptosis. They can activate the promoters of several p53 responsive genes including *$p21^{WAF1/CIP1}$, BAX, MDM2, GADD45, cyclin G, IGFBP3 and p53 R2*. ASPP1 and ASPP2, the activators of p53 also interact and activate p63 and p73 (Bergamaschi, Samuels et al. 2004). There is no evidence if the ING family proteins, which are one of the common activators of p53 also interact with p63 and p73.

1.5 ING Family

1.5.1 Structure of the ING proteins

The ING family comprises of five members, ING1, ING2, ING3, ING4 and ING5. The founder member of this family is $p33^{ING1b}$. Transcripts of the human ING1 gene are processed into several different splice isoforms. The ING1 gene has three exons that are alternatively spliced onto a common 3' exon thereby generating - $p47^{ING1a}$, $p33^{ING1b}$ and $p27^{ING1d}$. In addition to it, internal initiation at an ATG within the common exon generates $p24^{ING1c}$ (Garkavtsev 1999; Gunduz, Ouchida et al. 2000; Jager, Stockert et al. 1999; Saito, Furukawa et al. 2000).

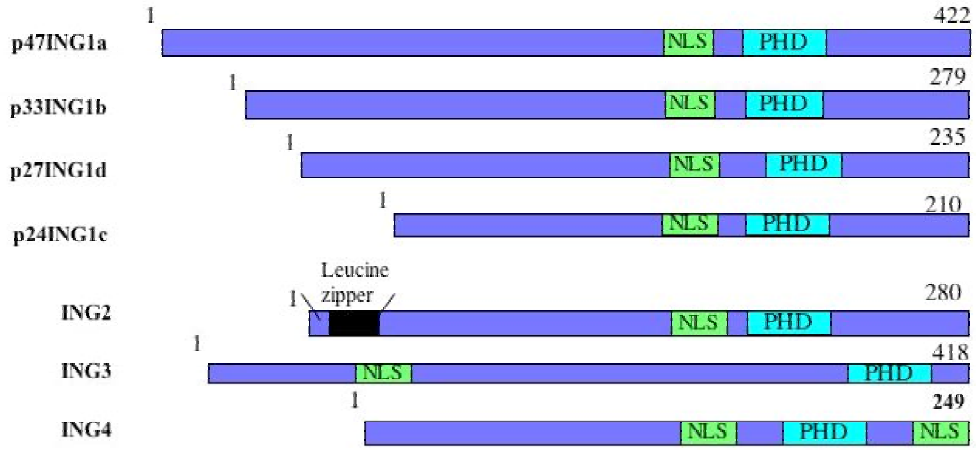

Figure 1.4: Schematic representation of the different members of the ING family and their functional domains. (PHD, the Plant Homeo Domain; NLS, Nuclear Localisation sequence).

ING2 was identified through a homology search of $p33^{ING1b}$ complementary DNA (Shimada, Saito et al. 1998) and encodes a 33 kDa protein. ING3 was subsequently identified through a sequence homology search and ING4 through a computational search of expressed sequence tag (EST) clones showing homology to *ING1* and *ING2* (Shiseki, Nagashima et al. 2003). All ING proteins share a highly conserved carboxy-terminal plant homeo domain (PHD), a Cys4-His-Cys3 pattern zinc finger motif implicated in chromatin remodelling and a nuclear localisation sequence (Fig 1.4).

In addition within the NLS of the $p33^{ING1b}$ protein there is a nucleolar targeting sequence, which targets it to the nucleolus upon DNA damage due to UV induction (Scott, Boisvert et al. 2001). In addition to these common features there are certain features specific for a gene or isoform.

ING2 has a leucine zipper, which promotes interaction with other leucine-zipper-containing proteins, including transcription factors (reviewed in Hai and Hartman 2001). The PHD finger motif and NLS of the ING proteins is the target of mutation in some types of cancer (reviewed in Feng, Hara et al. 2002).

1.5.2 Role of ING proteins

The ING proteins play a significant role in cell cycle regulation, apoptosis, DNA repair and transcripition**.**

1.5.2.1 Role of ING proteins in cell cycle arrest

$p24^{ING1c}$ co-operates with the tumour suppressor p53 to enhance transcription of the cyclin dpendent kinase inhibitor, *p21*$^{WAF1/CIP1\ \ Waf1}$ (Garkavtsev, Grigorian et al. 1998). $p33^{ING2}$ and $p47^{ING3}$ mediate G1 cell cycle arrest and inhibit colony formation in RKO colorectal carcinoma cells carrying wild type p53 (Nagashima, Shiseki et al. 2003); (Nagashima, Shiseki et al. 2001). The microarray analysis of $p33^{ING1b}$-regulated genes identified *CCNB1* (cyclin B 1 gene) as a downregulatory target of $p33^{ING1b}$ (Takahashi, Seki et al. 2002) thus suggesting the involvement of ING proteins in cellular G2/M check point. It has been observed that certain ING proteins indirectly enhance G2 arrest by acetylating p53 on its C-terminus (Nakamura, Gomyo et al. 2002). Further, it was observed that the high levels of cyclin E kinase activity in hepatocellular carcinoma cells (HCC) coincided with negative staining for $p33^{ING1b}$, where as this was not the case for HCCs that stained positive for $p33^{ING1b}$ (Ohgi, Masaki et al. 2002). Recently $p33^{ING1b}$ was shown to interact with DNA methyl transferase 1 (DNMT1) associated protein 1 (DMAP1) complex and a core sin3-HDAC1/2 complex to maintain histone acetylation and hypoacetylation in pericentric heterochromatin during late S phase of the cell cycle (Xin, Yoon et al. 2004). Thus the various ING proteins regulate the cell cycle progression.

1.5.2.2 Role in apoptosis

The senescent human fibroblasts were shown to express 10 fold more *ING1* mRNA compared to early passage fibroblasts, while antisense ING1 expression resulted in prolonged proliferative life span of these cells (Garkavtsev, Grigorian et al. 1998). The early passage fibroblast upregulated $p33^{ING1b}$ expression and entered apoptosis upon growth factor deprivation (Vieyra,

Loewith et al. 2002). $p33^{ING1b}$ interacts with p53 protein, enhances the transactivation of the Bcl2 family member *BAX* upon UV induction to induce apoptosis (Cheung and Li 2002). Overexpression of both $p33^{ING2}$ and $p47^{ING3}$ induced apoptosis in the in RKO cells that are wild type for p53, but not in RKO-E6 cells where p53 is inactivated, thus showing their dependency on 53 to induce apoptosis (Nagashima, Shiseki et al. 2003).

1.5.2.3 Role in DNA repair

Overexpression of $p33^{ING1b}$ protein enhanced nucleotide excision repair (NER) of UVC damaged exogenous plasmid DNA and UVB-damaged genomic DNA (Cheung, Mitchell et al. 2001; Cheung and Li 2002). Human melanoma cells over expressing $p33^{ING1b}$ repaired cyclo butane pyrimidine dimmers (CPDs) at a rate nearly twice as fast as the vector only transfected control. It was also observed that mutations at codon 102 and 260 of the *ING1b* gene are detrimental for $p33^{ING1b}$-enhanced nucleotide exchange reaction. $P33^{ING1b}$ binds to PCNA and competes with $p21^{WAF1/CIP1}$ for binding with PCNA (Scott, Boisvert et al. 2001). Therefore it was proposed that perhaps $p33^{ING1b}$ inhibits DNA replication resulting from $p21^{waf1/cip1}$ dissociation with PCNA (Waga, Hannon et al. 1994).

Thus the members of the ING family inhibit cell proliferation through cell cycle arrest and apoptosis. They also mediate the repair of damaged DNA. ING1 exerts its tumour suppressor functions by helping to maintain genomic stability. Therefore, ING proteins, which are down-regulated in a broad variety of cancer types, are able to restrict cell growth and proliferation to induce apoptosis and to modulate cell cycle progression, which strongly supports the notion that ING family proteins act as class II tumor suppressors (Feng et al., 2002).

1.6 Identification of ING5 in a screen for the substrates of cyclin E/CDK2

Given the importance of cyclin E/CDK2 it is surprising that the knowledge of its substrates is limiting. This lack in knowledge of the targets of cyclin E/CDK2 demands approaches that unravel the substrates of cyclin E/CDK2. One such novel approach was a screening technique that involves phosphorylation of immobilised proteins on high-density arrays with active kinase and radioactive ATP. The cyclin E/CDK2 complexes were expressed in insect cells and affinity purified. The clones on the arrays were preselected for high protein expression via His-tags using

specific antibodies. Around 37830 clones were screened in duplicates per filter. In total more than one hundred potential substrates were detected in this novel approach (unpublished data from Dr. Lilischkis). The known substrate, PRC1, regulator of cytokinesis one was amongst them thus ensuring the success of this technique. One cDNA isolated from this screen had an open reading frame of 240 amino acids. The homology searches suggested that it was a member of the ING family.

1.6.1 ING5

ING5 is a 28-kDa protein. It has two nuclear localisation sequences and a PH (Plant homeo) on the C-terminus. In addition, ING5 has a phosphorylation site for cyclin-dependent kinases on Threonine 152, as revealed by the *in vitro* and *in vivo* phosphorylation experiments. The phosphorylation of ING5 affected its stability as observed in the emetine chase experiments (unpublished data from Dr. Lilischkis).

ING5 inhibits growth of the osteosarcoma cell line, U2OS that are wild type for p53 as observed in the colony formation assays and transformation by Myc/Ras in the transformation assays.

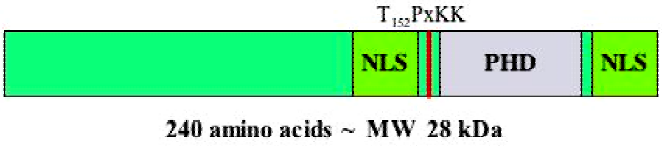

Figure 1.5: Schematic representation of the structure of the ING5 protein: NLS, nuclear localisation sequence; PHD, plant homeo domain; red band, pohsphorylation site, which is on threonine 152; T_{152}PxKK, sequence of the consensus phosphorylation site.

1.7 Objectives

ING5 is the fifth member of the ING family that was identified in a screen for the substrates for cyclin E/CDK2. ING5 inhibited the growth of the p53 wild type osteosarcoma cells in the cell culture but unlike any of its family members ING5 was phosphorylated by cyclin E/CDK2 at a single site. So the objectives for this thesis work were aimed at addressing the interaction of ING5 with p53 and the effect of cyclin E/CDK2 on this interaction. All the approaches adopted were aimed at testing three main points:

- The interaction of ING5 with p53 and the physiological relevance of this interaction.
- The effect of cyclin E/CDK2 on ING5 on the function of ING5
- The mechanism of ING5 action

The interaction of ING5 with was aimed to be addressed in the in the *in vi*vo Co-IP experiments and the *in vitro* GST pull down experiments and the effect of ING5 on p53–mediated transcativation in the luciferase assays. The physiological relevance of ING5-p53 interaction was aimed to be addressed using the apoptosis assays and the effect of cyclin E/CDK2 was to be tested in the same system.

To gain an insight into the mechanism of ING5 action the self-transactivation capacity of ING5, the other interaction partners of ING5 and the region of p53 and ING5 interaction were to be studied. The experiments aimed to address these points were the luciferase assays, the yeast two-hybrid screening and the GST pull down and *in vivo* Co-IP experiments, respectively.

2. MATERIALS AND METHODS Chapter 2

2.1 Materials

Common Reagents, chemicals and laboratory-ware were purchased from the firms Amersham, Amicon, Applichem, Baker, Biomol, Biorad, Boehringer, Difco, Du Pont, Eurogentech, Falkon, Fluka, Fermentas, Gibco BRL, ICN, Invitrogen, Merck, New England Biolabs, Promega, Riedel-deHaen, Roth, Santa Cruz, Seromed, Serva, Sigma and Stratagene. The laboratory-ware were from the firms Amesham, Beckman, Biozym, Costar, Eppendorf, Falcon, Fuji, Gilson, Greiner, Kodak, Macherey-Nagel, Olympus, Pharmacia, Qiagen, Sarstedt, Scleicher & Schuell and Whattman

2.1.1 Primers

Primer Name	Primer Sequence
Jas1cloneF	5'CAT GGC GAC CGC CAT GTA CTT 3'
Jas1cloneR	5' CTA CTT CTT CTT CCT CTT TTC CTG GAC ACA 3'
Jas1clonEcoF	5'GCA GAA TTC ATG GCG ACC GCC ATG 3'
Jas1clonBamR	5' CGA GGA TCC TAC TTC TTC TTC CTC TTT 3'
p53268_292 F	5'-TCC TGG CCC CTG TCA TCT TCT GTC- 3'
AttB1 Jas1F	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTC 3'
AttB Jas1R	5' GGGGACCACTTTGTACAAGAAAGCTGGGTC 3'

2.1.2 Vectors and Plasmid constructs

2.1.2.1 Cloning Vectors

Vector/Author	Description
pDonR 201-ING5	The ING5 cDNA, amplified with the attB attachment sites-ING5 cDNA specific primers was cloned into the gateway compatible, pDonR201 cloning vector by recombination. (Invitrogen Gateway Technology)

2.1.2.2 Eukaryotic expression vectors

2.1.2.2.1 Mammalian expression vectors and plasmids

Construct/Author	Description
pBluescript KSII+ Available from stratagene	The 2961bp pBluescript phasmid is a derivative of pUC19 with a ColE1 and F1 (+) origins of replication. It codes for ampicillin resistance and lacZ genes
pBlue-p53dbox-II [Dr Karen H Vousden (pCB6 p53 dboxII) and P. Basavarajaiah]	The p53 cDNA carrying a deletion of the box-II was excised from the original construct, pCB6-p53dboxII with the restriction enzymes EcoRI and BamHI and was cloned into the EcoR1 and BamH1 sites of pBS II KS+. The p53 cDNA here carried a 125 bp 5' UTR and a 712bp 3' UTR
pcDNA3	It is a eukaryotic expression plasmid under the control of the CMV promoter. The vector codes for Neomycin and ampicillin resistance genes, has an SV-40 and a bacterial (from pBR322) origins of replication.
pcDNA3-TA-p63γ (Roth and Dobbelstein., 1999)	Kindly provided by Dr. Dobbelstein. It expresses a C-terminally HA tagged version of p63 splice variant, gamma, under control of CMV promoter.
pcDNA3-TA-p73α (Dobbelstein et. al., 1998)	Kindly provided by Dr. Dobbelstein. The p73α cDNA was amplified with the primers that carried a HA-tag on the reverse primer and cloned into the BamH1 (that cuts just before the ATG) and the XbaI (that cuts immediately after the stop codon of the p73 protein) cloning sites of the pcDNA3 expression vector.
pcDNA3-TA-p73β (Dobbelstein et. al., 1998)	Kindly provided by Dr. Dobbelstein. It was cloned similarly as p73α.
GWpEVRF-O-HA (Dr.Richard Lilischkis)	Gateway compatible expression vector for expression of N-terminallly HA tagged proteins. under the regulation of the human CMV promoter and has an SV-40 origin of replication.

GWpHA-ING5 (P. Basavarajaiah)	The ING5 cDNA, amplified with the attB1 and attB2 primers was cloned into the expression vector, pEVRF-O-HA by Gateway cloning system.
pRc/CMV	5,5kb plasmid with F1, ColE1 and SV-40 origins of replication. Have sites for CMV, T7, SP6 and SV40 promoters. Codes for ampicillin and neomycin resistance genes
pRc/CMV-p53 dC30 (Dr. Moshe Oren)	30 amino acids on the C terminus that codes for the oligomerisation domain of p53 were deleted. This deletion mutant, was cloned at the HindIII-XbaI cloning site of the Rc/CMV vector that has already been described
pRc/CMV-p53 dN43 (Dr. Moshe Oren)	43 amino acids on the N-terminus that codes for the trans-activation domain of the full- length p53 were deleted and this deletion mutant was cloned into HindIII-XbaI site of the Rc/CMV vector
pRc/CMV-p53 dproAE (Kristen K. Walker and Arnold J. Levine., 1996)	Was kindly provided by Dr. Arnold J. Levine. Amino acids 63-93 coding for the proline rich region in p53 are deleted.
pRc/CMV-p53 d181-335 (P. Basavarajaiah)	The full-length p53 cDNA cloned at the HindIII+XbaI site of the Rc/CMV vector was restricted with Eco47III restriction enzyme and re-ligated. This gave rise to the deletion mutant where amino acids 181-335 were deleted.
cmv-cyclinE	There is no description available
cmv-CDK2	There is no description available
GWpGal	The Gal-O-HA linker plasmid was made gateway compatible by adding the cassette B at the SmaI site of the MCS of the vector. The vector has a Gal4 DNA binding domain (1-147 a.a.) and a SV 40 origin of replication.
GWpgal-ING5	pDonR-ING5 was recombined with Gwpgal to give Gwpgal-ING5

Gwpgal-ING5 (T152A)	ING5-T152A is a point mutant of ING5 where the bases representing amino acid, threonine at position 152 was mutated to alanine. pDonR-ING5 (T152A) was recombined with GWpgal to give rise to GwpGal-ING5(T152A)

2.1.2.2.2 Yeast vectors and plasmids

Vector/Plasmid name	Description
pGBKT7 (Clontech)	The pGBKT7 vector expresses proteins fused to amino acids 1-147 of the GAL-4 DNA binding domain from the constitutive ADH1 promoter. Transciption termination is by the signals from the T7 and ADH1 promoter. Contains a T7 promoter, c-myc epitope tag and a MCS. It carries a kanamycin resistance gene for selection in E. coli and TRP1 gene for selection in Yeast. Has a pUC19 and 2µ ori for replication in bacteria and yeast repectively
pGBKT7-ING5a (P. Basavarajaiah)	ING5 cDNA was amplified from pDonR-ING5 with ING5 specific pair of primers (Jas1 clon*Eco* F+ Jas1clon*Bam* R; refer primer list) including an *Eco*RI site in the forward primer and a *Bam*HI site in the reverse primer. ING5 cDNA restricted with *Eco*RI and *Bam*HI was cloned into the pGBKT7 expression vector at the *Eco*RI and *Bam*HI cloning sites.

2.1.2.2.3 Bacterial expression vectors and plasmids

Vector/Plasmid name	Description
GWpGST (Dr. R. Lilischkis)	The pGex4T2 vector codes for Glutathione-S-transferase enzyme (GST) and is in series with the restriction enzymes that are used to insert cDNAs. The expression of the fusion protein from the synthetic lac promoter could be induced with IPTG. The pGex4T2 vector was made gateway compatible by ligating the gateway cassette A into the *Sma*I

	cloning site
GWp GST-ING5 (P. Basavarajaiah)	The Gwpgex4T2 was recombined with the entry clone pDonR ING5 to give the GW GST-ING5 expression clone.

2.1.3 Antibodies

Antibody Name	**Description**
αEGFP	Gfp(FL) It is a Rabbit polyclonal antibody against GFP that was purchased from the company, Santa Cruz (sc-8334)
αHA-3F10	It is a monoclonal rat antibody that recognises the amino acids 98-108 in haem-agglutinin of the influenza virus. It was made and kindly provided by Dr. E. Kremmer
αING5 7A-11	It is a rat monoclonal antibody that recognises ING5. It was kindly made and provided by Dr. E. Kremmer
α ING5 3F9	It is a rat monoclonal antibody that recognises. It was kindly made and provided by Dr. E. Kremmer
α ING5 SA-8061. 030800	Purified Rabbit polyclonal antibody recognising ING5.
α p53 DO-7	Mouse monoclonal antibody that recognises the p53 protein was purchased from Novo Castra
α p53 sc-6243-G	It is a goat monoclonal antibody against p53 and was purchased from Santa Cruz.
α p53 DO-1	It is a mouse monoclonal human recognising the amino acids 11-25 of p53 protein and was purchased from Santa Cruz.
α p63 sc H-137	Rabbit polyclonal antibody against p63 protein, purchased from Santa Cruz (# sc-8343)
α p73 sc H-79	It is a rabbit polyclonal antibody against p73 protein that was purchased from Santa Cruz (# sc-7957)
α p21	It is a mouse monoclonal antibody against p21 that was purchased from BD Pharmingen (#556431)
α PUMA	It is a polyclonal rabbit antibody that recognises amino acids 180-193 of hPUMA and was purchased from Sigma

	(# P4618).
α Gal4-DBD	It is a rabbit polyclonal antibody against the gal4 DNA binding domain and was purchased from Upstate (# 06-262).
α GST-G9	It is a rat monoclonal antibody purchased from Upstate (# 06-262) against GST.

