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Rescue of mutant p53 family members by the low molecular weight compound PRIMA-1^{MET}/APR-246

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To my parents
To my supervisor Yuan Wei Jia

ABSTRACT

The tumor suppressor p53, guardian of the genome, is induced and activated by cellular stress signals such as DNA damage, hypoxia and activation of oncogenes. p53 upregulates downstream target genes, that are involved in cell cycle arrest, senescence, apoptosis, etc. Mutations in p53 occur frequently (around 50%) in many human tumors. Tumors with p53 mutations often show increased resistance to chemotherapy, since many anti-cancer drugs induce p53-dependent apoptosis though DNA damage. Thus restoration of wild type function to mutant p53 appears as an attractive approach for novel cancer therapy.

The low molecular weight compounds PRIMA-1 and PRIMA-1^{MET} were previously identified in our laboratory. We have shown that both PRIMA-1 and PRIMA-1^{MET} (as denoted APR-246) are converted to methylene quinuclidinone (MQ), that binds covalently to the DNA binding domain of mutant p53, restores its wild type function and triggers massive apoptosis in cancer cells. However the exact molecular mechanism of the mutant p53-dependent apoptosis induced by these compounds was not elucidated.

In paper I, we demonstrate that PRIMA-1^{MET}/ APR-246 triggers the mitochondrial apoptosis pathway in mutant p53 expressing cells. We show that early activation of caspase 2, along with induction of wild type p53 target genes PUMA and Bax are crucial for triggering mitochondrial apoptosis pathway. In paper II, we show that STMA-1, as a Michael acceptor, inhibits cell proliferation and induces apoptosis in mutant p53- expressing tumor cells, but not human diploid fibroblasts. The effect of STIMA-1 is dependent on thiol modification.

p53 family members p63 and p73, particularly their DNA binding domains share high structure similarity to p53. That prompted us to test whether the mutant p53-reactivating compound PRIMA-1^{MET}/ APR-246 could also rescue mutant forms of p63 and p73. In paper III and paper IV we show that PRIMA-1^{MET}/ APR-246 enhances mutant p63 DNA binding and restores pro-apoptotic functions to mutant p63γ and p73β in tumor cells. Mutations in p63 in humans cause several hereditary developmental syndromes with impaired limb development and skin differentiation (such as the EEC syndrome). We found that treatment with PRIMA-1^{MET}/ APR-246 promotes differentiation of mutant p63 expressing keratinocytes isolated from patients with EEC syndrome.

In conclusion, PRIMA-1^{MET}/ APR-246 restores wild type function to mutant p53 family members presumably through interaction with homologous structures in their DNA binding domain. Our studies shed further light on the rescue mechanism of mutant p53 family members and raise possibilities for treatment of mutant p63 carrying development syndromes such as EEC in the future.

LIST OF PUBLICATIONS

I. **Shen J.**, Vakifahmetoglu H., Stridh H., Zhivotovsky B., Wiman K. G.
PRIMA-1^{MET} induces mitochondrial apoptosis through activation of caspase-2.
Oncogene, 2008, 27(51): 6571-6580.

II. Zache, N., Lambert, J. M., Rökaeus, N., **Shen, J.**, Hainaut, P., Bergman, J., Wiman, K. G., Bykov, V.
Mutant p53 targeting by the low molecular weight compound STIMA-1.
Molecular Oncology, 2008, 2(1): 70-80.

III. Rökaeus N.*, **Shen J.***, Eckhardt I., Bykov V., Wiman K. G., Wilhelm M.
PRIMA-1^{MET}/APR-246 targets mutant forms of p53 family members p63 and p73. Oncogene, 2010, Epub ahead of print (* these authors contributed equally).

IV. **Shen J.,** Bykov V., van Bokhoven H., Wiman K.G., Zhou H.
PRIMA-1^{MET} targets endogenous mutant p63 and enhances keratinocyte differentiation (Manuscript).