2.1.4 Materials required for the modifications, resolution and recovery of DNA

2.1.4.1 Buffers and Reagents

1) Restriction enzymes: The restriction enzymes were purchased from MBI fermentas and New England Biolabs

2) DNA loading buffer: 50 mM Tris-HCl, pH 8.0
50 mM EDTA
50 % (v/v) Glycerine
0.25 % (w/v) Bromo phenol blue
0.25 % (w/v) Xylene cyanol

3) Electrophoresis buffer (TBE): 89 mM Tris-HCl, pH-8.0
89 mM Boric acid
2 mM EDTA

4) DNA extraction kit was purchased from QIAGEN

5) T4 DNA ligation kit was purchased from Roche and MBI Fermentas

2.1.5 Materials required for the bacterial work

1) Agar Plates:

LB Medium 1.5 % (w/v) Bacto-Agar (Difco)
100 mg/ ml Ampicillin

2) LB Medium: 1 % (w/v) Tryptone (Applichem)
0.5 %(w/v) Yeast Extract (Applichem)
1 %(w/v) NaCl pH 7.0

3) SOB medium:

Bacto tryptone	20 g/L
Bacto Yeast-extract	5 g/L
NaCl	0.5 g/L
1M KCl	2.5 ml/L

The medium was adjusted to pH 7 and autoclaved. The following components were added just before use:

2M $MgCl_2$	5 ml
2M $MgSO_4$	5 ml

4) SOC Medium: SOB medium containing 20 mM glucose

5) Bacterial Strains

DH5α	F^- (Φ80d Lac 2ΔM15) Δ(LacZYA-argF) U169end A1 rec1 hsdR17($r_k^- m_k^+$) deoR thi-1 supE44 gyrA96 relA1λ$^-$
XL-10 gold	TetrD (mcrA) 183, D (mcr CB-hsd SMR-mrr) 173, endA1, supE44 thi-1, recA1, gyr A96, relA1, lac, Hte, (F' proAB lacIqZDM15 Tn10(Tetr) Amy Camr)a
BL21	(DE) pLysS: F-, ompT, hsdsB, (rB-, mB-), dcm, gal (DE3), pLysS (Cmr)

2.1.6 Materials required for the Yeast-Two Hybrid work

1) YPD agar plates

Difco peptone	20 g/l
Yeast extract	10 g/l

pH 6.5 was adjusted and autoclaved. Glucose to 2 % of the final volume was added

Kanamycin 10 mg/L

2) SD Agar plates: Yeast nitrogen base

without amino acids	6.7 g/l
Adenine hemi-sulphate	0.2 %
Agar	20 g/l
Water	850 ml

Autoclaved and 10x amino acid drop out solution was added.

3) 10x Amino acid mix (1 L):

Adenine hemisulphate	200 mg
Arginine HCl	200 mg
Histidine HCl monohydrate	200 mg
Isoleucine	300 mg
Lysine HCl	300 mg
Methionine	200 mg
Phenyalanine	500 mg
Threonine	2000 mg
Tyrosine	300 mg
Uracil	200 mg
Valine	1500 mg

Depending upon the screening requirement either of the amino acids were dropped out to prepare the drop out media.

4) Yeast Strain:

Strains	Reporter gene	Origin of UAS	UAS regulated by	Origin of TATA sequences	Expression level induced
	HIS3	GAL1	GAL4	GAL1	High (tight)
AH109	ADE2	GAL2	GAL4	GAL2	High (tight)
	lacZ	MEL1	GAL4	MEL1	Low
Y187	Lac Z^d	GAL1	GAL4	GAL1	High

2.1.7 Materials required for the cell culture work

DMEM (Gibco) with 4.5 g/l glucose

MEM (Gibco) with 4.5 g/l glucose

RPMI 1640 (Gibco)

Mc Coy's 5A (Gibco)

PBS: 140 mM NaCl
2.6 mM KCl
2 mM Na_2HPO_4
1.45 mM KH_2PO_4
Penicillin/ streptomycin (Seromed)

Trypsin / EDTA (Seromed) 0.5 / 0.02 % (w/v)

FCS: Foetal Calf Serum

HS: Horse serum

Cell culture Petri dishes with the diameter of 6 and 10cm (Falcon)

Cell culture treated multi-well plates: 12 well, 24 well and 96 well plates (Falcon, Costar)

Cell culture treated flasks

Cryotubes (Nalgene)

Cell Lines:

293: Adenovirus Type 5 (Ad5) transformed embryonal kidney cells (ATCC CRL 1573) SAOS-2: Osteosarcoma cell line (ATCC HTB-85)
U2OS: Osteosarcoma cell line (ATCC HTB 96)
MCF-7: Human adenocarcinoma cell line from the mammary glands (ATCC HTB 22) HCT116

p53 $^{+/+}$: Human Colorectal carcinoma cell lines wild type for p53$^{+/+}$ (ATCC-CCL 247) HCT116 p53$^{-/-}$: Human Colorectal carcinoma cell lines that was made synthetically wild type for p53 $^{-/-}$ (ATCC- CCL 247)

2.1.7.1 Culturing conditions for the different cell-lines

All the cells lines were cultured in the presence of 1 % (w/v) penicillin / streptomycin and 10 % FCS. The different cell lines and their culturing medium are as follows:

293, U2OS, SAOS-2:	DMEM (Gibco)
MCF-7:	DMEM + 4 mM glutamin
HCT116:	Mc Coy' s 5A

The cells were incubated at 37°C with constant supply of 5 % CO_2

2.2 Methodology

2.2.1 RNA and DNA extraction and analysis

2.2.1.1 Extraction of RNA from cells in cell culture

The total RNA was extracted from cells in the cell culture using Qiagen's RNeasy kit according to the manufacturer's protocol.

2.2.1.2 Reverse transcriptase PCR (RT-PCR): First strand synthesis

The total RNA was incubated along with the random hexamers at 70°C and cooled on ice. Subsequently the dNTP and the ribonuclease inhibitor was added along with the reaction buffer and incubated at 42°C for 2 min. The MMLV reverse transcriptase was added and incubated at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min. The cDNA was used for amplification.

2.2.1.3 cDNA amplification using PCR

The Polymerase Chain Reaction (PCR) was adopted to amplify the cDNA. The cDNA obtained from RT-PCR was amplified with specific primers using the *Taq* polymerase or the *Pfu* polymerase depending upon the fidelity requirement. The annealing temperatures were

dependent on the Tm of the primers while the denaturation and the extension temperatures were 96°C and 72°C respectively.

2.2.1.4 Enzymatic modifications of DNA

The DNA was digested with the specific restriction enzyme according to the manufacturer's instruction, phosphorylated using T4 polynucleotide kinase enzyme, dephosophorylated using the calf intestinal phosphatase and ligation was done using DNA T4 ligase available from Roche or MBI Fermentas in the buffer delivered by the manufacturer.

2.2.1.5 Electrophoresis and DNA extraction from the gel

10x DNA loading buffer was added to the plasmid DNA or the linearised DNA or the PCR product and loaded onto the 0,8 %-4 % agarose gel and electrophoresed. The current was variable and the voltage, determined by the size of the gel chamber, was kept constant. The gel was subsequently stained with ethidium bromide and observed under UV light in the Chemi-Doc (Bio-Metra).

Ethidium Bromide Solution: 1µg/ml ethidium bromide staining solution.

Ethidium bromide stock:	10mg/ ml
Ethidium Bromide:	50 µl
TBE:	500 ml

The DNA band was excised from the gel and the DNA was extracted using the QIAGEN DNA extraction kit according to the manufacturer's instructions.

2.2.1.6 Gateway cloning

Gateway cloning Technology is a novel cloning system supplied by Invitrogen (Life technologies). It is based on the site-specific recombination system of bacteriophage lambda and is schematically represented as follows:

attB1-gene of interest-att B2 x *attP1-ccdB-attP2* ⇔ *attL1-gene-attL2* x *attR1-ccdB-attR2*
(expression clone or (pDonR 201) (Entry clone) (Destination vector) PCR product) The schematic representation is taken from Invitrogen manual.

Where att = attachment sites

CcdB = is the F plasmid encoded gene that inhibits growth of *E. coli*

The cDNA of ING5 amplified with the attB1 forward primer and attB2 reverse primer was cloned into the entry vector pDonR201 using the BP clonase enzyme reaction mix. The enzyme reaction mix that promotes *in vitro* recombination between the att B-PCR product and the attP containing pDonR vector contains bacteriophage lamda recombination protein Integrase (Int) and the *E. coli* encoded protein Integration Host Factor (IHF). The att B PCR product and the pDonR 201 vector was incubated in the BP clonase enzyme reaction at 25°C for 1hr. Proteinase K was added to terminate the reaction and the reaction was further incubated for 10 min at 37°C. The BP reaction (1 µl) was transformed into the DH5α competent *E. coli* bacterial cells.

LR cloning: Cloning of the gene of interest from the entry vector into the expression clone. ING5 was further cloned from the entry vector pDonR into destination vectors, which were made gateway compatible such as GWpgal, GWpGex4T2 and GWpEVRF-O-HA to generate the ING5 expression clones. The entry clone and the destination vector were incubated in the LR clonase mix, that contains lambda recombination proteins Int (Integrase) and Xis (excisionase) and the *E. coli* encoded protein IHF (Integration host factor) at 25°C for 1hr. Proteinase K was added and further incubated at 37°C for 10 min. The LR reaction (1 µl) was transformed into 50 µl of competent DH5α cells.

2.2.2 Bacterial work

2.2.2.1 Preparation of competent bacterial cells

The competent bacterial cells for transformation were prepared as described by Tang X. et al., (1994). 10-12 large colonies of the selected bacterial strain grown overnight at 37°C on LB plate were inoculated into 20 ml SOB medium, vortexed and the OD_{600nm} was read. When the reading was 0,01-0,03, the suspension was added to 230 ml SOB taken in a 1 L Schikane-flask and incubated at RT with constant shaking at 150 rpm until the optical density at 600nm reached 0,94. The cells were then centrifuged at 4,000 rpm for 10 min at 4°C. The pellet was re-suspended with 80 ml ice-cold transformation buffer and centrifuged again at 4,000 rpm for 10min at 4°C. The cells were then re-suspended with 20 ml ice-cold transformation buffer and 7

% DMSO (final concentration) was added under swirling. The cells were further incubated on ice for 10 min and aliquoted in 200 µl fractions in pre-chilled 2 ml Eppendorf tubes. The aliquots were quickly frozen in liquid nitrogen and later stored at –80°C.

Transformation Buffer:	Hepes	10 mM
	$CaCl_2$	15 mM
	KCl	250 mM
	$MnCl_2$	55 mM

All components except $MnCl_2$ were dissolved in water and the pH was adjusted to 6.7. Then the solid $MnCl_2$ was added and the transformation buffer was filter sterilised.

2.2.2.2 Transformation of *E. coli*

Transformation of bacterial strains with plasmid DNA was done as described by Inoue H., Nojima H. and Okayama H., 1996.

The DNA (1-5 µl) was mixed gently with 100-200 µl of the freshly thawed competent bacterial cells and incubated on ice for 30 min. The suspension was heat pulsed at 42°C for 30s without agitation and immediately transferred on to the ice bath again. The sterile SOC medium (800 µl) was added and incubated at 37°C for 1/2 an hour (in case of plasmids coding for ampicillin resistance) or 1 hr (as in case of plasmids coding for kanamycin resistance) under vigorous shaking. The suspension (200 µl) was then spread on to the LB/antibiotic plate and incubated at 37°C overnight.

2.2.2.3 Isolation of plasmid DNA from bacterial cells:

A single colony picked from the transformation plate was inoculated into 3 ml TB medium and incubated overnight at 37°C under constant shaking. The plasmid DNA was extracted from 1,5 ml of the overnight culture as mentioned in Qiagen's plasmid mini-prep protocol handbook.

2.2.3 GST fusion protein purification

Two different protocols were adopted to purify the GST-fused protein:

Protocol-1:

The BL-21 bacterial colonies, transformed with the desired pGex construct were inoculated into 100 ml LB medium and incubated overnight at 37°C with constant shaking at 200 rpm. The overnight bacterial culture was diluted 1:10 with LB medium and 0,5 mM $ZnCl_2$ was added and incubated further at 37°C. The cells were induced with 0,2-0,4 mM IPTG at OD_{600nm}-0.6 and further incubated at RT for four hours at 250 rpm. The bacterial culture was centrifuged at 6000 rpm for 10 min at 4°C and the pellet was re-suspended in NETN buffer. The cells were treated with lysozyme, incubated on ice for 30 min and sonicated for disruption. The suspension was centrifuged at 15,000 rpm for 10 min to remove the bacterial debris. The 50% slurry of the glutathon sepharose beads (500 µl) was added to the supernatant to capture the GST fusion protein. Subsequently, the beads were washed four times with 5 ml wash buffer and the GST fusion protein was eluted with the elution buffer. The concentration of the fusion protein was measured and the protein was stored in 50% glycerol and stored at –20°C for further use.

Protocol-2:

The BL-21 bacterial colonies, transformed with the desired pGex construct were inoculated into 100 ml LB medium and incubated overnight at 37°C with constant shaking at 200 rpm. The overnight bacterial culture was diluted 1:10 with LB medium, incubated at 37°C and induced with 25 µl of 1M IPTG when the OD600nm reached 0,6. The culture induced to over express the protein of interest was further incubated at RT. for 2 hrs at 250 rpm. The bacterial culture was spun down at 8600 rpm for 10 min at 4°C and the pellet re-suspended in 30 ml cold buffer. The bacterial suspension was sonicated until the pale-yellow suspension turned greyish and the suspension appeared less turbid. To this suspension 0,5 ml of 20 % triton-X-100 was added, incubated at 4°C for 1 hr and centrifuged at 15,000 rpm for 20 min at RT. The glutathione sepharose beads (500 µl) were added to the supernatant, incubated at 4°C for 1 hr and the beads were spun down at 1750 rpm for 1 min. The glutathione sepharose beads with GST fusion protein bound covalently on to it were washed thrice with 5 ml 1x cold PBS, containing the protease inhibitors (as in the wash buffer mentioned in the previous protocol). The suspension was centrifuged at 1750 rpm for 1 min after each washing step. The GST-fused protein was eluted by incubating the beads with 500 µl elution buffer for 1 hr at 4°C on the overhead turner.

The beads were centrifuged and the supernatant that is the first eluate was stored immediately at –20°C. The beads were further incubated twice with the elution buffer followed by the centrifugation step. The protein concentration was measured, aliquoted in 50 % sterile glycerol and stored at –20°C for long-term usage.

Reagents required for protocol-1

Lysozyme: 100µg/ml Lysozyme in TE buffer

NETN Buffer:	0.5 % NP-40
	20 mM Tris-Cl (pH-8.0)
	100 mM NaCl
	1mM EDTA pH 8.0
Added just before use	
Pepstatin A	1 µg/ml
Pefa bloc	1 mM
Trasylol	(approx 14µg/ ml aprotinin 1 % & 1.5mM NaCl)

Wash Buffer	100 mM TrisHCl pH8
	120 mM NaCl
Added just before use	
	5 mM PefaBloc

Elution Buffer:	For 11 ml	Wash buffer	10 ml
		Glutathion	20 mM

Glutathion Sepharose beads: The Glutathion sepharose beads were swelled in water for 1 hr and washed with NETN buffer twice

Reagents required for protocol-2

Cold Buffer:	1M TrisH Cl pH 7.5
	l50 mM NaCl
	0.5M EDTA

Elution buffer: 1M TrisHCl pH 8.0

20 mM Glutathion

2.2.4 Methods for Yeast-Two Hybrid screening

2.2.4.1 Preparation of competent Yeast cells and transformation:

Preparation of Yeast competent cells:

The AH109 yeast strain grown overnight on the YPDA plate was inoculated into 10 ml of SD medium and incubated overnight at 30°C with constant shaking at 150 rpm. The overnight yeast culture was diluted 1:50 with SD medium and incubated at 30°C for 20-24 hrs. It was then diluted in YPD medium to obtain an OD_{600nm} of 0.2- 0.3 and further incubated at 30°C with constant shaking (50 rpm) until OD_{600nm} reached 0.4–0.6. The cells were then transferred to a 50 ml falcon tube and centrifuged at 3000 rpm for 5 min at RT. The yeast pellet was washed once each with 10 ml water and 10 ml LiSORB followed by centrifugation at 3,000 rpm for 5 min. The yeast competent cells were then re-suspended in the desired amount of LiSORB (25µl yeast suspension/ transformation was used) and used for transformation.

Reagents:

Working concentrations:

1) LiPEG solution: Lithium acetate solution/ Polyethylene glycol

Prepared fresh prior use

40 %PEG 3350

1 x TE buffer

1 x LiAcetate

Autoclaved

2) LiSorb: Lithium acetate-sorbitol solution

100 mM Lithium Acetate pH 7.5)

1 M Sorbitol In TE buffer pH 7.5

Filter sterilised and used

Stock solutions:

1) Sterile 50 % PEG 3350 (MW: 3350, Sigma #3640)

2) 10xTE Buffer: 0.1M TrisHCl pH 7.5
10 mM EDTA pH 7.5

Autoclaved

3) 10x Lithium Acetate: MW: 102.0

1M Lithium acetate. Adjusted to pH 7.5 with diluted acetic acid and autoclaved

4) Sorbitol 2M: MW: 182.2

36.4 g/100 ml of water and autoclaved

Preparation of Carrier DNA mix: (Prepared just before use)

10µl (per transformation) of sonicated herrings testes carrier DNA (concentration = 10mg/ ml) procured from Clontech (# K1606-A) was boiled at 95°C for 2-3 min. Immediately LiSORB (20µl per transformation) was added, thoroughly mixed, ice cooled and the solution was let stand at RT until further use.

Transformation of Yeast Cells:

The plasmid DNA (1 µg), 25 µl of carrier DNA and 25 µl of yeast competent cells were mixed in a 2 ml Eppendorf tube and incubated at RT for 30 min. 450 µl of LiPEG was added and incubated at RT with periodic mixing (every 2min). DMSO (50 µl) was added, heat shocked at 42°C for 10 min without agitation and the suspension was centrifuged at 6000 rpm for 2 min. The pellet was re-suspended in water or SD and spread on suitable SD selection plates (drop-out medium plates)

2.2.4.2 Protein extraction from Yeast by Urea/SDS method:

The yeast colony grown on the appropriate selection media was inoculated into the SD selection medium and incubated at 30°C overnight. The overnight culture was vortexed to remove cell clumps and the whole suspension transferred to 50 ml YPD medium. The suspension was further incubated at 30°C with constant shaking at 220-250 rpm until an OD_{600} of 0,4 – 0,6. The OD unit was calculated by multiplying the culture volume with that of the OD reading. The culture was poured into a 500 ml pre-chilled centrifuge flask half way filled with ice, centrifuged at 4000 rpm for 5 min at 4°C and the pellet frozen on ice until the next step. The pellet was re-suspended with the cracking buffer pre-warmed at 60°C and the cell suspension was transferred to a 1,5 ml microfuge tube containing 80 µl of glass beads per 7,5 OD_{600} units of cells. The sample was heated at 70°C for 10 min and vortexed vigorously for 1 min to remove the membrane associated proteins. The sample was then centrifuged at 14,000 rpm for 5 min at 4°C and the supernatant stored on ice. The resultant pellet was further incubated on a 100°C boiling water bath for 3-5min and vortexed vigorously. The debris was removed by centrifuging at 14,000 rpm for 5 min at 4°C. The supernatant from this step* was mixed with the supernatant from the earlier step. The protein samples were boiled briefly, loaded onto a 12 % SDS-PAGE and analysed by western blotting. (the samples could be stored at –80°C as well)

Note: When there was no supernatant available from the second step, 100µl of cracking buffer was added and then boiled at 100°C.

Cracking buffer: For 100 ml stock solution

Urea	8 M
SDS	5 % w/v
Tris-HCl pH 6.8	40 mM
EDTA	0.1 mM
Bromophenol blue	0.4 mg/ml

Complete cracking buffer solution: Prepared just before use

To prepare 1.13 ml	Cracking buffer stock solution	1 ml
	ß-mercaptoethanol	10 µl
	Protease inhibitor solution	

2.2.4.3 Trans-activation assay in Yeast:

The bait plasmid (pGBKT7-ING5) was checked for self trans-activation by transforming the bait into the AH109 yeast strain and plating on the following dropout SD agar plates for testing the respective reporter gene activation:

SD – trp

SD – leu

SD - -leu/-trp

SD – His/ + 3 mM 3-AT

SD – Ade

SD – Ade/-His/ + 3 mM 3-AT

SD - -trp/ -leu/ -Ade/ -His/+ 3 mM 3-AT

All drop out plates contained X-gal. The plates were incubated at 30°C for 3-7 days.

2.2.4.4 Yeast-Two Hybrid Interaction screening by Yeast Mating:

A large colony of the bait was inoculated into 50 ml of the appropriate drop out medium and incubated at 30°C with constant shaking at 200-250 rpm until the OD_{600} reached 0,8 This required 20-24 hrs. The culture was centrifuged at 1500 rpm for 5 min and re-suspended in 5 ml of 2x YPD medium. The library aliquot was thawed at RT and inoculated into 2L Ehrlenmeyer flask containing 45 ml of the 2x YPD/ kanamycin medium and 5 ml of the bait culture. 10 µl of the library suspension was set-aside before inoculating into the bait mixture for library titering purposes. The library strain and the bait strain mixture, called the mating mixture was incubated at 30°C for 20 – 24 hrs with constant shaking at 30-50 rpm. The mating mixture was transferred to a sterile centrifuge flask and centrifuged at 2000 rpm for 10 min. Meanwhile the mating flask was rinsed twice with 50 ml 2x YPD/ Kan. The combined rinses were used to re-suspend the first pellet. The cells were centrifuged again at 2000 rpm for 10 min and the pellet was re-suspended in 10 ml of 0,5x YPD/ kan. The library mating mixtures were plated as follows:

100 µl of a 1:10, 1:100, 1:1000 and 1:10,000 dilution of the mating mixture was spread on 10cm SD/ -trp, SD/ -leu, SD/ -leu/ -trp, SD/ -leu/ -trp/ -Ade and SD/ -leu/ -trp/ -Ade/ -His/ plates to score for mating efficiency.

The remaining mating mixture was plated on 50, 150 mm SD/-trp/ -leu/ -Ade and SD/- leu/ -trp/ -His plates. The plates were incubated at 30°C for 8-10 days and started looking for the colonies after 3 days of incubation.The 10 cm plates where the mating mixture was titrated was scored for the number of colonies and the mating efficiency was calculated as follows:

Mating efficiency:

(# cfu/ ml of diploids) / (#cfu/ ml of limiting partner) x 100= % Diploid

The colonies on the library screening plates were picked and screened on the SD/ - His/ - leu/ - trp/ + X-gal and SD/ - His/ - Ade/ - leu/ - trp/ + X-gal plates for true positives. The colonies that grew on both the drop out plates or just on SD/ - His/ - leu/ -trp/ + X-gal were subsequently sorted on the SD/ - leu/ - trp/ + X-gal plates to eliminate library clones with more than one library plasmid. A mixture of white and blue colonies indicated segregation. The sorted colonies were confirmed for the right phenotypes by streaking subsequently on the SD/ -His/ - leu/ - trp plates. The clones were then grown on 10cm SD/ - His/ - leu/ - trp grided plates for 3-4 days at 30°C and stored at 4°C. The library plasmids were isolated from the clones and the cDNA amplified with PCR to isolate the clones bearing the same library plasmid. The selected clones were then transformed into *E. coli* by chemical transformation and the plasmid DNA was purified. The bait DNA and the library plasmid were transformed into the AH 109 strain to confirm the interaction. The survivors of this screening were sequenced and the sequences were analysed.

Pre-transformed Library:

The library used for screening was bought from Clontech LOT # 0051603. The normal bone marrow pooled from 51 male/ female Caucasians, aged 22-70 who died of sudden death were the source of mRNA. The mRNA was *Xho*I- $(dt)_{15}$ primed and was appended to the *EcoR*I-*Not*I-*Sal*I

adaptor sequence.The cDNA was cloned into the *Xho*I/ *Eco*RI sites of the pACT2 vector and transformed into the Y187 yeast strain.

2.2.4.5 Isolation of plasmid DNA from Yeast

The library plasmid was retrieved from the clones that survived the initial screening as follows:

The yeast clones were grown in 3 ml of the respective selection medium for 36-48 hrs at 30°C and 2 ml of this culture was centrifuged at 6000 rpm for 1 min at RT. The pellet was re-suspended in DB buffer and 40 µl of Lyticase (stock 20 mg/ ml in water) was added and incubated at 37°C for 1 hr with constant shaking. The turbid suspension was centrifuged at 6000 rpm for 1 min at RT and the supernatant was discarded. The pellet was treated with buffers P1, P2 and N3 available in the miniprep kit from Qiagen. After treating with buffer N3, the sample was centrifuged at 13,000 rpm for 15 min. The supernatant was loaded to equal volume of iso-propanol and centrifuged at 13,000 rpm for 15 min at 4°C. The DNA was dissolved in 150 µl TE buffer, 500 µl of 95 % ethanol and 8 µl of sodium acetate were added and incubated immediately at –80°C for 10 min. The DNA pellet was then centrifuged at 4°C for 10 min at 13,000 rpm. The plasmid DNA was eluted in 50 µl TE buffer.

DB buffer: 1 M Sorbitol
1 mM EDTA

2.2.5 Methods for cell culture

2.2.5.1 Cryo-conservation:

The adherent cells were trypsinised and centrifuged at 2000 rpm for 1 min at 4°C. The pellet was re-suspended in 1 ml FCS consisting of 10 % DMSO and incubated on ice. The cell suspension was transferred to cryo screw cap 1 ml tubes and cooled slowly overnight at –80°C. The tubes were then transferred to –150°C for long-term storage purposes. The cells were thawed in a 37°C water bath for 5 min and transferred onto a 10 cm plate containing the suitable medium. The medium from the plate was transferred after one hour to another plate and fresh medium added to the first plate. The medium in the plates was changed on the following day.

2.2.5.2 Transient Transfection:

Calcium phosphate and Ex-Gen transfection methods were used for transient transfection of the adherent cells.

1) Calcium-Phosphate transfection: (Chen and Okayama, 1988; Bousset et al., 1994)

The DNA co-precipitates with calcium phosphate in a complex and these complexes when provided are endocytosed by the cells and the DNA is extra chromosomally transcribed.

The cells ($1,5*10^5$ cells per 6 well plate or $1*10^6$ cells per 10 cm plate) were seeded a day prior to transfection and just two hours prior to transfection the medium with FCS was substituted with MEM containing HS. Meanwhile the DNA was pipetted into 500µl of 0,25 mM $CaCl_2$ (in case of 10 cm plate) or 200 µl of 0,25 mM $CaCl_2$ (in case of 6 cm plate) taken in a snap cap tube. Equal volume of Hebs was added and the sample mixed well by pipetting the mixture up and down. The snap cap tube containing the transfection mixture was tapped 17 times and the calicum-phosphate-DNA complex was pipetted onto the cell monolayer. Depending upon the cell lines the cells were either washed after 4-8 hrs of transfection or on the following day with HEPES Buffer.

Reagents :

1) 2 x Hebs-Buffer
274 mM NaCl
42 mM HEPES
9.6 mM KCl
1.5 mM Na_2HPO_4
Adjusted pH 7.1

2) HEPES-Buffer
142 mM NaCl
10 mM HEPES pH 7.3
6.7 mM KCl

3) 250 mM CaCl2

2) Ex-Gen transfection:

Ex-Gen is a transfection reagent available from MBI Fermentas. The cells were transfected according to the manufacturer's protocol. Ex-Gen was mostly used to transfect MCF-7 cells, these cells were washed with 1 x PBS 3-4 hrs after transfection**.**

2.2.4.3 Cell fixation on coverslips:

The cells were fixed on coverslips with 4 % paraformaldehyde for 22 min and washed thrice with PBS.

2.2.5.3 Reporter Gene Assay/ Luciferase assays:

The promoter of the gene of interest was conjugated to a luciferase reporter and co-transfected along with the other desired plasmids coding for the genes of interest. The promoter activation or repression, influenced by the other genes co-expressed, was measured in terms of luciferase activity. The transfection efficiency was standardised by co-transfection with a β-gal expression plasmid. The different steps involved are as follows:

1) Extraction of proteins for the Luciferase assay:

The cells transfected with the desired constructs were washed with 1x PBS, and 300 µl (per 6 cm plate) of extraction buffer added. The plates were incubated at 4°C for 10 min, the cells scraped from the plate into a 1,5 ml microfuge tube and centrifuged at 13,000 rpm for 10 min to remove the cell debris. The supernatant was transferred into a fresh Eppendorf tube.

2) Luciferase Assay:

The lysate (20 µl) and 100µl of the measuring buffer, were added into the wells of the white micro-titre plates. The substrate, luciferin (35µl) was auto injected just before measuring by the spectrophotometer, Victor and the luminescence was measured. The assay was performed in duplicates of each triplicate.

3) β-galactosidase assay:

20 µl of the lysate was added in duplicates into the micro-titre plates. The pre-mixed Z-buffer and ONPG was added and the samples were incubated at RT for 10 to 20 min. The reaction was stopped by adding 65 µl of sodium bi carbonate (Na_2CO_3) and the absorbance measured at 405 nm.

Reagents:

1.) 5x Extraction Buffer: 25 mM Tris pH 7.8with H_3PO_4
2 mM EDTA
10 %Glycerine
1 %Triton X 100

Diluted 1:5 just before extraction and 10 mM DTT was added

2) Measuring Buffer: 25 mM Glycin
15 mM MgSO4 x 7H2O

Just before measuring 5 mM ATP was added. Stored at 4°C

3) Luciferin stocksolution: 25 mM Luciferinin 3.6 ml of 25 mM NaOH Solution. Aliquot 50 ml in falcon tube, wrapped with aluminium foil and stored at –20°C. Made up with 50 ml measuring buffer just before use. Store at –20°C

4) Z-Buffer : 60 mM Na_2HPO_4
40 mM NaH_2PO_4 x H_20
10 mM KCl
1 mM $MgSO_4$

1L water was added. Autoclaved and Added just before Use 50 mM ß-Mercaptoethanol Stored at RT

5) ONPG Stock Solution: 4mg/ ml ONPG in Z-Buffer. Aliquoted and stored at -20°C

2.2.6 Methods for protein analysis

2.2.6.1 Preparation of the cell lysates for protein analysis:

The cells were lysed in the concentration of 4 x 10^6 cells per ml of the Frakelton buffer. The cells were incubated 10 min on ice with the F buffer, scraped into an Eppendorf tube and vortexed for 15 s. The cells were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was immediately used for further protein analysis or stored at –20°C.

The modified Frakelton buffer (F-buffer)

50 mM Hepes pH 7.5
0.2 % NP-40
50 mM NaCl
0.5 mM $ZnCl_2$
10 %Glycerin

Protease Inhibitor Mix:

1 mM EDTA
1.4 µg/ ml Aprotinin
0.5 µg/ ml Leupeptin
5 u/ ml α-Macroglobulin

Phophatase inhibitors:

1 mM Sodium fluoride
0.1 mM Orthovandate
10 mM ß-glycerophosphate

Added Just Before Use

5 mM PefablocPepstatin 1 µg/ ml

2.2.6.2 Denaturing Discontinuous Polyacrylamide Gel Electrophoresis: (Laemmli, 1970)

The denaturing SDS-PAGE was adopted to separate proteins electrophoretically based on their molecular size as they move through a polyacrylamide gel matrix toward the anode. A 5 % (w/v) (was the same throughout) stacking gel topped on a 10-15 % separating gel was secured in a Bio-Metra gel chamber. The protein sample was diluted 1:5 with the 5x Laemmli loading buffer that contained SDS, boiled at 95°C for 2 min and loaded into the individual wells of the 5 % stacking

gel, submerged in the Laemmli running buffer that was assembled in the Bio-Metra gel chamber. The proteins were separated electrophoretically at 25-30 mA and the voltage was variable.

Laemmli loading buffer:

Concentration	1x
TrisHCl pH 6.8	50 mM
DTT	100 mM
SDS	2 %
BPB	0,1 %
Glycerol	10 %

Running buffer: 50 mM Tris-Base
380 mM Glycine
0.1 % (w/v) SDS

Protein Ladder: Prestained protein ladder, 10-180 kDa, was purchased from MBI-Fermentas (#SM0671)

2.2.6.3 Coomassie staining of the protein gels

Quick Coomassie blue staining was used to detect GST fused proteins on a SDS PAA gel. The protein gel was fixed with the fixing solution for 20-30min and stained with Coomassie G-250 solution for 10 min to overnight at RT. It was de-stained with the de-staining solution for 1-2 hrs to remove the Coomassie stain bound to the polyacrylamide of the gel. In case of proteins labelled with a radioactive isotope the de-staining step was followed by incubation in the amplifier for 10 min. The de-stained gel was placed on a thick Whattmann filter paper and dried at 60°C under vacuum for 2-3 hrs. An autoradiogram was laid on the radioactive gels and incubated at –80°C for varying periods.

Reagents:

Rapid coomassie blue G-250: 10 % (v/v) Acetic acid
0.006 % (w/v) Coomassie brilliant blue G-250

Fixing solution: 25 %Isopropanol
10 %Acetic acid

De-staining solution: 10% Glycerol

Amplifier: From Amersham Biosciences (Code Number: NAMP100)

2.2.6.4 Western Blotting and immuno-detection of target proteins:

Western blotting was performed to transfer proteins from the SDS-PAA gel onto the nitrocellulose or PVDF membrane. The protein gel was cut into the size to be blotted; two thick Whatman filter papers and a nitro-cellulose membrane were cut into the dimension of the gel. The nitrocellulose membrane and the Whatmann filter papers were soaked in the transfer buffer before being used. The gel was laid on the first Whatman filter paper in a blotting chamber, the nitrocellulose membrane was placed over the gel and the second Whatman filter was placed over the membrane, such that the proteins moved from the negative cathode (gel) to the positive anode (the membrane). Any trapped air bubbles between these layers were removed by rolling a glass pipette over them and was blotted for 1-1, 1/2 hr at 0,8-2 mA per sq cm area of the membrane. The membrane was removed from the gel chamber, incubated with ponceau staining solution for 1-2 min and washed with water twice. The ponceau staining served as a control for equal loading of samples. The membrane was blocked with 5 % milk in PBS-T for 30 min at RT or overnight at 4°C and was further immuno-blotted. Immuno-blotting involved the incubation of the membrane with the primary antibody (recognising the antigenic epitopes on the target protein) for 2 hrs at RT or overnight at 4°C. and the blot was washed 3x 5 min each with 1x PBST buffer to remove any unbound antibody. The bound antibody was detected with the appropriate secondary antibody, conjugated to the horseradish peroxidase enzyme. The primary and secondary antibodies were diluted in 5 % milk. The blot was incubated with the luminogenic substrate available from PICO for a min and the chemi-luminescent signal was captured by Las 3000, from Fugi.

10 x Semi-dry buffer for nitro-cellulose

250 mM Tris Base pH 8.3-8.8

1.92 M Glycine

1 x Semi-dry buffer: 10 x semidry buffer

20%Methanol

Blocking buffer: 1 x PBS

5 % Milk powder

Wash Buffer: 1x PBS

0.05 % (v/v) Tween-20

Ponceau S solution: 0.5 g Ponceau S was dissolved in 1.0 ml glacial acetic acid and the volume was made upto 100 ml.

2.2.6.5 Co-ImmunoPrecipitation: Co-IP

Co- Immunoprecipitation was used to detect protein-protein interactions in cells growing in the cell culture. The cells expressing the proteins of interest were lysed with F-buffer and centrifuged at 13,000 rpm for 10 min. The target protein was pulled down by its interacting partner, that was captured by specifc antibody coupled on to the protein A or G sepharose beads depending upon the Ig subclass of the antibody. The interaction mixture was incubated at 4°C for 2 hrs and washed with F-buffer 2-3x. The beads were centrifuged after each washing step at 9,000 rpm for 1.5 min. Sample buffer (1x) was added to the beads and boiled at 95°C for 2 min and loaded on to the SDS-PAA gel. Western blotting was performed and the blot was developed with the specific antibody against the pulled down protein.

2.2.6.6 Invitro Transcription and Translation:

A TNT-T7 quick coupled transcription/ translation system available from Promega Corp was used. It is a system used for transcription and translation of genes cloned downstream from the T7 RNA polymerase promoter. The kit is stored at –80°C. All reagents are thawed at RT just before use and stored on ice after thawing. 1 µg of the plasmid DNA was added to 16 µl of the

TNT-T7 mix, 0,5 µl of enhancer provided in the kit was used to enable enhanced transcription and 6-8 µ Ci of radioactively labelled methionine was added. The total volume was made up to 20 µl with nuclease free water and incubated at 30°C for 90 min. The in-vitro translated protein was either stored at –20°C or used up immediately for the GST pull down experiments

2.2.6.7 GST pull down assays

It is an in vitro technique used to detect interaction between two proteins for ex:-protein A and protein B. Protein A was transcribed and translated *in vitro* in the presence of radioactive methionine and incubated with a mixture of, a 50 % slurry of glutathione sepharose beads, 1 µg of GST fused protein B and 200 µl of GST pull down buffer for 2 hrs. The beads were washed 4 times with the GST pull down buffer and the protein samples were separated on a SDS-PAA gel. A coomassie staining of this gel was used as a control for equal loading of the GST fused proteins. The coomassie stained gel was de-stained, amplified with NAMP 100, and dried under vacuum for 2 hrs at 60°C. An autoradiogram was laid over the gel and incubated at –80°C for variable periods. The interaction was detected in terms of the radioactivity captured by the autorad.

GST-pull down buffer:

20 mM Hepes
50 mM NaCl
0.1 %NP-40
1.5 mM EDTA
1 mM DTT

Protease inhibitors Pepstatin 1 µg/ ml
5 mM Pefabloc
Leupeptin 0.5 µg/ ml

2.2.6.8 Apoptotic Assay:

Apoptosis was scored in terms of the morphological appearance of the cells. The cells (2×10^4) were seeded on cover slips measuring 18 mm in diameter. The cells were co-expressed with the protein of interest along with the green fluorescent protein (EGFP). The GFP was used as a

marker for transfected cells. The cells were fixed 24 hrs after transfection with 4 % PFA and permeabilised by treating with 0,1 % triton-X-100. The nucleus was stained with Hoechst 33258 diluted to 1:1000 in PBS and washed thrice with PBS to remove any unbound stain. The cover slips were mounted onto the slides with mowiol, air dried in dark o/n and sealed with nail enamel to avoid complete drying of the cover slips. The cells were then observed under fluorescence microscope and the number of transfected apoptotic cells was counted. Percentage apoptosis was calculated.

4 % (w/v) Paraformaldehyde in PBS

0.1 % (v/v) Triton X-100 in PBS

Moviol 4-88 (Hoechst) with Isopropylgallate

1 mg/ml Hoechst 33258 in PBS.

3.1 Interaction of ING5 with p53

3.1.1 ING5 activates p53

The objective of this thesis is the characterisation of the role of ING5 in regulating p53. The first step towards achieving this was to test the effect of ING5 on p53-mediated transactivation of its target promoters. The blast search of the ING5 sequence revealed that ING5 is highly homologous to its family member, $p33^{ING1}$. The fact that $p33^{ING1}$

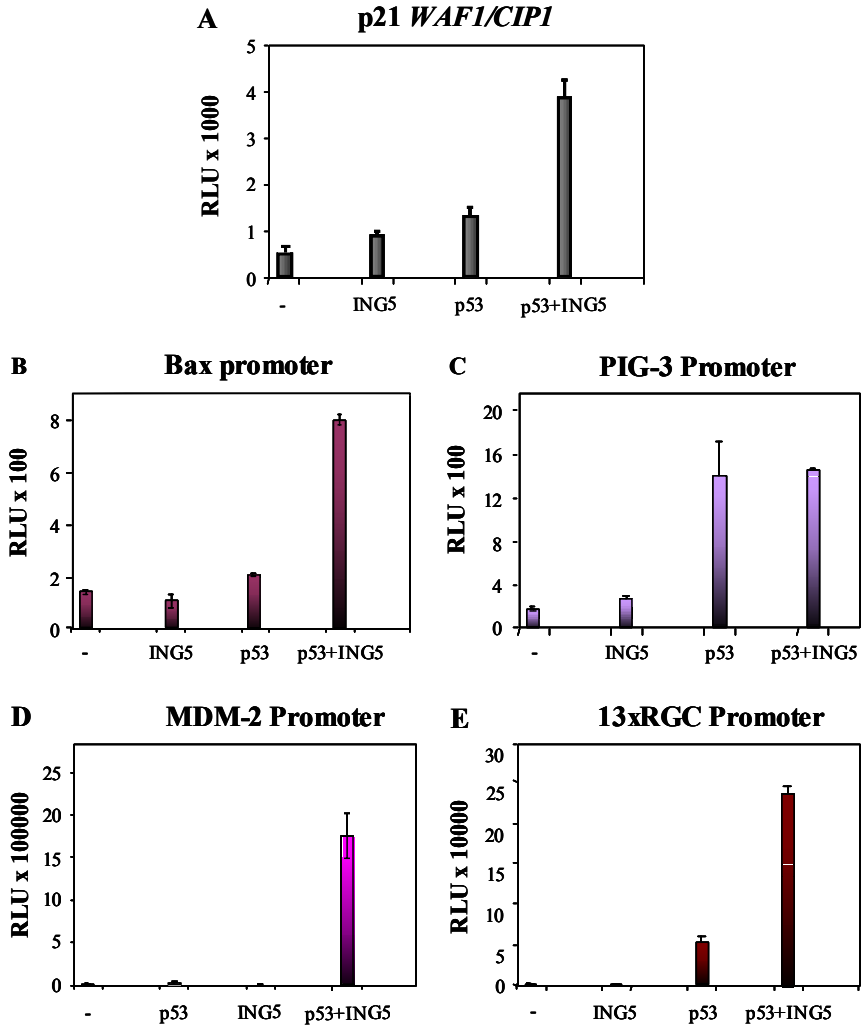

Figure 3.1: ING5 stimulates p53 dependent transactivation. For the reporter gene assays the promoter-luciferase constructs (0,5 µg per 6 cm dish) were co-transfected with the p53 (0,5 µg) and ING5 (1,5 µg) constructs into the 293 cells. (A) ING5 enhances the transactivational capacity of p53 on the promoter of the *p21*gene. (B) ING5 potentiates p53 mediated transactivation on the promoter of the pro-apoptotic gene, *bax*. (C) ING5 unlike in case of p21 and bax does not significantly enhance the transactivational

capacity of p53 on the promoter of the *pig3* gene.(D) and (E) ING5 could also stimulate p53 mediated activation of transcription on the MDM2 and 13xRGC promoters respectively. -, empty vector; RLU, relative light units

enhances the transcriptional activation of p53 on one of its target promoter, *p21* as reported by Garkavtsev et al., and the results that ING5 could inhibit colony formation in a p53 wt cell line, U2OS, as revealed from the works of our group, suggested that ING5 perhaps plays a similar role in the transcriptional activation of the p53 target promoters as p33ING1. This possibility was tested in reporter gene assays. The ING5 and p53 constructs were co-transfected into 293 cells along with the promoters of the *p21, bax, pig3* and *mdm2* genes, which were conjugated to the luciferase reporter. The p53-mediated activation of the transcriptional activity of the aforementioned promoters was measured in terms of luciferase activity and the effect of ING5 on this activation was determined.