CONTENTS

LIST OF ABBREVIATIONS

ARE	AU rich element
ARF	Alternative reading frame
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
Bax	Bcl2 associated X protein
Bcl2	B cell lymphoma 2
BH3	Bcl-2 homology domain 3
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
EEC	Ectrodactyly, ectodermal dysplasia, and cleft lip/palate
G1	Gap1
HuR	Human antigen R
MDM2	Murine double minute 2
iPS	Induced pluripotent stem cells
kDa	kilo Dalton
$\Delta\psi m$	Mitochondrial membrane potential
MQ	Methylene quinuclidinone
LIF	Leukemia inhibitory factor
MIRA-1	Mutant p53-dependent induction of rapid apoptosis
miRNA	MicroRNA
mRNA	Messenger ribonucleic acid
NCI	National Cancer Institute
PRIMA-1	p53 reactivation and induction of massive apoptosis
PUMA	p53 upregulated modulator of apoptosis
pRb	Retinoblastoma protein
siRNA	Small interfering RNA
STIMA-1	SH targeting compound that induces massive apoptosis
UV	Ultra violet
Wig-1	Wild type p53 induced gene 1
WRAP53	WD40 encoding RNA antisense to p53

INTRODUCTION

Cancer

Cancer is one of the major causes of death worldwide. Tumor cells arise from normal cells and acquire proliferation advantages through mutations, deletions, amplifications, chromosomal fusions and epigenetic changes such as DNA methylation and histone modification, allowing escape from cell cycle checkpoints and tumor suppressor genes (pRb, p53), and get unlimited replication potential. At later stages, tumors acquire capacity of sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg 2000).

Tumor development is a multi-step process with similarities to the Darwinian evolution theory. The accumulation of several critical genetic alternations (three hits) gives cells survival and growth advantage, which lead to clonal expansion and tumor development. Based on that model, all cells in the tumor are equal in their capacity to maintain the neoplastic growth. Recently developed cancer stem cell model suggests that genetic alternations may occur in stem cells. The cancer stem cells harboring critical mutations in their genome are capable of both self renewal and they are responsible for maintaining of the population pool of growing tumor (Reya *et al.,* 2001).

Factors such as chemical carcinogens, ultra violate light, hereditary mutations, viral infection, inflammation and reactive oxygen species produced during metabolism contribute to malignant transformation. Major components of a solid tumor are stroma cells, immune cells and blood vessels, that could be manipulated by tumor cells and create an interactive tumor microenvironment which supplies the tumor with growth factors, oxygen and nutrients.

The frequency of different cancer types varies vastly due to environmental factors, diet and life style and genetic factors. Highest incidence of breast cancer occurs in Nordic Europe, which is associated with many factors including diet, higher child-bearing age, and small number of children per woman. Liver cancer is most common in Asia and sub-Saharan Africa due to consumption of food contaminated with aflatoxin produced by molds growing in nuts and corn and due to chronic hepatitis B and C infections. Melanoma is most frequently in Caucasians, caused by lack of skin pigmentation which renders skin poorly protected from solar radiation (IARC, http://www-dep.iarc.fr/).

Cancer therapy

Surgery, chemo therapy, radiation therapy, combination of radiation and bone marrow transplantation and hormone therapy are the most well known and established cancer treatment. Cancer cells in general proliferate faster than normal cells. Chemotherapeutic drugs mainly target proliferating cells. These drugs can be divided into alkylating agents, anti-metabolites, mitotic poisons, topoisomerase inhibitors, and other anti-tumor agents. They target DNA replication, inhibit synthesis of new DNA strands, prevent microtubule assembling/disassembling or they are interfering with DNA supercoiling. The disadvantage of chemo therapy and radiation therapy is lack of specificity, thus causing severe side effects. Efforts have also been devoted to the

development of targeted cancer therapy, including small molecules or antibodies that target cancer associated proteins, for instance activated kinases.

Examples of targeted drugs include: Erlotinib or Tarceva (Roche), a tyrosine kinase inhibitor, prevents EGF receptor autophosphorylation, subsequently inhibiting downstream cell proliferation signaling cascade (application in non-small lung cell cancer) (Shepherd *et al.,* 2005). Gleevec or STI571 (Norvatis), a tyrosine kinase-Bcr-Abl inhibitor, blocks it's ATP binding site and stabilizes it as an inactive conformation (application in CML) (Weisberg and Griffin 2000). Herceptin, a monoclonal antibody, binds and blocks a tyrosine kinase receptor, Her2 (application in advanced breast cancer) (Goldenberg 1999).

p53 and its family members p63 and p73

In late 1979, p53 was identified as a cellular protein which coimmunoprecipitates with the SV40 large T-antigen and it was named as p53 since its molecular weight was estimated to be around 53 kDa. The p53 gene is located on the short arm of chromosome 17p13 (Isobe *et al.,* 1986; Kern *et al.,* 1991; Matlashewski *et al.,* 1984). Originally p53 was regarded as an oncogene, since it was able to transform cells together with Ras. Eventually it was realized that a mutant form of p53 was used in the original experiments and that wild type p53 is rather a tumor suppressor. Loss of p53 heterozygosity and mutation of p53 was often observed in many cancers (Baker *et al.,* 1989; Olivier *et al.,* 2002).