The effect of ING5 on the p53 responsive synthetic promoter, *13xRGC* (13 times ribosome gene cluster) conjugated to the luciferase reporter was also tested. As shown in Fig. 3.1 ING5 enhanced the transactivational capacity of p53 on the promoters of the genes *p21, Bax, and MDM2* and also on the 13xRGC promoter in varying degrees however not on the PIG-3 promoter. The fold enhancement in the activation of the p53 responsive promoters by p53 obtained upon overexpression of ING5 is as shown in the Tab 3.1.

Table 3-1: Representation of the average fold increase in p53-mediated transactivation of its target promoters upon ING5 overexpression. The table is not a representation of the figure 3.1. (* no significant activation)

Promoter	Average fold activation over p53
p21	4.2
bax	6.8
pig3	1.4*
mdm2	114
13xrgc	6

To be concise, ING5 enhances the transactivational capacity of p53 on promoters of the proapoptotic gene like *bax*, and also on the promoter of the p21 gene that is involved in the cell

cycle arrest. The further experiments were aimed at testing the interaction of ING5 with p53 *in vitro* and *in vivo*.

3.1.2 ING5 binds to p53 both *in vitro* and *in vivo*

The strong effect of ING5 on the transactivation function of p53 emphasized a possible interaction of p53 with ING5. To test this, *in vitro* GST pull down experiments and *in vivo* co-*immunoprecipitation* experiments were employed. In the *in vitro* experiment, the p53 protein obtained by transcription and translation in the presence of radioactively labelled methionine was precipitated with GST fused ING5 protein bound to the glutathione sepharose beads. p53 bound specifically to ING5, as p53 could not be precipitated with just the GST protein. This shows that the interaction of p53 with ING5 is direct (Fig. 3.12).

Further the interaction of p53 with ING5 was confirmed *in vivo* in cell culture with Co-IP experiments. HA-ING5 was overexpressed in increasing concentrations in 293 cells. Anti-HA antibody was used to precipitate overexpressed ING5 (carrying an N-**terminal HA** epitope) and associated proteins from the 293 whole cell lysate under low stringency conditions.

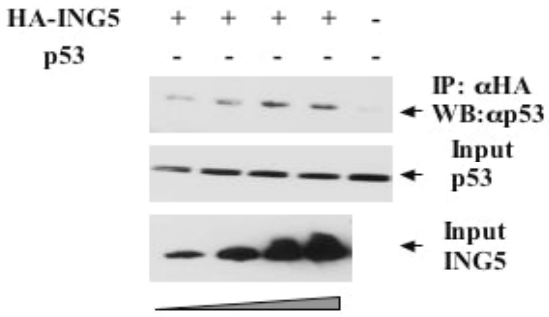

Figure 3.2: ING5 interacts with p53 *in vivo*. The upper panel shows the endogenous p53 precipitated with increasing amounts of HA-ING5 detected by the αp53 DO-7 antibody. The middle panel is the input control for the expression of endogenous p53 and the lower panel is the input control for HA-ING5 detected with the HA-3F10 antibody.

The presence of endogenous p53 in the precipitate was monitored by immunoblotting for p53 with the Do-7 monoclonal antibody. p53 could not be precipitated from the cell lysates where ING5 was not overexpressed, thus confirming the specificity of the interaction (Fig. 3.2). The *in vtro* interaction of p53 with ING5 suggests that p53 binds to ING5 directly.

3.2 ING5 mediates apoptosis

ING5 like its family member ING1 inhibited cell proliferation in a p53 dependent manner in colony formation assays and transformation assays (R. Lilischkis and C Cerni, unpublished data). Overexpression of ING5 inhibited proliferation in U2OS cells, which are wild type for p53 whereas this inhibition was not observed in case of p53 null SAOS cells. Functionally inhibition of cell proliferation could mean either a cell cycle arrest or apoptosis. ING5 as shown in Fig. 3.1, enhanced significantly p53-dependent activation of both the promoters of the genes responsible for cell cycle arrest and apoptosis namely p21 $^{(WAF1,\ Cip\text{-}1)}$ and bax1, respectively. p21 (El-Diery et al., 1993) binds to several cyclin-CDK complexes to inhibit kinase activity and block cell cycle progression. Bax (Miyashita and Reed, 1995) binds to Bcl2, antagonizes its ability to block apoptosis and releases cyctochrome c to activate a mitochondrial apoptotic pathway. To test if ING5 influenced p53-mediated apoptosis, apoptotic assays were performed in MCF-7 **cells,**

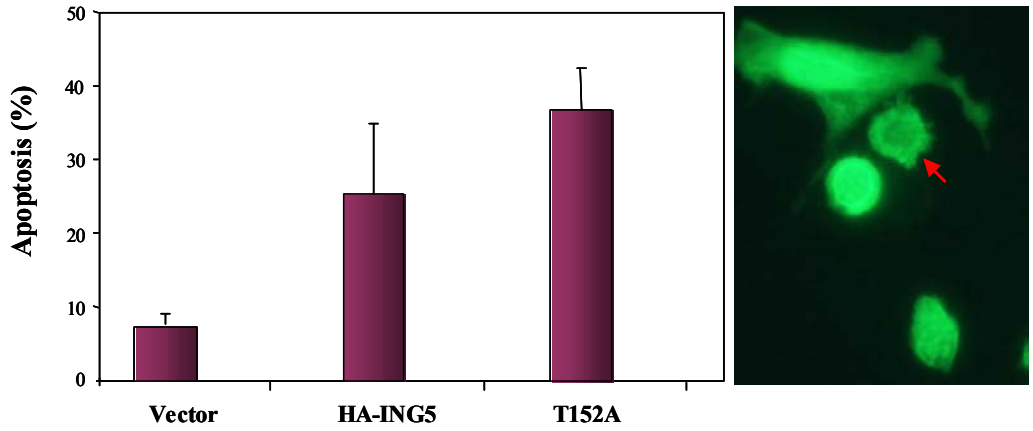

Figure 3.3: ING5 induces apoptosis in the MCF-7 cells. For the apoptotic assays, 1µg of HA-ING5 and 50 ng of EGFP constructs were transfected into MCF-7. The left panel indicates the percentage apoptosis observed in the MCF-7 cells upon treating the cells with the above mentioned parameters. The result shown is the mean of five experiments and in each experiment 100 transfected cells were counted. T152A is the non-phosphorylatable mutant of ING5. The figure on the right panel is a representative of the EGFP transfected MCF-7 cells. The cell highlighted with the red arrow is a typical apoptotic cell.

that are reported to have wild type p53. HA-ING5 along with EGFP as a transfection marker was overexpressed in MCF-7 cells. The cells were fixed 24 hrs later and the number of transfected apoptotic cells was counted under fluorescence microscopy. Apoptosis induced due to transfection procedure was controlled for by overexpressing the empty vector along with EGFP. Considerable amount of apoptosis was seen in cells with overexpressed ING5. The non-phosphorylatable mutant of ING5, T512A was more potent in inducing apoptosis than the wt ING5.

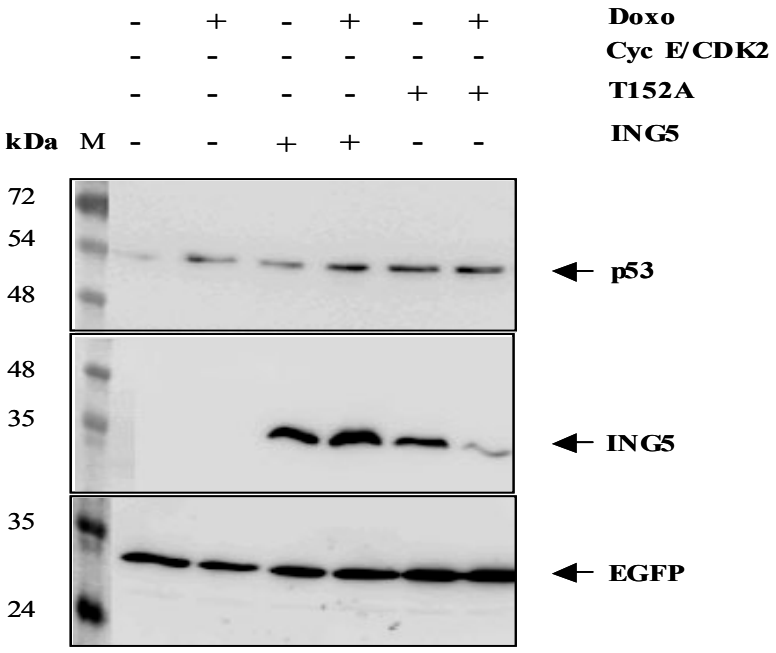

Figure 3.4: Expression of p53 and ING5. The cells transfected with ING5 and EGFP were analysed for the expression of ING5, p53, EGFP and also for the expression and induction of endogenous p21. The upper panel shows that the expression of p53 is slightly induced upon doxorubicin treatment. The panel below it shows the expression of HA-ING5 in the lanes where ING5/T152A has been overexpressed. The 3rd panel shows the expression of endogenous p21. The lower panel shows the expression of EGFP, which in the apoptotic experiments was used as a transfection marker.

The expression of ING5 and p53 with and without induction with 0,5 μM doxorubicin in this cell line was controlled by western blotting. 0,5 μM doxorubicin was used to induce the expression of endogenous p53. Since the initial experiments indicated that the doxorubicin treatment of the cells did not enhance apoptosis in these cells (data not shown), doxorubicin treatment of the cells was dropped in the subsequent experiments. EGFP was used as an expression control marker (Fig. 3.4).

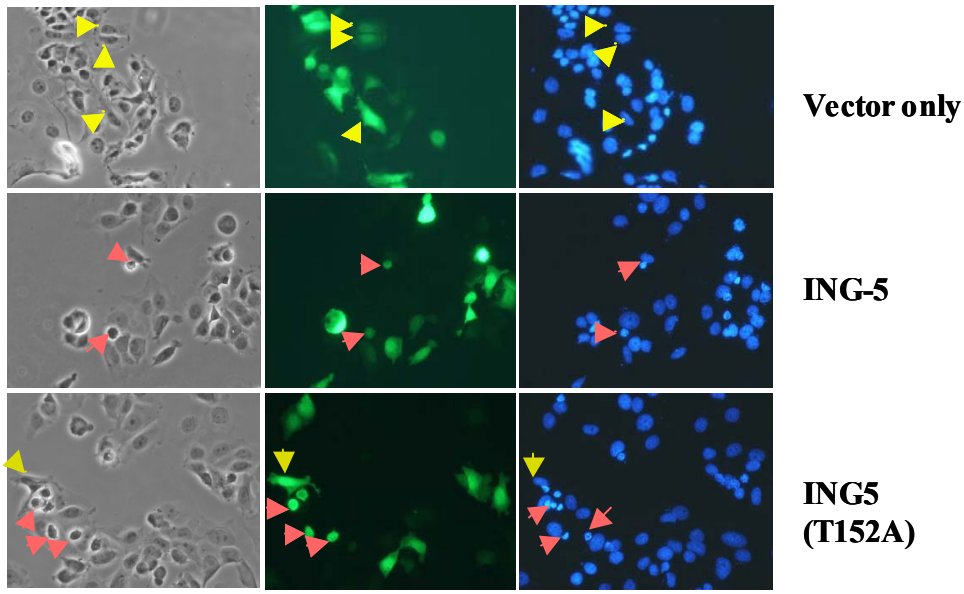

Figure 3.5: Morphological appearance of the cells in the apoptotic assays. The leftmost column shows the appearance of cells under phase contrast microscopy. The middle column shows the cells that are transfected with EGFP and ING5 which are then counted for apoptotsis. The third column shows the nucleus of these cells, which is stained with the DNA binding dye, Hoechst. The cells identified by a yellow arrow indicate the healthy cells and that with the red arrow indicate the apoptotic cells.

These results suggest that ING5 mediates apoptosis in the MCF-7 cells. The non-phosphorylatable form of ING5, the T152A mutant where the threonine at the 152 position is mutated to alanine, is more potent in inducing apoptosis indicates that perhaps the function of ING5 is regulated by phosphorylation. Therefore the effect **of** phosphorylation on the function of ING5 has been addressed in the subsequent experiments.

3.3 The function of ING5 is regulated by cyclin E/CDK2

A member of our group identified ING5 in a screen for the substrates of cyclin E/CDK2 and characterised the phosphorylation site on ING5 to threonine 152. ING5 as observed by our group in the emetine chase experiments, was an unstable protein whereas the non-phosphorylatable mutant, T152A was more stable suggesting that phosphorylation affected the stability of the ING5 protein (Dr. Lilischkis, unpublished data). In the following experiments, the effect of cyclin E/CDK2 on the function of ING5 was addressed. So far, there are two functions of ING5 that have been studied in this work; the activation of p53 and ING5-mediated apoptosis.

The effect of cyclin-E-CDK2 on the activation of p53 was tested in the reporter gene assays and on apoptosis in the apoptotic assay system that has been described already. In the reporter gene assays, performed in 293 cells, the 13 x RGC promoter luciferase construct was co-transfected with the ING5, p53 and cmv-cyclin E constructs. The T152A mutant was co-expressed in the parallel set of experiments and the luciferase activity measured 36 hrs later.

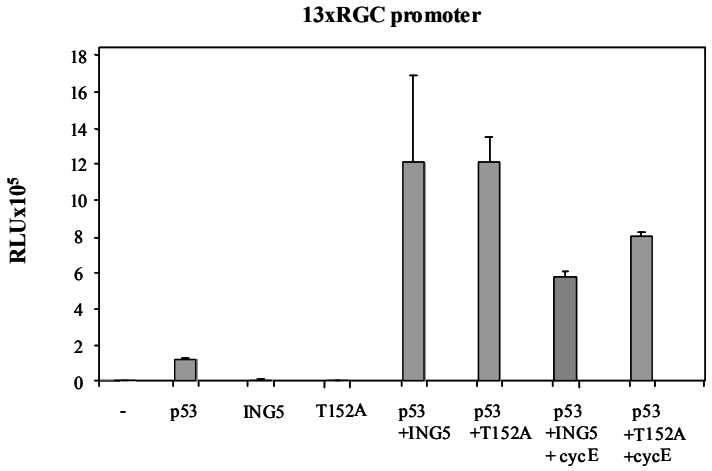

Figure 3.6: ING5-mediated activation of p53 is negatively regulated by cyclin E. For the reporter gene assays the promoter-luciferase constructs (0,5 µg per 6 cm dish) were co-transfected with p53 (0,50)µg and ING5/T152A (1,5 µg) constructs into the 293 cells. (-, empty vector)

The enhanced activation of transcription at the 13 x RGC promoter by p53, instigated by ING5 was counteracted to certain extent by the ectopic expression of cyclin E. The T152A mutant also enhanced the activation of p53 on the 13xRGC promoter but this activation was not counteracted to the same extent as that of ING5 upon cyclin E overexpression showing that the phosphorylation of ING5 is responsible in negatively regulating the activation of p53 by ING5 (Fig. 3.6).

In the apoptotic assays (Fig. 3.7 and 3.8), performed similar to the earlier experiments, co-expression of cyclin E/CDK2 with HA-ING5 in the MCF-7 cells reduced significantly the amount of apoptosis induced by ING5.

The reduction in the amount of apoptosis induced by the T152A mutant upon cyclin E/CDK2

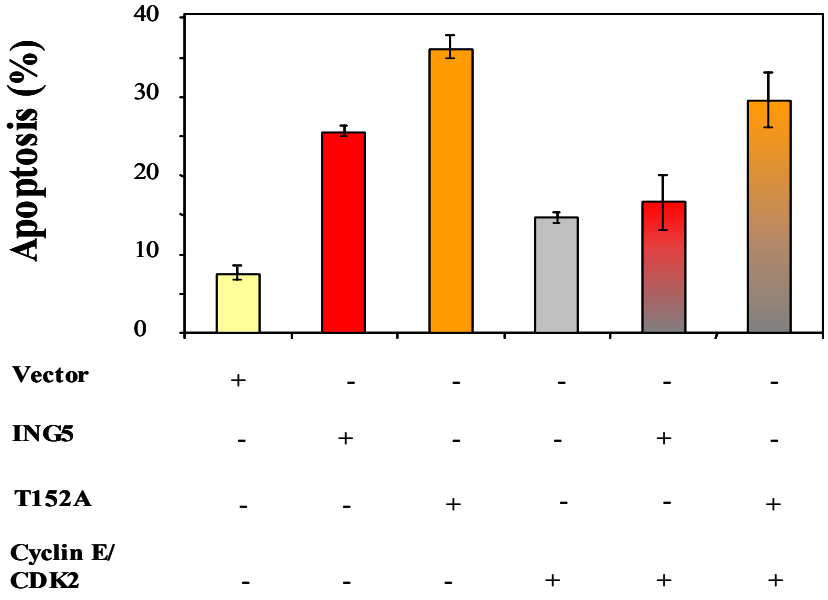

Figure 3.7: ING5 mediated apoptosis is counteracted by cyclin E/CDK2. Representative of the results of the apoptotic assays. The graph indicates the percentage of apoptosis observed in the MCF-7 cells. ING5 mediated apoptosis is counteracted by overexpression of 30 ng of cyclin E/CDK2 where as the apoptosis mediated by the T152A mutant of ING5 is not significantly due to cyclin E/CDK2 overexpression.

over expression was almost negligible once again demonstrating that phosphorylation of ING5 negatively regulated its function.

The results of the reporter gene assays and the apoptotic assays are consistent with each other.

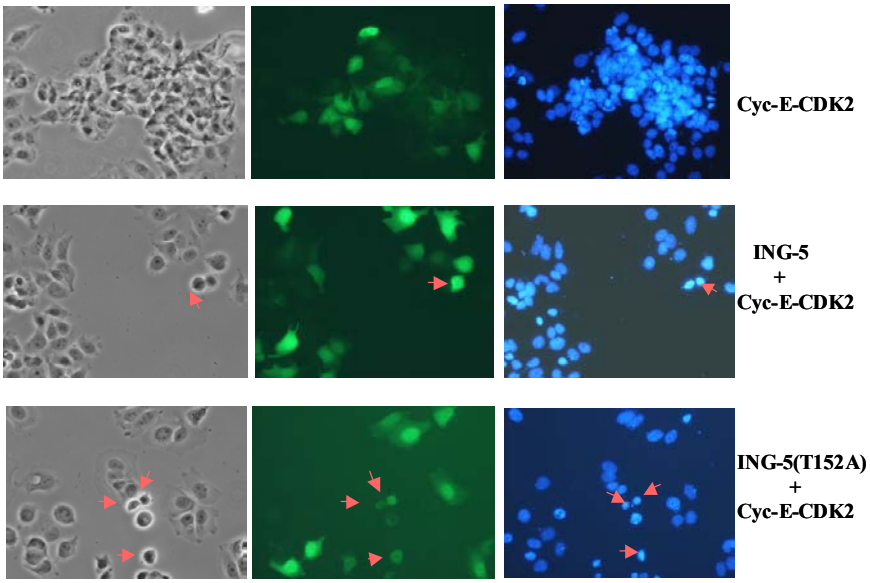

Figure 3.8: Representative of the morphological appearance of the cells in the apoptotic assays. The upper panel represents the cells where cyclin E/CDK2 was overexpressed along with EGFP. In the middle panel ING5 is co-erexpressed along with cyclin E/CDK2 and in the lower panel T152A mutant of ING5 is co-expressed with cyclin E-CDK2.

3.4 Mechanism of ING5 action

Though the function and regulation of ING5 has been studied so far, the mechanism of ING5 action has been incomprehensible. ING5 activates p53 but the mechanism, how it activates p53 is to be studied. It is not clear if ING5 is a transactivator on its own from what has been discussed already. Several mechanisms can be envisaged how ING5 might affect the function of p53

- DNA binding
- MDM2 inactivation
- Cofactor recruitment to modify p53 post-translationally (phosphorylation/ methylation/ acteylation)
- Affecting the stability of p53

To shed some light into the mechanism of ING5 action, the following approaches were adopted.