Almost 20 years later, p73 and p63 were discovered. p73 is located on chromosome 1p36, which exhibits frequent loss of heterozygosity in human cancers (Versteeg *et al.,* 1995). p63 is located in a region on chromosome 3q27-ter that is actually amplified, not lost, in various cancers (Hibi *et al.,* 2000; Yang *et al.,* 1998). Evolution analysis suggests that p63 is the ancestor of the p53 family and that p53 is the youngest family member. In contrast to p53, mutations in p63 and p73 are rare in tumors. Indeed, p63 or p73 null mice showed defects in neurological, inflammatory or defect in hair follicles, limb and skin development. (Belyi *et al.,* 2010; Yang *et al.,* 2000; Yang *et al.,* 2002).

Structure of p53

The genomic sequence of p53 is transcribed into an mRNA transcript composed of 11 exons with first two non-coding exons. mRNA then is translated to a 393 amino acid nuclear protein. Studies have also shown p53 has several isoforms due to its alternative promoters in the N-terminus and splicing in the C-terminus (Marcel and Hainaut 2009).

The p53 protein contains a transactivation domain, a proline-rich domain, a DNA binding domain and an oligomerization (tetramerization) domain (figure 1). The transactivatin domain (amino acids 20 to 60) is composed of two subdomains and responsible for p53 transactivation activity. There is also a nuclear localization signal situated between the DBD and oligomerization domain. A nuclear export and C-terminal regulatory domains are located at the end of C terminus (Levine and Oren 2009).

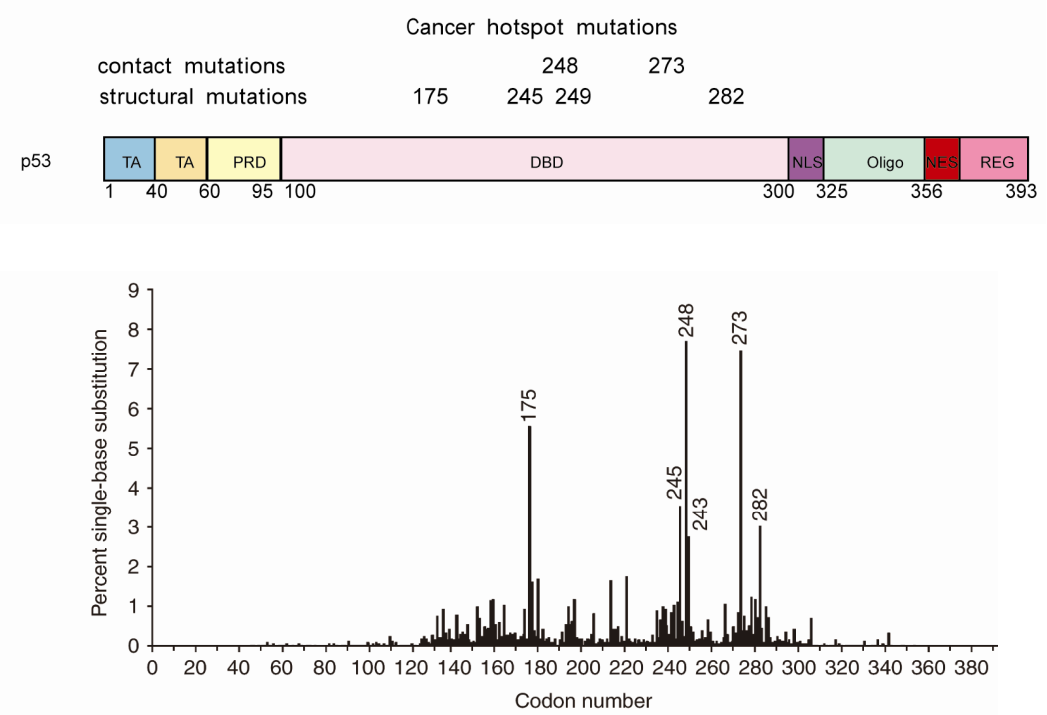

Figure 1. p53 protein structure Adapted and revised from Hainaut *et al*, 2010, Cold Spring Harbor Perspectives in Biology.