3.4.1 ING5 is not a transactivator

Though the analysis of the ING5 protein sequence did not reveal similarity to any of the known transactivation domains, nevertheless it was probed for transactivation experimentally. The ING5 cDNA sequence was conjugated to the Gal4 DNA binding domain (1-147) sequence and the resultant construct was co-expressed in the 293 cells along with the reporter in which multiple Gal-responsive elements regulate transcription of the luciferase from a minimal TK-promoter. The activation or repression of the reporter was measured in terms of the luciferase activity. If ING5 were a transactivator, the Gal4-ING5 fusion protein would be expected to activate the Gal4 responsive promoter but as shown in the Fig. 3.9. ING5 does not activate transcription at the Gal4tk promoter.

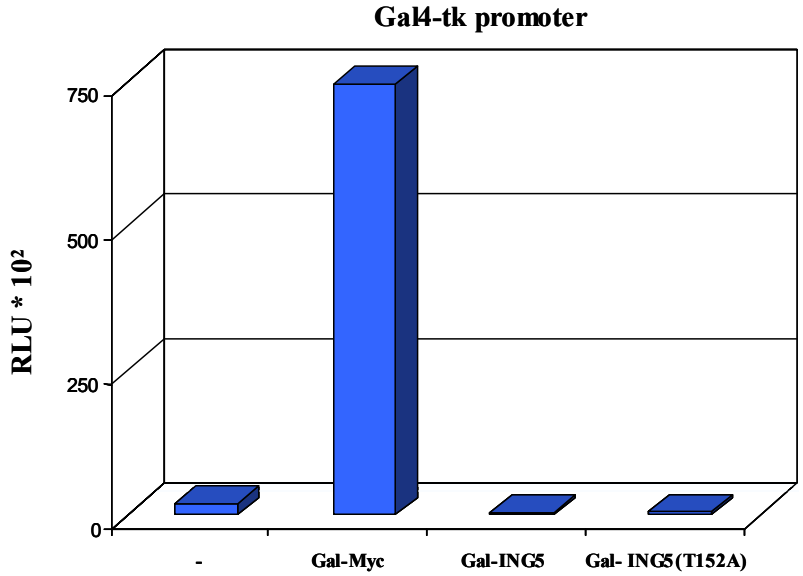

Figure 3.9: ING5 does not transactivate in the reporter gene assays. The Gal4 fused ING5/T152A was co-expressed along with the gal4 responsive promter-luciferase constructs. The gal4-fused Myc was used as the positive control for transactivation. The transcription at the promoter was measured in terms of luciferase activity, which was standardised with the ß-gal activity. ß gal activity was measured to control transfection efficiency.

The Gal4-ING5 was titrated in different amounts to avoid the possibility of concentration being a limiting factor (data not shown). Nevertheless ING5 did not transactivate in the transactivation assays. In order to better understand the mechanism of ING5 action two different approaches were employed:

In the first approach, ING5 was screened for other interaction partners so as to unravel the pathway, in which ING5 was involved, the possible co-factors that it recruited to activate p53. The yeast two-hybrid system was used for screening

In the second approach, an effort to map the interaction sites of p53 and ING5 was made. It was hypothesised that ING5 bound to the same site as MDM2 on p53 and therefore competed with MDM2 for binding to p53. To test this hypothesis, the GST pull down experiments were utilized.

3.4.2 Screening for interaction partners

The ING1 protein to which ING5 is highly homologous is known to be involved in complex with several cell cycle regulating and apoptosis mediating proteins. Knowing the versatile proteins with which ING1 is involved, it was interesting to know the interaction partners of ING5. Although p53 turned out to be one of the interaction partners of ING5, the mechanism of their interaction was not very clear. The fact that ING5 was not a transactivator on its own, insinuated that ING5 perhaps recruited a co-factor in order to activate p53 or it repressed the activity of a repressor of p53 in order to activate p53. Therefore in search of other interactors of ING5, a

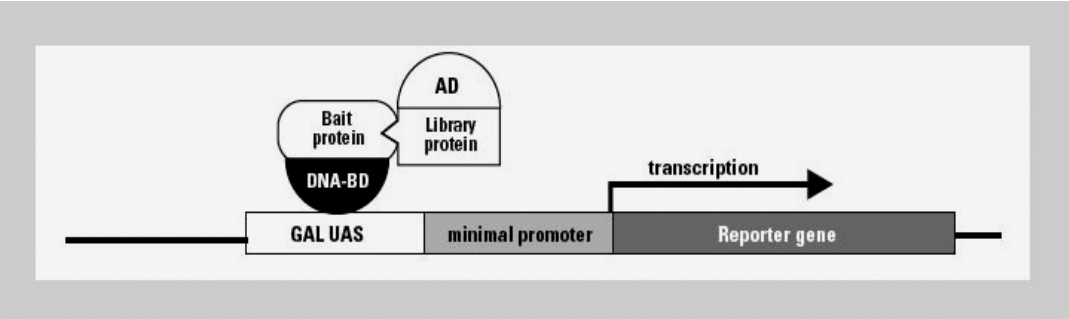

Figure 3.10: Schematic representation of the principle involved in yeast two-hybrid screening. AD, activation domain; DNA-BD, DNA binding domain; UAS, upstream activating sequence.

yeast two-hybrid screening was performed. The yeast two-hybrid system is a eukaryotic *in vivo* system used for the detection of protein-protein interactions. Since ING5 was not a transactivator by itself, the gal4 based matchmaker system was used. Gal4 protein is a yeast transcription factor that normally controls the genes responsible for galactose metabolism. Each Gal4-responsive element contains a target site called an upstream activating sequence or UAS and when Gal4 binds the UAS; transcription is activated from a downstream promoter.

In the yeast two-hybrid assays the yeast strains where the wild type Gal4 gene is eliminated and the gal UAS is linked to the metabolic genes *ade2, his3, mel1* and *lacz* were developed. In the gal4-based system, the bait gene is expressed as a fusion to the Gal4 DNA binding domain (DBD), while the prey cDNA is expressed as a fusion to the Gal4 activation domain. When the bait and the prey fusion proteins interact, the DNA binding domain and the activation domain are brought into proximity, thus activating the transcription of the four reporter genes *ade2, his3, mel1* and *lacz.* Although the DNA binding domain can bind the gal UAS, it cannot activate the transcription. Activation of transcription occurs only when the other half, that is, the activation domain (AD) joins the DBD at the UAS (Fig. 3.10). In the matchmaker systems, the AD consists of amino acids 768-881 of the Gal4 protein, the DBD, amino acids 1-147.

3.4.2.1 Construction, transformation and the expression of the Gal4 fused ING5 in Yeast.

The ING5 cDNA amplified from pDonR-ING5 was cloned into the *Eco* R1 and *Bam* H1 sites of the MCS of the pGBKT7 vector, in frame with the 3' end of the Gal4 DBD. The fusion protein was expressed from the constitutive ADH1 promoter (P_{ADH1}). The ING5 construct was transformed into the bait strain AH109. The AH109 strain was Ade^-, His^-, Leu^-, and Trp^- and

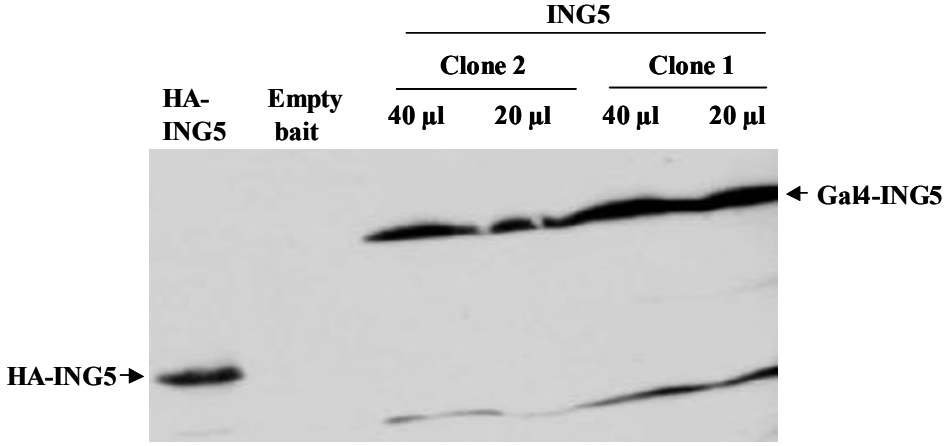

Figure 3.11: ING5 is expressed in Yeast. The clone 1 and clone 2 of pGBKT7-ING5 constructs were transformed into the AH109 cells and the protein extracted was separated on a SDS-PA gel and western blotted. ING5 was detected with the monoclonal ING antibody, 7A-11. HA-ING5 overexpressed in 293 cells was used as a positive control.

cannot grow on SD minimal media lacking these nutrients. It can grow on SD/-Leu/-Trp only if functional *trp1* and *leu2* genes were introduced. The transformed yeast was recognised with the

change in their phenotype as they could synthesize tryptophan and hence were capable of growing on tryptophan negative media. The total protein was extracted and separated by SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane by western blotting and the expression of ING5 was detected by immunoblotting. ING5, as seen in the Fig. 3.11 was well expressed in yeast.

3.4.2.2 ING5 does not transactivate in the yeast transactivation assays

Although ING5 did not transactivate in the reporter gene assays discussed already, nevertheless the ability of ING5 to transactivate was tested in the yeast two-hybrid system. If ING5 were a transactivator, it had to activate the expression of the reporter genes namely *ade2, his3, mel1* and *lacZ.* The yeast expressing ING5 was plated on the SD–Trp/+X-gal, SD-Leu/+X-gal, SD-Trp/-Ade/+X-gal, SD-Trp/-His/+X-gal/+3AT. The empty vector was used as the negative control. p150K1, p150K2, p150K3 and p150K4, fragments of PARP10 (Yu et al., 2005), which were known to transactivate as shown by Dr. Walsemann, a member of our group, were used as positive controls. These clones transactivated and hence were used as positive controls in these experiments (the data of p150K2, K3 and K4 are not shown). The yeast strain transformed with ING5 did not grow on the SD-Leu/+X-gal, SD-Trp/-Ade/+X-gal, SD-Trp/-His/+X-gal/+3AT plates. Adding 2,5 mM 3-AT suppressed the growth of background colonies due to the leaky expression of the *his3* gene. The concentration of 3-AT required was determined by titrating different concentrations of 3-AT. The lowest concentration that could suppress leaky growth on the SD/-His/-Trp plates was used in the subsequent experiments. Though ING5 transformed yeast grew on the SD–Trp/+X-gal, the colonies did not turn blue indicating that the expression of the *LacZ* reporter gene was not turned on. The negative control that is the yeast transformed with the empty bait vector, pGBKT7, did not grow on the SD-Leu/+X-gal, SD-Trp/-Ade/+X-gal, SD-Trp/-His/+X-gal/+3AT whereas the positive controls grew on the SD-Trp/-Ade/+X-gal, SD-Trp/-His/+X-gal/+3AT. It was concluded that ING5 does not transactivate in the transactivation assays performed in yeast and hence is not a transactivator. The following is a schematic representation of the transactivation assay results.

Table 3-2: Tabular representation of the transactivation result. The different probes and their growth on the different SD drop out plates are showed. The –Trp plate selects for the bait vector/ construct, -Leu for the library construct, -Ade and –His for the self-transactivators. (+, growth; –, no growth)

Probe	-Trp	-Leu	-Ade	-His (+3-AT2,5, mM)	X-gal
PGBKT7-ING5	+	-	-	-	-
PGBKT7	+	-	-	-	-
PGBKT7-P150K1	+	-	+	+	+

Since ING5 did not transactivate, it was used to screen for interaction partners against a pre-transformed matchmaker library.

3.4.2.3 Library Screening of ING5

The screening procedure involved the mating of the bait strain AH109 transformed with the bait construct, ING5, with the pre-transformed library strain, Y187. The bait strain was grown to a concentration of greater than 1 x 10^9 cells/ml. The pre-transformed library strain stored at –80°C was thawed just before use. The bait strain and the library strain were combined and the resultant mating mixture was incubated at 30°C overnight. The mating mixture was screened for interaction partners by plating on the SD/-His/-Leu/-Trp and SD/-Ade/-Leu/-Trp 15mm dropout plates after the washing, centrifuging and the re-suspending steps. Different dilutions ranging from 1:10, 1:100, 1:1000, 1:10,000 of the mating mixture was plated on SD/-Trp, SD/-Leu and SD/-Leu/-Trp 10 cm plates in order to calculate the mating efficiency. The number of colonies on these plates and the mating efficiency was calculated after 5 days. The number of colonies on the different dilution dropout plates is as follows:

	1:10	**1:100**	**1:1000**	**1:10,000**
SD/-trp	**>2000**	**>2000**	**948**	**400**
SD/-leu	**500**	**234**	**40**	**10**
SD/-leu/-trp	**412**	**50**	**30**	**10**

The Cfu of each of the mating partners and the diploid cells was calculated using the formula**:**

(Cfu x 100 µl/ml) / (Vol. Plated (µl) x dilution factor) = number of viable cfu/ml

The average Cfu/ml of the mating partners and the diploids was as follows:

Dropout plate	**Cfu/ml**
SD/-trp	**24,74,0000**
SD/-leu	**421,000**
SD/-leu/-trp	**347,800**

The mating efficiency was calculated as follows:

$$\frac{\text{Number of cfu/ml of diploids}}{\text{Number of cfu/ml of limiting partner}} \times 100$$

Therefore the mating efficiency in this case was:

$$\frac{347{,}800}{421{,}000} \times 100 = 82.6\ \%$$

The numbers of clones screened were 3.9×10^6. The colonies on the screening plates were checked after 3, 5 and 8 days of incubation. There were several colonies on the SD/-Ade/-Leu/-Trp plates, many of these colonies turned red after 8 days, which is an indication of being false positives on the –Ade plates. Only the colonies those were white on the SD/-Ade/-Leu/-Trp after 8 days were selected for further screening. There were several colonies on the SD/-His/-Leu/-Trp plates, only the colonies that were robust after 8 days were selected for further screening procedures. In total 76 colonies were picked for the subsequent screening procedures. These colonies were subsequently tested on the SD/-His/-Leu/-Trp/+X-gal, SD/-Ade/-Leu/-Trp/+X-gal, and SD/-His/-Ade/-Leu/-Trp/+X-gal plates. The clones that survived this round of screening are as follows:

Table 3-3: Represents the result from the initial re-testing of the Yeast-Two Hybrid clones. Growth on QDO indicates strong interaction, growth on SD/ - His/ - Leu/ -Trp and SD/-Ade/-Leu/-Trp plates indicates interaction, growth on SD/-Leu/-Trp indicates the presence of both the library and the bait plasmids in the growing colonies, growth on SD/-Trp indicates the presence of the bait and growth on SD/-Leu indicates the presence of the library plasmid. The clones that showed very little growth did not survive in the subsequent screening steps except for clone 58. (+, growth/ bluish appearance of the colonies; –, no growth; quadruple dropout plates, SD/-His/-Ade/-Leu/-trp plates)

Clone No.	SD/-Ade/-Leu/-trp/+X-gal	SD-His/-leu/-trp/+X-gal	QDO+X-gal	SD-His/-Leu/-trp (Master plates)
1	+	+	-	+
2	+	+	-	+
3	+	+	-	+
4	-	+	-	+
5	+	+	-	+
6	+	+	-	+
7	+	+	-	+
8	+	+	-	+
9	+	+	+	+
10	+	+	-	+
11	+	+	+	+
12	+	+	-	+
13	+	+	+	+
14	+	+	+	+
15	+	+	+	+
16	+	+	+	+
17	+	+	+	+
18	+	+	+	+
19	+	+	+	+
20	+	+	+	+
21	+	+	+	+
22	+	-	-	-
23	+	+	-	+
24	+	+	-	+
25	-	-	-	-
26	-	-	-	little growth
27	-	-	-	little growth
28	-	-	-	little growth

Clone No.	SD/-Ade/-Leu/-trp/+X-gal	SD-His/-leu/-trp/+X-gal	QDO+X-gal	SD-His/-Leu/-trp (Master plates)
29	+	+	-	+
30	-	-	-	-
31	-	-	-	-
32	+	+	-	+
33	-	-	-	-
34	+	+	+	+
35	-	-	-	-
36	-	-	-	-
37	+	+	+	+
38	-	-	-	little growth
39	+	+	-	+
40	+	+	-	+
41	-	-	-	+
42	-	-	-	+
43	-	-	-	-
44	-	-	-	-
45	-	-	-	-
46	-	-	-	-
47	-	+	-	+
48	+	+	+	+
49	+	+	+	+
50	+	+	+	+
51	-	+	-	+
52	-	-	-	+
53	+	+	-	+
54	+	+	-	+
55	+	+	-	+
56	-	+	-	+
57	-	+	-	+
58	-	+	-	very little growth
59	+	+	-	+
60	+	+	-	+
61	+	+		+

Clone No.	SD/-Ade/-Leu/-trp/+X-gal	SD-His/-leu/-trp/+X-gal	QDO+X-gal	SD-His/-Leu/-trp (Master plates)
62	+	+		+
63	+	+	-	+
64	+	+		+
65	+	+	+	+
66	-	-	-	-
67	+	+	-	+
68	-	+	-	+
69	-	-	-	-
70	+	+	+	+
71	+	+	-	+
72	+	+	-	+
73	+	+	+	+
74	+	+	-	+
75	+	+	+	+
76	+	+	+	+

The clones that grew on all the 3 drop out plates or just on SD/ - His/ - leu/ -trp/ + X-gal were subsequently sorted on the SD/ - Leu/ - Trp/ + X-gal plates to eliminate library clones with more than one library plasmid. A mixture of white and blue colonies indicated segregation. The sorted colonies were confirmed for the right phenotypes by growing them subsequently on the SD/ - His/ - Leu/ - Trp plates. The clones that turned blue in the SD-Leu/-Trp/+X-gal were confirmed for the right phenotype by growing them on the SD/-His/-Leu/-Trp plates. The clones that did not turn blue in the two rounds of screening on SD/-Leu/-Trp/+X-gal were nevertheless carried on for the further screening steps, but such clones were selected from the SD/-His/-Leu/-Trp master plates. The library plasmids were then isolated from the faster growing clones and transformed into the E. *coli* strain DH5α by chemical transformation. The library plasmids were isolated from the bacteria and the cDNA amplified by PCR, restriction digested with the *Alu* 1 enzyme and resolved on a 4 % agarose gel to isolate the clones harbouring the same library plasmid, based on their restriction patterns. The clones that were of the same length were identified and a representative of this group was carried onto the last step of screening that is the confirmation of

interaction by co-transformation of the bait and the library plasmids. The following table shows the clones that were of the same length on a 4 % agarose gel and grouped together.

Table 3-4: Represents the clones that were grouped together due to similarity in restriction pattern.

Group no.	Similar/ independent clones
1	1, 3, 16, 29
2	5,10, 50
3	19, 20, 21
4	13, 59, 60
5	34
6	51
7	53, 68
8	62
9	63, 76
10	67, 72
11	71
12	74
13	2
14	32
15	48

The library plasmids of the clones, 1, 2, 5, 19, 32, 34, 48, 51, 53, 59, 62, 63, 67, 71, 74, 76, were then co-transformed with the bait, pGBKT7-ING5 construct into the AH109 yeast strain and the interaction was confirmed on the SD/-his/-leu/-trp/, SD/-ade/-leu/-trp/, SD/-his/-ade/-leu/-trp/ selection plates. The activation of the *lacZ* reporter gene due to the postive interaction between ING5 and the library clone was confirmed by ONPG assays. In parallel the clones were sequenced and searched for homology. The clones 1, 2, 32, 34, 54, 71 and 74 turned positive upon re-screening. The following table represents the results of sequence analysis and homology search of the positive clones.

Table 3-5: Representation of the results from re-screening.

Clone #.	In Frame?	Homology searches	Locus	Re-screening result
1	No	J4-07 mitochondrion	no info	positive
2	Yes	Homo sapiens Uveal autoantigen with coiled coil repeats and ankyrin repeats (UACA)	15q22-24	positive
32	No	J4-07 mitochondrion	no info	positive
34	No	J4-07 mitochondrion	no info	positive
54	No	Homosapiens actinin,alpha 2	1q42-q43	positive
71	No	Homosapeins hepatocyte growth-factor regulated tyrosine kinase substrate alpha isoform 2	17q25	positive
74	No	Homosapeins hepatocyte growth-factor regulated tyrosine kinase substrate	17q25	positive

The sequencing analysis revealed that most of the clones analysed were out of frame. Therefore uveal autoantigen was the only survivor of this yeast two-hybrid screening**.** The following table represents an overview of the Yeast Two-Hybrid Screening results.

Table 3-6: An overview of the results of the Yeast Two-Hybrid Screening. In the above table + represents "growth" in case of the selection media and in case of re-screening + means the "clones turned positive" in the re-screening steps. (-) Represents no growth/ no colonies/ turned negative in the re-screening. * these clones did not survive in the subsequent rounds of screening

Clone No.	Master plates (SD-His/-Leu/-trp)	Segregation (SD-leu/-trp)	Retransformation (E. coli)	PCR fragment (bp)	BLAST analysis	In frame	Re-screening
1	+	No	30	550	J4-07 mitochondrion	No	+
2	+	No	5	1000-1500	uveal autoantigen	Yes	+
3	+	Yes	lots	500-750			
4	+	Yes	2	2000			
5	+	No	1	750-1000	DNA directed RNA polymerase	No	-
6	+	No	20	no product			
7	+	Yes	11	no product			
8	+	No	-				
9	+	Yes	-				
10	+	Yes	1	750-1000			
11	+	Yes	1	1000			
12	+	Yes	13	no product			
13	+	Yes	3	1500-2000			
14	+	No	3	750			
15	+	No	-				
16	+	No	34	500			
17	+	Yes	-				
18	+	Yes	3	no product			

19	+	No	30	1500-2000	SYNE1 (spectrin like protein)	No	-
Clone No.	Master plates (SD-His/-Leu/-trp)	Segregation (SD-leu/-trp)	Retransformation (E. coli)	PCR fragment (bp)	BLAST analysis	In frame	Re-screening
20	+	No	15	1500-2000			
21	+	Yes	13	1500-2000			
22	-	No					
23	+	Yes	5	no product			
24	+	No					
25	-	No					
26	little growth*	No growth					
27	little growth*	No growth					
28	little growth*	No growth					
29	+	Yes	6	approx 500			
30	-	-					
31	-	-					
32	+	Yes	13+4	250-500	J4-07 mitochondrion	No	+
33	-	-					
34	+	No	70	500	J4-07 mitochondrion	No	+
35	-	-					
36	-	-					
37	+	No	-				

38	little growth*	No growth					
39	+	Yes	could not be revived				
Clone No.	Master plates (SD-His/-Leu/-trp)	Segregation (SD-leu/-trp)	Retransformation (E. coli)	PCR fragment (bp)	BLAST analysis	In frame	Re-screening
40	+	Yes	could not be revived				
41	-	No					
42	+	Yes	could not be revived				
43	-	-					
44	-	-					
45	-	-					
46	-	-					
47	+	Yes	-				
48	+	Yes	3	700	TOM1	No	-
49	+	Yes	lots	no product			
50	+	Yes	5	750-1000			
51	+	Yes	36	approx. 1100	chromosome 11q	No	-
52	+	No					
53	+	Yes	lots	~ 650	Bad sequencing		
54	+	Yes	15	~ 1500	homosapiens actinin alpha 2	No	-
55	+	No	1	no product			
56	+	No					
57	+	Yes	3	3000			

58	very little growth*	Yes	could not be revived				
59	+	No	slow growers	1500			
60	+	No	slow growers	1500			
61	+	Yes	-				
62	+	No	9	1100	glutathion peroxidase 1(transcript variant 1)	No	-
63	+	Yes	~100	1100	glutathion peroxidase 1(transcript variant 1)	No	-
64	+	Yes	-				
65	+	Yes	-				
66	-	-					
67	+	Yes	~ 100	1100	glutathion peroxidase 1(transcript variant 1)	No	-
68	+	No		750			
69	-	-					
70	+	Yes	3	800			
71	+	Yes	55	2000-2500	*Homo sapiens* hepatocyte growth factor regulated tyrosine	No	+

					kinase 1		
Clone No.	Master plates (SD-His/-Leu/-trp)	Segregation (SD-leu/-trp)	Retransformation (E. coli)	PCR fragment (bp)	BLAST analysis	In frame	Re-screening
72	+	Yes	4	1400	*Homo sapiens* hepatocyte growth factor regulated tyrosine kinase 1	No	-
73	+	No	lots	2500			
74	+	No	28	750	*Homo sapiens* hepatocyte growth factor regulated tyrosine kinase 1	No	+
75	+	No	17	no product			
76	+	Yes	lots	1000			

In the yeast two-hybrid screening, the procedure per se was highly successful with a mating efficiency of 82.6%. It was highly promising to obtain 7 independent clones after re-screening. It is also interesting to note the fact that the only survivor, uveal auto antigen with coiled coil repeats, was one amongst the three clones that appeared after 3 days of incubation from the day of plating.

3.4.3 Mapping the interaction sites

3.4.3.1 ING5 binds to the DNA binding domain of p53

As mentioned already to gain insight into the mechanism of ING5 action, an effort was made to map the interaction sites of ING5 and p53. The hypothesis was that ING5 interacted with p53 and binds at the same site as MDM2. Thus competing with MDM2 to interact with p53. MDM2 binds specifically to p53 and promotes the covalent conjugation of ubiquitin residues to it, leading to subsequent p53 degradation by the 26s proteasome (Michael and Oren, 2003). So, if ING5 bound to the same region as MDM2 on p53, the MDM2 mediated degradation of p53 would be prevented and in turn p53 would be stabilised. To detect the interaction site of ING5 on p53, *in vitro* GST pull down assays were employed. The functional p53 protein is categorised into five domains, starting from the N-terminus, the transactivation domain (1-50), the proline rich domain (63-97), the DNA binding domain or the core domain (102-292), the tetramerization

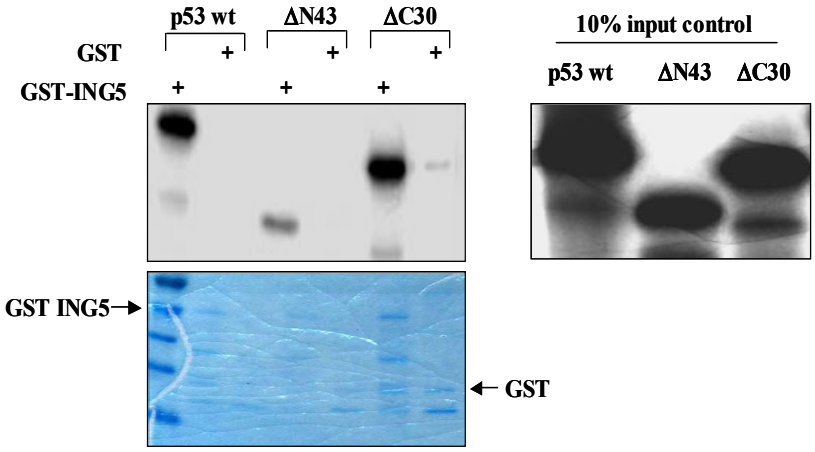

Figure 3.12: GST-pull down assays. The upper left panel shows the specific binding of p53 and its deletion mutants to ING5. The upper right panel is the input control of the in vitro transcribed/translated proteins of p53 and its deletion mutants. The lower panel is a coomassie stained gel, which indicates loading of GST/GST-fused proteins.

domain (323-356) and the regulatory domain (363-393). In the initial step, three mutants of p53, the ΔN43, where the 43 amino acids in the N terminus were deleted, the ΔC30, where the 30 amino acids on the C terminus were deleted and J7, where the three amino acids in the oligomerization domain were point mutated, were tested. The full-length p53 and the three aforementioned mutants were *in vitro* translated

in the presence of radioactively labelled methionine and precipitated with the GST fused ING5 or just GST protein bound to the glutathione sepharose beads. The precipitated proteins were

separated by SDS-PAGE and the presence of GST fused proteins was detected by Coomassie staining. It was surprising that the ΔN43 mutant of p53 bound to ING5. The ΔC30 and the J7 mutants as well bound to ING5 (Fig. 3.12). The full-length p53 was used as a positive control for interaction. So, ING5 does not bind to the same site as MDM2, as MDM2 binds to the N terminus of p53. The evidence that ING5 did not bind to the transactivation domain or the oligomerisation or the regulatory domain suggested that ING5 perhaps bound to the DNA binding domain or the proline rich domain of p53. The proline rich domain, with striking similarity to SH3-binding proteins is known to be required for p53-mediated apoptosis in some experimental systems as described by Sakamuro *et al*., 1997 for suppressing tumour cell growth (Walker and Levine, 1996). The significance of the central DNA binding domain has already been described in the introduction. Given the importance of the core domain of p53, and the fact that ING5 activated p53 (although it was not a transcriptional activator and did not bind to the same region as MDM2 on p53), it was probable that ING5 bound to the central region of p53 to stabilise p53 by modulating its binding to the target promoters. The proline rich regions are responsible for protein-protein interaction, the mutants carrying deletions of the proline rich region and certain regions of the DNA binding domain were tested for interaction in the GST pull down experiments. The DNA binding domain mutant Δ181-335 was created by deleting the DNA representing amino acids Δ181-335 of the full-length p53 by restriction digesting the pRc/CMV-p53 construct with *Eco* 47III and re-ligating it. The Δbox II was procured from Dr. Karen Vousden and the Δproline AE mutant from Dr. Arnold J. Levine. The Δ181-393 was created by a member of our group by restriction digesting the pRc/CMV-p53 construct with *Eco* 47III and *Xba* 1 and re-ligating it. The deletion mutants, Δproline AE, Δ181-335, Δbox II and the Δ181-393 were tested in the GST-pulldown experiments for interaction with ING5. All the mutants tested bound to ING5 very well. For unknown reasons the transcription/translation efficiency of the Δproline AE mutant was very less, nevertheless the binding was observed (Fig. 3.13).

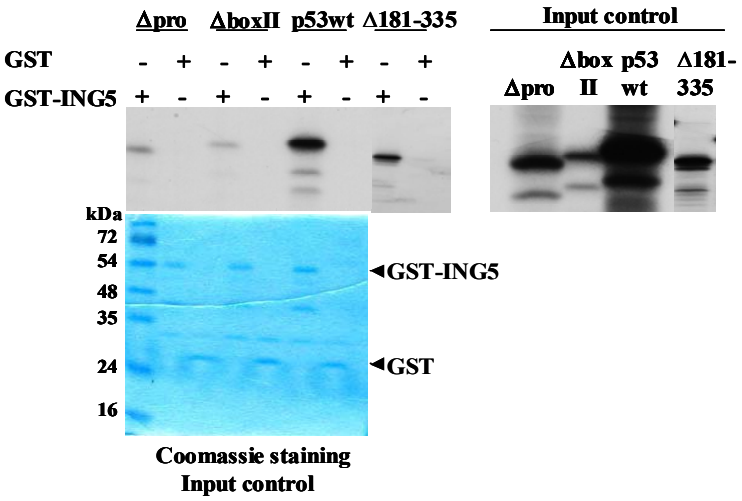

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Figure 3.13: Represents the GST pull down experiments. The Δproline mutant (Δpro), Δbox II and Δ181-335 of p53 bind to GST-ING5 specifically (upper left panel). The binding of wild type p53 to GST-ING5 is used as the positive control. On the right panel is the input control of the *in vitro* transcribed and translated protein of p53 and its deletion mutants. The lower panel is a coomassie-stained version of the upper left panel indicating the presence of the GST/- fused proteins. kDa, kilo Daltons

The interaction of ING5 with all of the deletion mutants tested so far, might suggest the region of interaction to be amino acids 143-181, which is in the central core domain. In the process of making the deletion mutants, a Δ137-159 was created where the DNA representing the amino acids 137-159 was deleted. This mutant that was assumed to be a deletion of the amino acids from 137-159 did not bind to ING5 in the GST pull down experiments. The sequencing results of this mutant revealed that there was a cloning error in the creation of this mutant, in other terms, the mutant harboured no stop codon and was expressed as a fusion with the vector sequence. In order to create a similar mutant a colleague deleted the amino acids 137-393. This mutant could not be pulled down by GST-ING5 in the GST pull down experiments (data not shown) whereas the full-length p53 could be pulled down. This result supports our suspicion that p53 interacts with ING5 through its DNA binding domain.

3.4.3.2 p53 binds to the PH domain of ING5

To detect the site of interaction of p53 on ING5, the *in vivo* Co-IP experiments were adopted. The ING5 deletion mutants, ΔPHD, where the PH domain was deleted, ΔN32, where the amino acids 1-32 were deleted and ΔC13 where the amino acids 1-13 were deleted were tested for interaction with p53 in the Co-IP experiments. The HA-ING5 and its deletion mutants, HA-ΔPHD, HA-ΔN32, HA-ΔC13 were over expressed in 293 cells and ING5 or its mutants containing complexes were precipitated with αHA antibody.

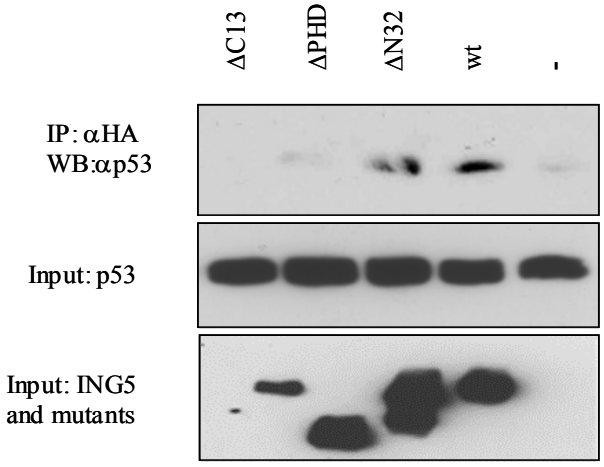

Figure 3.14: p53 binds to the PH domain of ING5. HA-ING5 and its deletion mutant constructs (1.5 μg) were overexpressed in the 293 cells. (A) The binding of endogenous p53 to ING5 and its deletion mutants was tested in the Co-IP experiments. The upper panel shows the binding of p53 to wild type ING5 and the ΔN32 mutant of ING5, no binding is observed in case of the ΔPHD and ΔC13 mutants of ING5. The middle and the lower panel show the expression of p53 and ING5, respectively. (B) schematic representation of ING5 (see section 1.6.1.).

The presence of endogenous p53 in the precipitates was monitored by immunoblotting for p53 with the monoclonal anti DO-7 antibody. The endogenous p53 could be pulled down with the full-length ING5 and the ΔN32 mutant, but not with the ΔPHD and ΔC13 mutants. The expression of the ΔC13 mutants was very low and hence could be accounted for the negative binding result. The ΔPHD mutant, though expressed well did not pull down p53 (Fig. 3.14). This suggests that p53 binds to the PH domain of ING5.

3.5 p63 and p73 also interact with ING5

3.5.1 ING5 modulates the transactivational capacity of p63 and p73

p63 and p73 are the recently identified members of the p53 family. They are known to perform certain "p53-like activities". Similar to p53 they form homo oligomers, bind DNA, activate transcription from p53-responsive genes, and induce apoptosis (Yang et al., 1998, Osada et al., 1998 and Jost et al., 1997). p63 and p73 can activate the

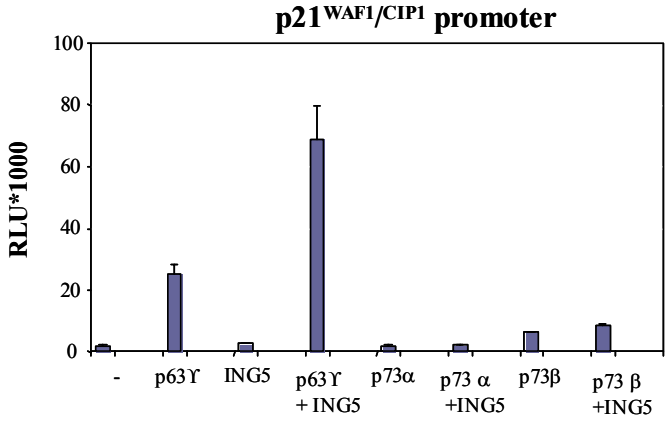

Figure 3.15: The reporter gene assays performed in the 293 cells. The promoter-luciferase constructs were transfected along with the HA-ING5 constructs and the HA tagged p63 and p73 constructs into 293 cells. The transactivation of the promoter was measured in terms of luciferase activity.

promoters of several p53 responsive genes like *p21, bax, mdm2* and others (Yang et al., 1998, Zhu et al., 1998). So it was tested if ING5 influenced the transactivation of the promoters of the *p21, pig3* and *Bax* genes by p63 and p73 in the reporter gene assays. The HA-ING5, the TA-p63γ, the TA-p73α and the TA-p73β constructs were co-transfected in the 293 cells along with the promoters of the *p21/bax/pig3* genes fused to luciferse ORF. The transcriptional activity of the *luciferase* gene was under the control of the aforementioned promoters. The transfection efficiency was normalised with ß-gal expression. TA-p63γ and TA-p73β activated the transcriptional activity of the promoters of the *bax, p21* and *pig3* genes. ING5 enhanced the transactivation by TA-p63γ on the promoters of the *bax* and *p21* genes. The transactivation by TA-p63γ was stronger on the p21 promoter (Fig. 3.15) rather than on the promoter of the *bax* gene (Fig. 3.16).

TA-p73β activates the promoters of the *Bax* and *PIG3* (Fig. 3.17) genes more strongly than the promoter of the *p21* gene.

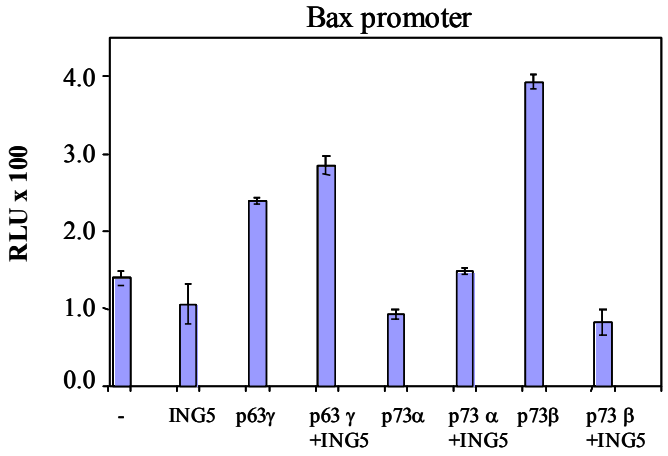

Figure 3.16: Effect of ING5 on p63 and p73 mediated transactivation of the *Bax1* gene.

ING5 repressed the activation of TA-p73β on the promoters of the *bax* and *pig3* genes whereas it slightly enhanced the activation of p73β on the promoter of the p21 gene. p73α was a weaker activator than p73β consistent with the observations of De Laurenzi et al., 1998 and Lee et al., 1999. p73α activated the promoter of *pig3* gene but not that of the *bax* and *p21* genes. Over expression of ING5 slightly enhances the activation by p73α on the *bax* promoter. Surprisingly, ING5 overexpression leads to a repression of

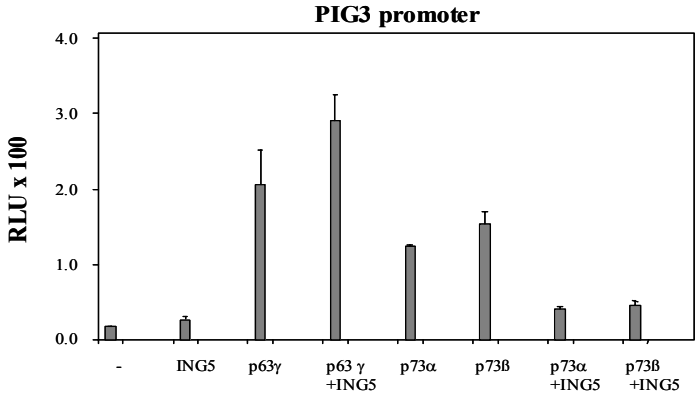

Figure 3.17: Effect of ING5 on p63 and p73 mediated transactivation of *pig3*. (RLU, relative luicferse activity; -, empty vector)

p73α-mediated pig3 promoter activation. ING5 does not influence the transactivation by p73α on the p21 promoter.

3.5.2 ING5 interacts with p63 and p73 physically

The interaction of TA-p63γ, TA-p73α and TA-p73β was tested *in vitro* in the GST pull

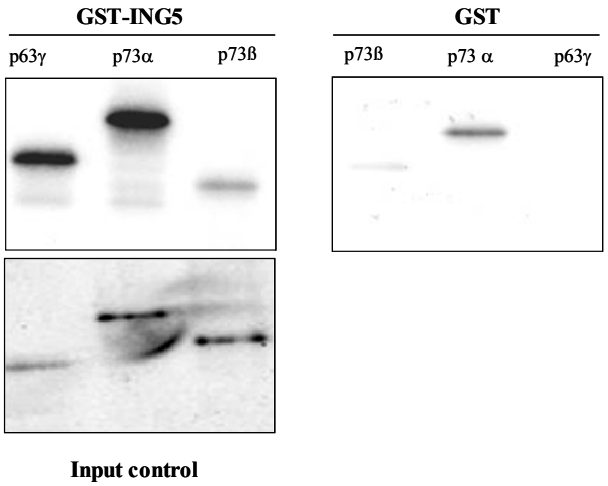

Figure 3.18: p63 and p73 bind to ING5 in the in vitro GST pull down experiments. The *in vitro* transcribed/translated TA-p63γ, TA-p73α and TA-p73β (TA, transactivation) bind specifically to GST-ING5 as shown in the upper left panel. The upper right panel indicates the binding of TA-p63γ, TA-p73α and TA-p73β to GST, the negative control. The lower panel indicates the input of the *in vitro* transcribed and translated proteins.

down experiments and *in vivo* in the Co-IP experiments. *In vitro*, TA-p63γ, TA-p73α and TA-p73β was transcribed and translated in the presence of radioactively labelled methionine and precipitated with the GST fused ING5 that was covalently bound to the glutathione sepharose beads. TA-p63γ, TA-p73α and TA-p73β interacted specifically with ING5, as they could not be precipitated with just the GST protein. Thus TA-p63γ, TA-p73α and TA-p73β bind to ING5 directly (Fig. 3.18).