The prolin-rich domain consists of five repeats "PXXP" is shown to be implicated in the p53 dependent apoptosis (Sakamuro *et al.*, 1997). The oligomerization domain (amino acids 320 to 360) is required for formation of dimers and tetramers. Formation of tetramer allows higher affinity between p53 and DNA (Chene 2001). The basic domain (last 30 amino acids) contains two nuclear localization signals. Post translational modifications of this region increase the stability and transactivation activity of p53 (Appella and Anderson 2001). Several residues in the C-terminus are known to be phosphorylated (Ser315 and 392), acetylated (Lys320, 373 and 382), or SUMOylated (Lys386) in response to DNA damage. The N-terminus is more prone to be phosphorylated (Ser15 and Ser23). Post translational modifications of p53 may affect stability, protein-protein interactions and transcription activity.

DNA binding domain of p53 is located from amino acids 100 to 300. It recognizes a DNA binding motif of two PuPuPuC (A/C)(A/C) GPyPyPyPy palindromes separated by a 0 to 21 base pair spacer, where Pu is a purine and Py is a pyrimidine (Hoh *et al.*, 2002). p53 can transactivate specific genes by binding to p53 response elements in their promoter regions. The DNA binding domain is composed of three loops (Cho *et al.*, 1994). The whole structure is maintained by a zinc ion, that binds to the core cysteine residues Cys176, 238, 242, and histidine His 179 in the first loop. There are in total 10 cysteine residues in the DNA binding domain. Strikingly, p53 mutations frequently occur in human tumors within the DNA binding domain- hot spots mutations. These mutations are structural or DNA contact mutant (figure 1). In most cases, these mutations disrupt p53 transcription. This indicates an essential role of p53-dependent transcription for p53 as a tumor suppressor.
p63 and p73 share high structural homology with p53, especially in their DNA-binding domains (>60% amino acid identity) (Kaghad *et al.*, 1997; Osada *et al.*, 1998; Schmale

and Bamberger 1997; Yang et al. 1998). All three proteins are expressed as several isoforms due to alternative promoter usage in the N-terminal region and splicing in the C-terminal region (figure 2). The α isoforms of p63 and p73 contain a sterile α motif (SAM) in their C-termini, that exerts an autoinhibitory effect on p63/p73 transactivation and a transcription inhibitory domain, both of which are not present in p53 (Belyi *et al.,* ; Takada *et al.,* 1999).

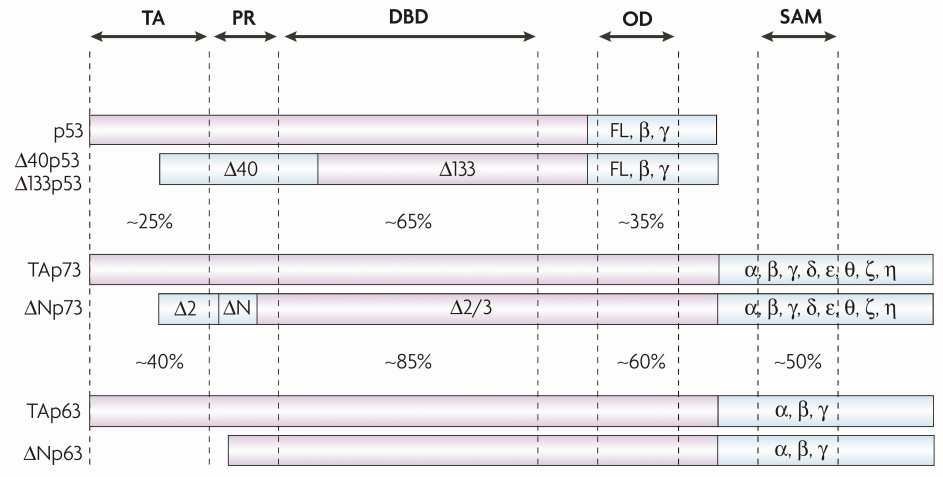

Figure 2. Structure of p53 family members. Adapted from Stiewe *et al*, 2007, Nature review.