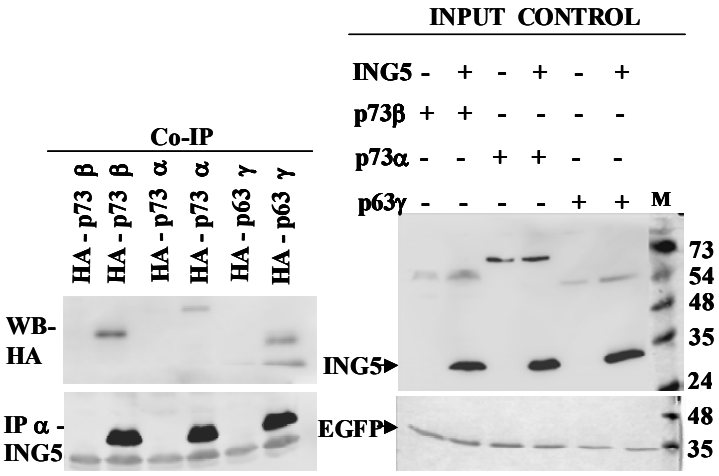

Figure 3.19: p63 and p73 bind to ING5 in vivo in the 293 cells. Both ING5 and the HA-tagged p63 and p73 constructs were overexpressed in the 29 3cells. ING5 was immuno precipitated with the monoclonal ING5 antibodies and the presence of p63 and p73 was detected in the immuno-precipitates with the HA-3F10 antibody. p63 and p73 bind to ING5 as shown in the upper left panel and the lower left panel shows that ING5 was immunoprecipitated with the monoclonal ING5 antibody. The upper right panel is the expression control for p63, p73 and ING5 where as the lower panel showing the expression of EGFP indicates equal loading of protein samples. M, marker(180 kDa).

In vivo, the TA-p63γ, TA-p73α and TA-p73β were co-expressed along with the HA-ING5 in 293 cells. ING5 was precipitated from the whole cell lysate with a combination of two ING5 specific monoclonal antibodies, αJas 7A-11 and αJas 3F9 antibodies. The presence of TA-p63γ, TA-p73α and TA-p73β in the precipitates was analysed by immunoblotting for TA-p63γ, TA-p73α and TA-p73β with the HA-3F10 antibody as all the aforementioned constructs were HA-tagged (Fig. 3.19). These results indicate that ING5 specifically interacts with p63 and p73 *in vitro* and *in vivo*.

4. DISCUSSION Chapter.4

Cyclin E is expressed during the late G1 phase of the cell cycle until the end of the S phase. Cyclin E binds and activates the kinase, CDK2 and by phosphorylating its substrates initiates a cascade of events that leads to the expression of S-phase specific genes ((Moroy and Geisen 2004). High levels of cyclin E have been associated with the initiation or progression of several cancers. In particular cyclin E has been linked to a poor prognosis in breast cancer (Porter et al 1997; Keyomarsi et al 2002). In order to better understand the role of cyclin E/CDK2, it is essential to know the substrates or the down stream targets of this complex. Therefore a member of our group performed a high-density protein array screening with the recombinant kinase. ING5 was identified as a potential cyclin E/CDK2 substrate.

The current report deals with the study of the cyclin E/CDK2 substrate, ING5, which belongs to the ING family of proteins that are known to suppress cell proliferation through interaction with p53. This work was aimed at addressing three significant points, which are as follows:

- Interaction of ING5 with p53 and its physiological relevance
- Effect of phoshorylation on ING5 mediated biological activity
- Possible interaction partners of ING5

4.1 Interaction of ING5 with p53 and its physiological relevance

The identification of ING5 from a screen for the substrates of cyclin E/CDK2 raised the questions such as:

- What is the physiological relevance of ING5?
- Does ING5 like the other members of its family, interact with p53?
- Does it modulate p53-mediated transactivation?
- If so what could be the mechanism of their interaction?
- What is the effect of phosphorylation on ING5?

The results of the colony formation assay and the transformation assays indicated that ING5 inhibits growth, thus answering the first question.

4.1.1 ING5 enhances the transactivational capacity of p53

Garkavtsev *et al* (Garkavtsev, Grigorian et al. 1998) reported that the activation of transcription from the $p21^{WAF1}$ promoter, a key mechanism of p53 mediated growth control, depends on the expression of ING1. The suppression of colony formation by ING5 may be either due to cell cycle arrest or due to apoptosis. One way of achieving cell cycle arrest is by inducing the expression of $p21^{WAF1/CIP1}$, which inhibits cyclin E/CDK2 to prevent G1-S progression of the cell cycle. Since ING1, which is highly homologous to ING5 co-operated with p53 to activate transcription at the p21 promoter, there was high possibility that ING5 played a similar role as ING1. When tested the effect of ING5 on the transactivational capacity of p53 on the promoter of the *p21* gene, it was observed that ING5 enhanced the transcriptional activation mediated by p53 whereas ING5 alone had just a minor effect on the transcriptional activity of the promoter. It was intriguing to know if ING5 had similar effect on the other target promoters of p53. So the effect of ING5 on the p53-mediated transcriptional activation of the promoters of the *bax, pig3* and *mdm2* genes was tested. ING5 enhanced the transcriptional activation mediated by p53 on the promoters of *bax*, and the *mdm2* genes whereas ING5 did not significantly influence the p53 mediated transcriptional activation of the promoter of the *pig3* gene. ING5 also enhanced p53-mediated transcriptional activation of the synthetic promoter *13xRGC*. This incongruity of ING5 activating p53 on certain target promoters and not on others is probably dependent on the functional p53 response element (PRE) present in these promoters. The specific interaction of p53 with its target promoters is tightly regulated. Promoter recognition by p53 is determined by the presence of PREs, which share homology with the consensus sequence 5'-$[PuPuPuC(A/T)(T/A)GPyPyPy]_n$-3' which is a decamer (el-Deiry, Kern et al. 1992). The naturally occurring PREs differ significantly from the optimised version. Typically only one decamer (termed "half site") stringently conforms to the consensus sequence whereas the other decamer(s) deviate from the consensus to a varying extent. There are also PREs whose bases violate the consensus rule completely for example the PRE within the *pig3* promoter. The PREs of the above tested promoters are as follows:

Table 4-1: Comparison of the functional PREs in different p53 responsive promoters: The decamers that conform to the consensus sequence are denoted in bold italicised and underlined letters. The bases that violate the consensus rule are denoted in small letters.

Promoter	p53 responsive element
p21 (El-Deiry et al., 1993)	**GAACATGTCC cAACATGTT**
Bax1 (Miyashita, T., and Reed, J. C. 1995)	**tcACAAGTTa AGACAAGCCT GGGCgTGggC**
Mdm2 (Juvel et al., 1993)	**GGTCAAGTTg GGACAcGTCC-N17 GAGCTAaGTCC tGACATGTCT**
PIG3 (Contente et al., 1997)	**TGCCC TGTCC TGTCC TGCCC TGCCC TGTCC TGTCC TGCCC TGCCC TGCCC TGTCC TGTCC TGCCC TGCCC TGCCC**

As seen above only the promoters of the p21 gene and the bax1 gene harbour a decamer that conforms completely to the consensus PRE where as the promoter of the MDM2 gene does not. In case of the p21 and the bax gene only one of the decamers comply with the rule where as the other decamers do not. What is noteworthy here is that the fold activation induced by ING5 upon p53 is consistent with the number of decamers in the PREs. For example the promoter of the bax1 gene has 3 decamers, where as the promoter of the p21 gene has 2 decamers and ING5 activates p53 by 6.8 fold on the promoter of the bax1 gene where as it activates p53 by 4.2 fold on the promoter of the p21 gene.

The microsatellite sequence in the pig3 promoter, $(TGYCC)_n$ where Y= C/T mediates the induction of p53 clearly indicating that it is deviating from the canonical PRE (Contente, Dittmer et al. 2002). Interestingly the PIG3 microsatellite is polymorphic and the size of the microsatellite directly co-relates with the extent of promoter activation by p53. ING5, in these experiments had a very minor effect on p53-mediated transactivation on the pig3 promoter. The Table 4.1 clearly indicates that the functional PRE in the pig3 promoter is totally different from the consensus PRE. It has also been reported that pig3 evolved its p53 responsiveness during the most recent stages of evolution (Contente, Dittmer et al. 2002). Reports suggest that the binding of p53 to its target sites is strongly dependent on DNA structure. The PREs are presented to p53 in form of different secondary structures (Gohler, Reimann et al. 2002) Therefore it could be

possible that the binding of p53 to the pig3 promoter covers the site of ING5 interaction and hence ING5 does not affect the transcriptional activation mediated by p53 upon the promoter of the pig3 gene.

To summarize, ING5 enhances p53-mediated transactivation of the promoters of p21, bax and mdm2 genes whereas ING5 does not activate p53 on the promoter of the pig3 gene.

4.1.2 ING5 interacts with p53 both *in vitro* and *in vivo*

In order to investigate the mechanism of interaction between p53 and ING5 it was necessary to know if ING5 and p53 interacted and if so whether the interaction was direct or indirect.

So the GST pull down experiments were performed to test the possibility of direct interaction. In the GST pull down experiments the in vitro transcribed/translated protein and the GST fusion protein (purified) are brought together in the GST pull down buffer. If the two proteins recognise each other then they would bind. A suitable control verifying the interaction between the in vitro translated protein and just the GST protein eliminates the possibility of false interaction. As shown in figure 3.7 p53 interacts with ING5 *in vitro*.

The interaction was further confirmed in vivo in cell culture with the Co-IP experiments. As shown in Fig. 3.2, ING5 precipitates endogenous p53 in the 293 cells. The 293 cells express significant amount of endogenous p53.

Therefore it could be inferred that ING5 interacts with p53 directly.

4.1.3 Phyisological relevance of p53 and ING5 interaction

The p53 gene has been shown to be a key regulator in a wide range of cellular processes such as cell cycle control, DNA repair, genome stability, programmed cell death, differentiation (Rotter, Aloni-Grinstein et al. 1994), senescence (Wynford-Thomas 1996) and angiogenesis (Vojta and Barrett 1995); (Bouck 1996). Since ING5 induces p53-mediated activation of the transcriptional activity at the promoter of the bax gene, the possibility of ING5 enhancing p53-mediated apoptosis was tested. ING5 was overexpressed in the MCF-7 cells that are wild type for p53. The morphological appearance of the cells was observed after fixing the cells 24 hours from the time

of transfection. The characteristic features of apoptosis such as blebbing and condensed chromatin were observed. It is interesting here to note that overexpression of ING5 alone was sufficient to mediate apoptosis in these cells. Initially 0,5 µM doxorubicin was used to induce the expression of endogenous p53 but since overexpression of ING5 alone was sufficient to see apoptosis in these cells, the doxorubicin treatment of the cells was avoided in the subsequent round of experiments. The fact that overexpression of ING5 alone without any induction for p53 induces apoptosis in MCF-7 cells could be reasoned out in several ways:

p53 is normally mutated in the breast cancers, but in case of those breast cancers which retain wild type p53, the mechanism of its inactivation is through nuclear exclusion (Moll et al., 1992). p53 in such breast cancers is present in the cytoplasm. The cells used in the apoptotic experiments here as well is a p53 wild type breast cancer cell line. So here in these experiments, it could be possible that overexpression of ING5 lead to the recruitment of p53 to the nucleus resulting in p53-mediated apoptosis.

Another possibility would be that ING5 mediates apoptosis independent of p53, by interacting with other members of the p53 family.

A third possibility could be technical reasons such as transfection, which cannot be ruled out. The transfection procedure by itself induces certain amount of stress, as observed in the empty vector control. So it could be possible that the transfection procedure by itself induces for low-level p53 expression and this minute amount of p53 is sufficient for ING5 to interact and activate to induce apoptosis.

To conclude ING5 induces apoptosis in the p53 wild type, breast cancer line, MCF-7.

4.1.4 Mechanism of ING5-p53 interaction

The interaction of ING5 with p53 and the effect of ING5 on the transactivational capacity of p53 raised the questions such as: How does ING5 activate p53? What is the mechanism behind this activation? One possibility was that ING5 transactivated p53 or it recruited a transcription factor to activate p53. The other possibility was that it competed with an inhibitor of p53 to interact with p53. One such inhibitor of p53 is MDM2. So it was suspected that ING5 competed with

MDM2 to interact with p53. To answer the above questions and test these possibilities two approaches were adopted:

The transactivation ability of ING5 was tested

The interaction sites of p53 and ING5 were mapped to gain an insight on the mechanism of their interaction

4.1.4.1 Is ING5 a transactivator?

Although the protein sequence comparison of ING5 with that of the known transactivation domains of several proteins showed no similarity, nevertheless, the possibility was tested as it was hypothesised that ING5 recruited a transcription factor to transactivate p53. The ability of ING5 to transactivate was tested in a Gal-4 based system. ING5, as shown in Fig.3.6, does not transactivate in this experiment where as the transactivation domain of c-myc fused to the DBD of gal4 did transactivate very strongly thus providing a positive control for the experiments with ING5. This important result became the foundation of the yeast two-hybrid Screening, which is discussed in the later part of this section of the thesis.

Two important conclusions could be derived from these experiments; ING5 does not transcativate and it does not recruit a transcriptional activator to transcativate**.**

4.1.4.2 Mapping the interaction sites

4.1.4.2.1 ING5 binds to the DNA binding domain of p53:

p53 is negatively regulated by MDM2 protein. The MDM2 protein binds to the N terminus that is the trans-activation domain of p53 and inhibits the ability of p53 to stimulate transcription (Momand, Zambetti et al. 1992); (Oliner, Pietenpol et al. 1993). This MDM2-p53 interaction leads to rapid proteasomal degradation of p53 (Haupt, Maya et al. 1997); (Kubbutat, Ludwig et al. 1998). Since ING5 function was antagonistic to that of MDM2, it was hypothesised that ING5 competed with MDM2 to interact with p53. To support this hypothesis, there were reports that ING1b the founder member of the ING family stabilised p53 by disrupting the p53-MDM2 interaction (Leung, Po et al. 2002). The first step towards probing the same was to test if ING5 bound to the same region on p53 as MDM2. The GST pull down experiments were performed

where interaction between the mutants of p53, namely the ΔN43, ΔC30 and J7 and the GST fused ING5 was tested. All the three mutants bound to ING5. The binding of the ΔN43 mutant where the 43 amino acids on the N terminus of p53 were deleted dispelled our hypothesis that ING5 competed with MDM2 for binding on p53. The consistent binding of the ΔC30 mutant and the J7 mutant where 30 amino acids on the C-terminus was deleted and where 3 amino acids on the C-terminus oligomerization domain were point mutated respectively, suggested that perhaps ING5 bound to the DNA binding domain or the proline rich domain of p53. Therefore, the mutants carrying deletions in the proline rich region and the DNA binding domain were tested in the GST pull downs. The ΔProAE, the Δ181-335, Δbox II and Δ181-393 mutants of p53 bound to ING5 whereas the deletion mutant Δ137-393 did not any more bind to ING5 narrowing down the region of p53-ING5 interaction on p53 to amino-acids 143-181. The amino acids 171-181 in p53 represents box III, which is one of the conserved regions in p53. It has been observed that 90% of mutations involve the core domain of p53. There are a total of 73 residues in the DNA binding domain that are 100% conserved across all species. Mutations resulting in amino acid substitution occur to these 73 conserved residues. Amongst the amino acid residues in this conserved region that are most frequently mutated are 158, 159, 161, 163, 164, 172, 173,175, 177, 178 and 179 which are of our interest. The DNA binding domain, is the domain that recognises and binds the target sequences (el-Deiry, Kern et al. 1992). The crystal structure of the p53 protein bound to its cognate sites reveals that the core domain consists of a large β sandwich that acts as a scaffold for three loop based elements. The scaffold anchors the loops and participates in head to tail dimerization. The first loop L1, which corresponds to box II binds to DNA within the major groove where as the second loop L2 corresponding to box III binds to the minor groove of the target sequence. The third loop L3 corresponding to the box IV packs against L1 and stabilises it. A zinc atom tetrahedrally co-ordinated on amino acids Cys 176, His 179, Cys 238 and Cys 242 connects the L2 and L3 (Cho, Gorina et al. 1994). The core domain is resistant to proteolysis by enzymes such as thermolysin and subtilisin. The Zinc binding of p53 is essential for the function of p53. Therefore any mutation to these residues would render p53 non functional. The residues cysteine176, His179, Cys238 and Cys242 are all involved in Zinc binding. It must be noted that the residues cysteine176 and His179 are suspected to be part of the region of ING5–p53 interaction thus emphasizing the significance of this interaction.

Given the importance of the central core domain of p53, one direct question that rises is what is the significance of ING5 binding to this region. The immediate answer would be perhaps ING5 stabilises p53 binding to its cognate promoters. It could be possible that ING5 interacts with p53 to modulate its binding to its respective cognate sites. ING5 could play a possible role in stabilising p53, by binding to L2 (loop 2), which represents box III of the conserved region in p53 that binds to the minor groove of the cognate sequence. This could be tested by looking at the binding of p53 to its target sites in the presence and absence of ING5 in assays like oligo-pull down or EMSA.

It is also possible that the zinc finger motif in the PH domain of ING5 interacts with the zinc finger of p53 to stabilise the binding of p53 to its cognate sites.

The other significance of this interaction could be the retention of p53 in the nucleus as already discussed in section 4.1.3.

To infer, ING5 binds to the DNA binding domain of p53 and enhances p53-mediated transcriptional activity of its target promoters. Therefore any mutation to this region of p53 would disrupt p53-ING5 interaction and thereby ING5 mediated activation of p53. The observation that more than 90% of mutations occur in the central DNA binding domain of p53 in cancers where p53 is non-functional further substantiates the significance of p53-ING5 interaction.

4.1.4.2.2 p53 binds to the PH domain of ING5

The site of interaction of p53 on ING5 was mapped in vivo with Co-IP experiments. Three mutants of ING5, ΔPHD, ΔN32 and ΔC13 were tested for binding with endogenous p53 in the 293 cells. p53 bound to both the ΔN32 mutant of ING5 and wild type ING5 where as no binding was observed in case of ΔPHD and ΔC13 mutants of ING5. The expression of the ΔC13 construct was quite low and hence a negative binding could be observed. Therefore p53 binds to the PH domain of ING5.

The PH (Plant Homeo) domains have been proposed to function in protein-protein and protein-DNA interactions. The PHD motif is a conserved Cys4-His-Cys3 orphan zinc finger domain

present throughout eukaryotic proteomes (Aasland, Gibson et al. 1995). A large number of chromatin regulatory factors contain PHD fingers including the acetyl transferase proteins CBP/p300, the chromatin remodelling protein ACF and the ING family of putative tumour suppressors (Feng, Hara et al. 2002); (Fyodorov and Kadonaga 2002); (Kalkhoven, Teunissen et al. 2002) . Feng et al and Pascual et a (Pascual, Martinez-Yamout et al. 2000) reported that mutations in the PHD fingers were found in tumour tissues and were responsible for various genetic disorders. There were reports that a subclass of putative PHD fingers were shown to have E3 ubiquitin ligase activity. Until recently there were no known binding partners for the PHD motifs, recently Gozani et al reported the binding of phosphoinositides to the PHD motif of the ING2 protein, which is another member of the ING family. This interaction influenced the sub-cellullar localisation of ING2 (Gozani, Karuman et al. 2003). They also observed that the PHD fingers from different chromatin associated factors bind to phosphoinositides suggesting that PHD fingers might function as a general nuclear phosphoinositide receptors. It is not known if the PHD motif in ING5 interacts with the phosphoinositides.

Once again the functional significance of this interaction could be that of stabilising p53 as already discussed in the previous section. Given the importance of the PH domains of various proteins it would not be surprising if the PH domain in ING5 played a major role in stabilising p53.

The mapping of interaction domains of p53 and ING5 is the first of its kind in the sense so far no one has mapped the interaction domains of any of the ING family members with that of p53. It would be interesting to know if the other members of the ING family also interact through their PH motifs with p53 as all the members of ING family have the PH domain. The mapping also would in future possibly unveil a new mechanism of p53 regulation.

To conclude, the PH domain of ING5 interacts with the DNA binding domain of p53.

4.2 Effect of phosphorylation on the function of p53

Dr. Lilischkis, a member of our group identified ING5 in a screen for the substrates of cyclin E/CDK2 and confirmed the phosphorylation of ING5 on the amino acid residue, threonine152, *in vitro* and *in vivo*. To test the effect of phosphorylation on the stability of the protein he

performed emitine chase experiments and observed that the wild type ING5 was unstable whereas the non-phosphorylatable mutant of ING5, where the threonine 152 was mutated to alanine was stable (unpublished data). This indicated that phosphorylation by cyclin E/CDK2 of ING5 lead to its degradation. Therefore the effect of cyclin E/CDK2 on ING5 mediated activation of p53 and apoptosis was tested. The enhanced transactivation mediated by p53 upon ING5 overexpression was reduced to half upon co-expression of cyclin E whereas that of T152A mutant was not significantly affected suggesting that phosphorylation negatively influenced ING5 function. Further the effect of cyclin E/CDK2 on the apoptotic function of ING5 was tested in the apoptotic assays. Overexpression of cyclin E/CDK2 almost completely knocked out ING5 mediated apoptosis and the non-phosphorylatable mutant, T152A was totally unaffected once again emphasizing the significance of phoshorylation of ING5.

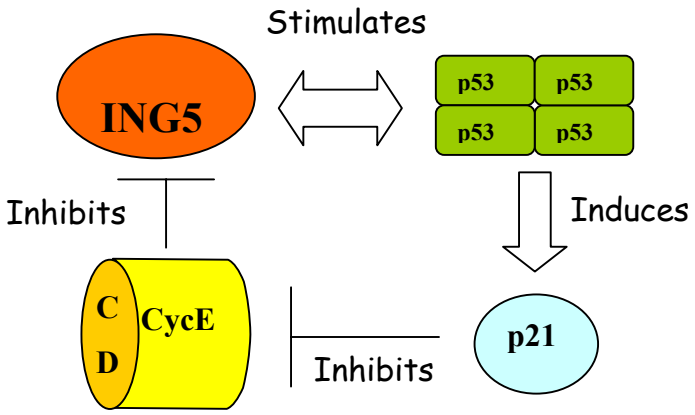

Figure 4.1: Hypothesis. In the forward loop, ING5 interacts with p53 to induce the expression pf p21, thereby inhibiting the activity of its negative regulator, cyclin E/CDK2. In the back loop, ING5 is phosphorylated by cyclin E/CDK2 and degraded; thereby the enhanced activity of p53 and p53-induced expression of p21 is abrogated.

Cyclin E is expressed during the late G1 phase of the cell cycle until the end of the S phase. Cyclin E binds and activates the kinase, CDK2 and by phosphorylating its substrates initiates a cascade of events that leads to the expression of the S-phase specific genes (Moroy and Geisen 2004),. The cyclin/CDK complexes induce two processes of the cell cycle, duplication of centrosomes and DNA. The cyclin E/CDK2 complex is required for the G1/S transition of the cell cycle (Resnitzky et al., 1995). Since ING5 interacts with p53 to possibly arrest the cell cycle at S phase (transactivation results), it becomes necessary for cyclin E/CDK2 to down regulate

this arrest. So it could be possible that cyclin E/CDK2 achieves this by down regulating the expression of ING5 during this phase. The hypothesis adopted by our group is as follows (figure 4.1)

To conclude phosphorylation of ING5 negatively regulates the function of ING5. ING5 is the only member of the ING family that is known to be phosphorylated. There is no evidence of phosphorylation of any of the other members of the ING family.

4.3 The interaction partners of ING5

4.3.1 Yeast Two Hybrid Screening

In order to understand the mechanism of ING5 action it was necessary to identify other possible interaction partners of ING5. Xin *et al* reported that p33ING1b interacted with the DNA methyl transferase 1 (DMNT 1) associated protein 1 (DMAP1) complex and a core Sin3 HDAC1/2 complex to maintain histone methylation and hypoacetylation in pericentric heterochromatin during the late S phase of the cell cycle. P33ING1b interacts with PCNA through its PIP box following UV exposure (Xin *et al., 2004)*. ING2 is reported to interact with phosphoinositides, which influence the cellular localisation of ING2 (Gozani, Karuman et al. 2003). ING4 physically interacts with p65 (Rel A) subunit of nuclear factor NF-kB and regulates brain tumour angiogenesis through transcriptional repression of NF-kB responsive genes (Garkavtsev, Kozin et al. 2004). p29ING4 interacted with p300 to acetylate p53 (Shiseki, Nagashima et al. 2003). Therefore the idea of looking for the other interaction partners was pursued. The best, unbiased way of looking for the interaction partners of ING5 was to perform a screen with ING5 as the probe. The yeast two-hybrid system (Y2H) was selected, as it provided an opportunity to screen for interaction partners in a eukaryotic system. Since ING5 did not transactivate transcription from a Gal4 based promoter on its own, which is discussed in the earlier section, the Gal 4 based Y2H system was used. In this system the bait protein, which in this case is ING5, was fused to the Gal4 DNA binding domain and an oligo dT primed library derived from the human bone marrow was searched for interaction partners. Out of the 15 independent clones from the initial screening procedures, 7 of them turned positive in the final re-screening procedure. The rest were false positives. Scientists working with the yeast two-hybrid screening procedure have been constantly troubled by the occurrence of these false positives. There is no genuine reason to

explain their occurrence. Some of the commonly known false positives are hsp's, ribosomal proteins, cytochrome oxidase, mitochondrial proteins, proteasome subunits, ferritin, tRNA synthase, collagen-related proteins, Zn finger proteins and Vimentin. Cytochrome oxidase, ferritin, tRNA synthase, collagen-related proteins and Vimentin have never occurred as real interactors in the Yeast two hybrid assays (Van Criekinge and Beyaert 1999). It was observed that the positive clones appeared mostly within the first five days of plating the mating mixture. The clone 2 appeared 3 days after plating the mating mixture, clones 32, 34 and 54 after 5 days, the clones 71 and 74 appeared after 8 day. The clone 34, which turned positive in the re-screening, was a strong interactor as it grew on the quadruple drop out plates as well. Sequence analysis of these clones showed that six out of seven clones were out of frame. The only surviving clone was "Uveal autoantigen with coiled coil repeats and ankyrin repeats".

Though the occurrence of out of frame clones is "normal" but it is "rare". The manual of clontech, from whom the Yeast two Hybrid kit was purchased describes that only one-third of the library clones carry in-frame cDNA. But it is surprising how these out of frame clones survive to form proteins. There are reports that the translation machinery in both yeast and higher eukaryotic cells can overcome frame shifts with low to moderate frequency (Dinman and Wickner 1992; Farabaugh 1993).

The other possibility could be that these proteins are true interactors of ING5. This idea is supported by the evidence that the bifunctional protein DCOH binds to HNF1. DCOH again was an out of frame prey protein that interacted with the bait, HNF1 in the yeast two-hybrid screening performed by David J. D. (Sourdive, Transy et al. 1997). Though DCOH was an out of frame clone they went ahead to characterize the interaction, as they knew from other procedeures that DCOH interacted with HNF1. They suggest that the yeast translation machinery is able to perform compensatory frame-shift leading to the synthesis of limited amounts of the in-frame fusion between the Gal-4 activation domain and DCOH, which has already been described in *S. cerevisiae* (Dinman and Wickner 1992). These truly in-frame fusion driving preys were probably excluded due to too limited growth and absence of visible colonies. Thus the data from Sourdive *et al* proves to be an important piece of data for researchers interested in performing yeast two-hybrid screening. This suggests that the positive out of frame clones in my case should still be considered as potential interactors of ING5.

The only in-frame clone that interacted with ING5 was "Uveal autoantigen with coiled coil repeats and ankyrin repeats" (UACA). It is expressed in almost all tissues and the highest level of expression is obtained in the skeletal muscle (Yamada, Senju et al. 2001). It is a 166 kD protein and contains 6 ankyrin repeats and coiled coil domains including a motif of leucine zipper pattern. Ankyrin repeat elements are 31-33 amino acid motif present in a number of proteins that are implicated in protein-protein interactions (Lux, John et al. 1990). In NF-kB, ankyrin repeats play important roles in protein-protein interactions that regulate localisation and activity of NF-kB (Blank, Kourilsky et al. 1992). Coiled coil domains are involved in self-aggregation or interaction with other proteins and have been noted in many auotantigens (Kaghad, Bonnet et al. 1997); (O'Rand, Richardson et al. 1992); (Pollard and Cohen 1990) (Amati, Alevizopoulos et al. 1998). The leucine zipper motif promotes dimerization through α helical coiled coil formation and it is able to bind to DNA (Nagase, Kikuno et al. 2000); (Surette and Stock 1996). Thus suggesting UACA to be involved in protein-protein interactions. The peptide fragment of UACA ^{1029}ENDKLKKE^{1036} is 7/8 identical to the peptide fragment ^{40}ENAKLKKE^{47} of segment VP6 of Banna virus (Attoui, Billoir et al. 2000). Banna virus belongs to the coltivirus type species and has been isolated from the patients suffering from meningoencephalitis (Chin, Pomerantz et al. 1998); (Chen and Tao 1996). UACA is a possible target autoantigen in Vogt-Koyanagi-Harada disease (VKH). VKH is an autoimmune systemic disorder occurring in multiple organs containing melanocytes, including uvea, skin, central nervous system and inner ears Kazuhiro Yamada *et al*., 2000). Although there is information about the expression of this protein there are no functional implications known. It could be possible that the PH domain in ING5 is responsible for the interaction between ING5 and UACA, as the PH domains are also known for protein-protein interactions. The current information on UACA is not sufficient enough to speculate on the functional significance of this interaction. To gain an insight into the significance of this interaction it would be important to know the effect of UACA on ING5 mediated activation of p53 responsive promoters and ING5 mediated apoptosis.

The other previously identified proteins that were derived as interaction partners from the yeast two-hybrid screening are actinin alpha –2 and hepatocyte growth factor regulated tyrosine kinase substrate.

Alpha actinin-2: Actinins belong to the spectrin gene family and are an ancient family of actin binding proteins. They play a regulatory role in cytoskeletal organisation and muscle contraction. Alpha actinin-2 is known to be specifically expressed in muscle cells. Actinins are also known to interact with integrins, thus implying a possible role for ING5 in integrin signalling (Barkebusch and Fassler. 2003).

Hepatocyte growth factor regulated tyrosine kinase substrate (HRS): It is a FYVE zinc finger protein phosphorylated at tyrosine residues in response to PDGF, HGF and other growth factors. This protein is implicated in cell signalling and intracellular membrane trafficking. HRS binds to phosphoinositol 3 phosphate and is localised in the endosomes. HRS was identified to be interacting with neurofibromatosis-2 (NF-2) in a yeast two hybrid screening. Although the functional significance of this interaction is not conclusive it was observed that NF-2 colocalised with HRS at the endosomes. It is also implicated to play a role in JAK-STAT signalling (Scoles et al., 2000). The obvious question would be what is the significance of ING5 interaction with HRS. First of all it must be noted that HRS is a protein with FYVE zinc finger domain. It is possible that the zinc motif in the PH domain interacts with that of the HRS protein. The PH domain of ING2, a member of the ING family functions as a nuclear phosphoinositide receptor. Since HRS interacts with phosphoinositidol 3 phosphate, it implies that ING5 also possibly interacts with phosphoinositides. Thus probably conferring a new role to ING5, as a phosphoinositide receptor.

To conclude, UACA, alpha actinin 2 and hepatocyte growth factor regulated tyrosine kinase substrate are novel potential interactors of ING5.

4.3.2 p63 and p73 are the other interactors of ING5

p63 and p73 are the most recently identified members of the p53 family. p63 and p73 can activate the promoters of several p53 responsive genes like p21, Bax, MDM2 and others (Yang, Kaghad et al. 1998); (Zhu, Jiang et al. 1998). The immediate question that arose was does ING5 activate p63 and p73 similar to p53. So p63 and p73 mediated transcriptional activation of the promoters of the p21, Bax and the PIG3 genes were tested in the luciferase assays. The results obtained were not so straightforward as in case of p53. The result of these assays is summarised in table 4.2**.**

Table 4-2: p63 and p73-mediated transactivation of the promoters of p21, Bax and PIG3 and the effect of ING5 on it. +, activation; -, no effect

Promoter	p63γ	p73α	p73β	ING5	p63γ+ING5	p73α+ING5	p73β+ING5
p21	+	-	+	-	+	-	-
Bax	+	-	+	-	+ (very little activation)	+	Repression
PIG3	+	+	+	-	+	Repression	Repression

TA-P63γ was used as the representative of p63 and TA-p73α and TA-p73β represented p73. Both TA-p63γ and TA-p73β transcativated the promoters of the Bax, p21 and PIG3 genes. This is in accordance with the earlier published reports (Zhu et al., 1998 and Yang et al., 1998). ING5 enhanced the activity of TA-p63γ on the promoters of Bax, and PIG3 genes though not as significantly as it did the promoter of the p21 gene. It needs to be tested if ING5 also activates the expression of the endogenous bax and p21 proteins. ING5 did not affect the transactivation of TA-p73β on the promoter of the p21 gene whereas it repressed TA-p73β mediated transcriptional activity on the promoters of the PIG3 and Bax genes. p73α transactivates the promoter of the PIG3 gene whereas it does not transactivate the promoters of the p21 and Bax genes. The transactivation of p73α on the promoter of the PIG3 gene is repressed by ING5 whereas ING5 enhances slightly the transactivation of p73α on the promoter of the Bax gene. The relationship between ING5 and PIG3 seems to be highly mysterious. ING5 dos not influence p53-mediated activation of the promoter of the PIG3 gene where as it does activate in case of all the other promoters tested and here, ING5 represses p73α and p73β-mediated activation of the promoter of the PIG3 gene. It is amazing to see how ING5 handles so differentially the two isoforms of p73.

On the whole ING5 stimulates the transactivation of p63 on the promoters of the Bax and p21 just as in case of p53. p73α on its own cannot activate transcription at the promoter of the Bax gene. It needs ING5 to do it.

Further the interaction of ING5 with p63 and p73 was checked in vitro in the GST pull down experiments and in vivo in the Co-IP experiments. ING5 interacted with TA-p63γ, TA-p73α and TA-p73β both in vitro and in vivo.

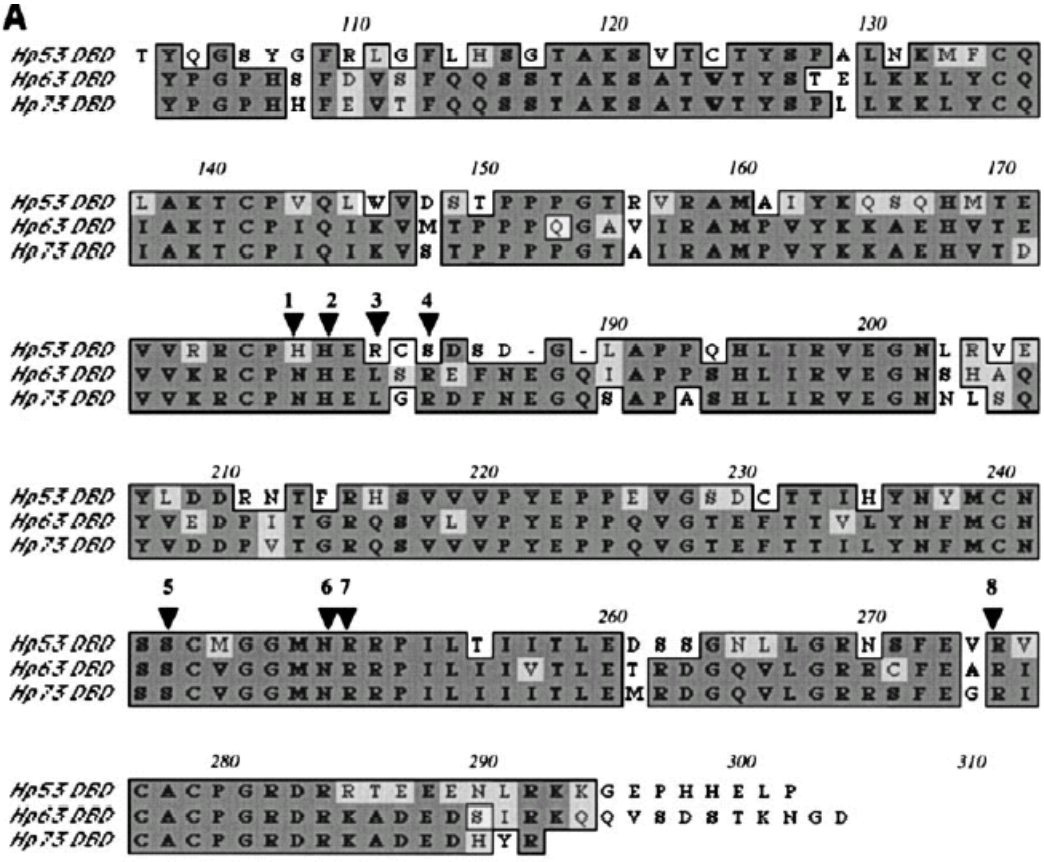

Figure 4.2: Sequence alignment of the DNA binding domains of p53, p63 and p73 (Bergamaschi, Samuels et al. 2004). The amino acids blocked indicate the identical amino acids. Arrows; amino acids of p53 that bind to ASPP1 and ASPP2.

This study shows that ING5 not only interacts with p53 but also interacts with its family members p63 and p73. So far there are no reports of any of the members of the ING family interacting with p63 and p73. ING5 is one amongst the three proteins that are known to be the common interactors of the p53 family. The first common interactors of the p53 family known are ASPP1 and ASPP2 (Bergamaschi, Samuels et al. 2004). Both ASPP1 and ASPP2 interact with p63 and p73 to specifically induce apoptosis just as in case of p53. But ING5 seems to play a more complicated role than the ASPP proteins. The coincidence is that both ING5 and ASPP proteins bind to the DNA binding domain of p53 and they also interact with p63 and p73.

Sequence comparison of the DNA binding domains of p53, p63 and p73 show that they are highly homologous to that of each other (Bergamaschi, Samuels et al. 2004). It must be observed that amino acid residues cys176 and his179 that are involved in zinc binding in case of p53 are identical to that of p63 and p73. This is also part of the region where ING5 is suspected to bind to p53. Hence it could be possible that ING5 interacts with both p63 and p73 through their DNA binding domains.

Some of the most obvious questions that needs to be answered are:

- What is the functional significance of ING5-p63/ING5-p73 interaction?
- Do the differences observed in the ING5 mediated activation/repression of the promoters of the different genes tested here apply to the endogenous expression of these proteins?
- How does ING5 decide to interact with either members of the p53 family? Or does it form complexes with the different p53 family members?
- Does ING5 mediate apoptosis by interacting with p63 or p73 in the p53 negative cells?

To conclude, ING5 is an activator of the tumour suppressor p53 and is regulated by cyclin E/CDK2. It interacts with p53 to induce apoptosis in the MCF-7 breast cancer cells with an intact p53 pathway where p53 is inactivated by nuclear exclusion thus suggesting that it probably recruits p53 to the nucleus in these cells to induce apoptosis. The PH domain of ING5 binds to the DNA binding domain of p53. Since the binding of ING5 to p53 is suspected to be in the region, which is involved in sequence specific DNA binding of p53, it is possible that the binding of ING5 to p53 stabilises p53. ING5 also interacts with p63 and p73 and regulates the two isoforms of p73 differentially. ING5 interacts with these two family members of p53 to probably induce cell cycle arrest and apoptosis. In such a case, ING5 would be a suitable target in treating tumours where p53 is inactivated but there is functional p63 or p73. Thus ING5 due to its functional importance and complexity demands attention from the researchers.

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6. Appendix

Abberivations

%	percentage
°C	degree celsius
µCi	micro curie
µl	micro litre
3AT	3- amino-1,2,4-triazole
ade	Adenine
Amp	Ampicilin
bp	base pair
BPB	Bromo phenol blue
cDNA	Complementary DNA
Cfu	Colony forming units
Co-IP	Co-immunoprecipitation
conc.	concentration
dd	Double distilled
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl suphoxide
DNA	Deoxyribo nucleic acid
DO	Dropout
DTT	Dithriothreitol
EDTA	Ethylene diamine tetra acetate
FAT	Factor acety transferase
FCS	Foetal calf serum
g	Gram
Gal1	Galactose1
GAL4	Galactose4
GST	Glutathion sepharose transferase
HAT	Histone acetyl transferase
Hebs	Hepes buffered saline

Hepes	N-2-Hydroxyethyl-Piperazine-N'-2-Ethano sulphonic acid
his	Histidine
hr	hour
Ig	Immunoglobulin
IP	Immunoprecipitation
IPTG	Isopropylthio-beta-D-galactoside
kan	kanamycin
L	litre
leu	leucine
mA	milli ampere
MEL1	Melanose 1
MEL2	Melanose 2
MEM	Modified Eagle medium
mg	milligram
Min	minute
mRNA	messenger RNA
ONPG	Orthonitrophenylpyranogalactoside
PEG	Poly ethylene glycol
PFA	Para-formaldehyde
RLU	Relative light units
RNA	Ribonucleic acid
Rpm	Rotations per minute
RT	Room temperature
s	second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
ß-gal	β galactosidase
Taq	*Thermus aquaticus*
TE	Tris-EDTA
Tm	Melting point
trp	tryptophan

v/v	volume/volume
w/v	veight/volume
WB	Western Blot
wt	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

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Declaration / Erklärung

Herewith I declare that I have written this PhD thesis myself, using only the referenced literature.

Hiermit versichere ich, dass ich die vorliegende Doktorarbeit selbstständig verfasst und keine anderen als die angebenden Hilfsmittel und Quellen verwendet habe.

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