Regulation of p53

The p53 protein has very short half life, due to its negative feedback loop regulator MDM2, which is an E3 ligase and is upregulated by p53. MDM2 gene contains two p53 binding elements (Barak and Oren 1992; Chen *et al.,* 1993; Momand *et al.,* 1992). It could mono- or poly-ubiquitinate the N-terminus of p53 and transport p53 from the nucleus to the cytosol. The ubiquitinated p53 will be recognized by the proteasome and get degraded. MDM2 also binds to the p53 transactivation domain, thus inhibiting its function (Haupt *et al.,* 1997; Kubbutat *et al.,* 1997; Wu *et al.,* 1993). Deletion of MDM2 in mice is lethal during early embryogenesis due to massive apoptosis. This phenotype is rescued by double MDM2 and p53 null mice (Jones *et al.,* 1995; Montes de Oca Luna *et al.,* 1995). This highlights the critical regulation of p53 by MDM2. MDM2 family member MDMX also negatively regulates p53 transcription. It forms heterodimers with MDM2, thus stabilizing MDM2 (Kawai *et al.,* 2007). MDMX null mice are also lethal and can be rescued by p53 inactivation (Migliorini *et al.,* 2002; Parant *et al.,* 2001).

p14ARF, an alternative reading frame protein encoded by INK4A locus, prevents the ubiquination of p53 through binding to MDM2 and thus stabilizes p53. p14ARF also sequesters MDM2 in nucleoli and prevents the interaction of MDM2 with p53 in nucleoplasm (Chin *et al.,* 1998; Gallagher *et al.,* 2006).

In addition to the regulation of p53 protein outlined above, p53 mRNA can be regulated by several factors. The p53 target Wig-1 and the human antigen R (HuR) bind to AU-rich elements (ARE) in the 3'UTR and stabilize the p53 mRNA (Vilborg *et al.,* 2009; Zou *et al.,* 2006). Wrap53 (WD40-encoding RNA antisense to p53) is located on

chromosome 17 and overlaps the first exon of p53. Wrap RNA can stabilize p53 mRNA by interacting with the 5' UTR (Mahmoudi *et al.,* 2009).

Another aspect is the regulation of p53 mRNA levels by microRNAs and siRNAs. MicroRNAs are endogenous RNAs. They are transcribed as precursor microRNAs that go through several cleavages to become matured microRNAs. They are incorporated in to the RISC complex, containing an Ago protein and guide the RISC complex to target mRNAs, whose 3'UTRs are partially complementary to the miRNA. siRNAs (about 22 nt), which are complementary to the target mRNAs, cleave and degrade them directly. The 3'UTR of p53 is regulated by several microRNAs, such as miR-125a, -125b, -504, -30, and -1285 (Hu *et al.,* ; Le *et al.,* 2009; Li *et al.,* ; Tian *et al.,* ; Zhang *et al.,* 2009).

Activation of p53

Stress signals such as DNA damage, hypoxia, metabolic stress, unstable ribosomal biogenesis, or activation of oncogenes cause stabilization of the p53 protein (Vousden and Prives 2009; Vousden and Ryan 2009; Zhang and Lu 2009).

When DNA damage occurs, protein complexes accumulate and get activated at the site of damage, which leads to activation of kinases. PI3-kinase family members ATM and ATR are activated following DNA double or single strand breaks, phosphorylate Chk2 and Chk1 and subsequently phosphorylate p53, which prevents the interaction between p53 and MDM2 , thus preventing degradation of p53 (Bartek and Lukas 2003). DNA-PK, another member of the PI3-kinase family, can also be activated following DNA strand breaks induced by ionizing radiation and stabilizes p53 by phosphorylating the MDM2 binding site on p53 (Lees-Miller *et al.,* 1992; Morozov *et al.,* 1994; Shieh *et al.,* 1997).

As a gate keeper, an important role for p53 is its activation by oncogene expression and abnormal cell proliferation. E2F is a cell proliferation-promoting transcription factor. The activation of E2F induces genes involved in proliferation. p14ARF is transcribed by E2F and stabilizes p53 protein through its interaction with MDM2 (Bates *et al.,* 1998; Gallagher et al. 2006). Moreover oncogene activation can probably lead to aberrant DNA replication and thus activate p53 via the DNA damage response pathway that involves ATM/ATR and Chk2/Chk1 (Bartkova *et al.,* 2005; Gorgoulis *et al.,* 2005).

Function of p53

Cell cycle arrest and DNA repair

Stress signals, such as DNA damage induces cell cycle arrest which allows DNA repair. Classical p53 target genes which trigger cell cycle arrest are p21 and 14-3-3σ (el-Deiry et al., 1993; Hermeking et al., 1997). p21 binds to and inhibits function of cyclin dependent kinase 2 (CDK2), which forms a complex with cyclin E and phosphorylates pRb. Once phosphorylated, pRb releases E2F, that will transcribe genes involved in DNA replication. Thus, cells can transit from G1 phase to S (DNA synthesis) phase (Abbas and Dutta 2009). 14-3-3σ inhibits CDK1(Cdc2) and thus prevents G2/M cell cycle transition (Taylor and Stark 2001).

In addition, p53 also plays a role in several types of DNA repair upon strand breaks and DNA adduct formation, including nucleotide excision repair (NER) and base excision repair (BER) (Ford and Hanawalt 1997; Zhou *et al.,* 2001).

Apoptosis

p53 induces several target genes involved in extrinsic (death receptor) and intrinsic (mitochondrial) apoptosis pathways. Fas receptor is a p53 target gene. Upregulation of Fas (the death receptor) by p53 binds with Fas ligand, further activates caspase-8 and eventually activates caspase-3, an effector caspase. Caspase-3 together with other effector caspases cleave cytoskeleton and cellular proteins and DNA, resulting in DNA fragmentation and formation of apoptotic bodies (Muller *et al.,* 1998). p53 induced genes, that activate intrinsic apoptosis pathways, include BH3 family apoptotic proteins, such as Bax, PUMA and Noxa (Miyashita and Reed 1995; Nakano and Vousden 2001; Oda *et al.,* 2000). Noxa and PUMA help Bax translocate to the surface of mitochondria from the cytosol and prevent binding of BCL2/ BCLXL to Bax. Bax oligomerizes or forms complex with Bak on the surface of mitochondria, it permeabilizes the outer mitochondrial membrane, which leads to release of pro-apoptotic proteins such as cytochrome c and AIF from mitochondria,. Eventually cytochorome c forms complex with procaspase-9 and Apaf-1, that activates caspase-9 which caspase-3 (Fulda and Debatin 2006). p53 also induces PIDD, whose C-terminal cleavage product PIDD cc forms PIDDsome complex with RAIDD and pro-caspase 2 (Tinel and Tschopp 2004). The PIDDsome complex activates caspase-2 that will also target mitochondria (Lassus *et al.,* 2002; Robertson *et al.,* 2002).

In addition, p53 can trigger apoptosis independent of transcription. This involves p53 translocation to mitochondria by the help of MDM2 and direct targeting mitochondrial membrane(Mihara *et al.,* 2003; Speidel 2010).

Senescence

p53 also is involved in senescence, an irreversible cell cycle arrest that can be caused by a loss of replicative ability due to shortened telomeres, or premature senescence signals, such as DNA damage, oxidative stress, nutrients starvation and oncogene activation. Cells are still metabolically active, but do not proliferate. Cells become enlarged and flattened (Dimri 2005; Goldstein and Singal 1974). Shortening of telomeres activates ATM and ATR that will activate p53. Several other p53 target genes, including p21 are involved in triggering senescence (Beausejour *et al.,* 2003; Evan and d'Adda di Fagagna 2009; Helton and Chen 2007; Zuckerman *et al.,* 2009).

Differentiation and fertility

p53 can also prevent stem cell self renewal and induce stem cell differentiation, thus eliminating defect stem cells that can contribute to the development of tumor (Zhao and Xu 2010). It also has been shown that the activation of p53 is one of the bottlenecks for reprogramming somatic cells to induced pluripotent stem cells (iPS) (Kawamura *et al.,* 2009).

Recently p53 has implicated in fertility. LIF, the gene encoding leukemia inhibitory factor, a cytokine critical for embryonic implantation, was recently identified as a p53 transcription target gene. Knocking down p53 decreases both the level and function of

LIF in the uterus and decreases embryonic implantation, pregnancy rate and litter size in female mice (Hu *et al.,* 2007).

p53 is also involved in such process as metabolism and preventing pathological angiogenesis (Folkman 2006).

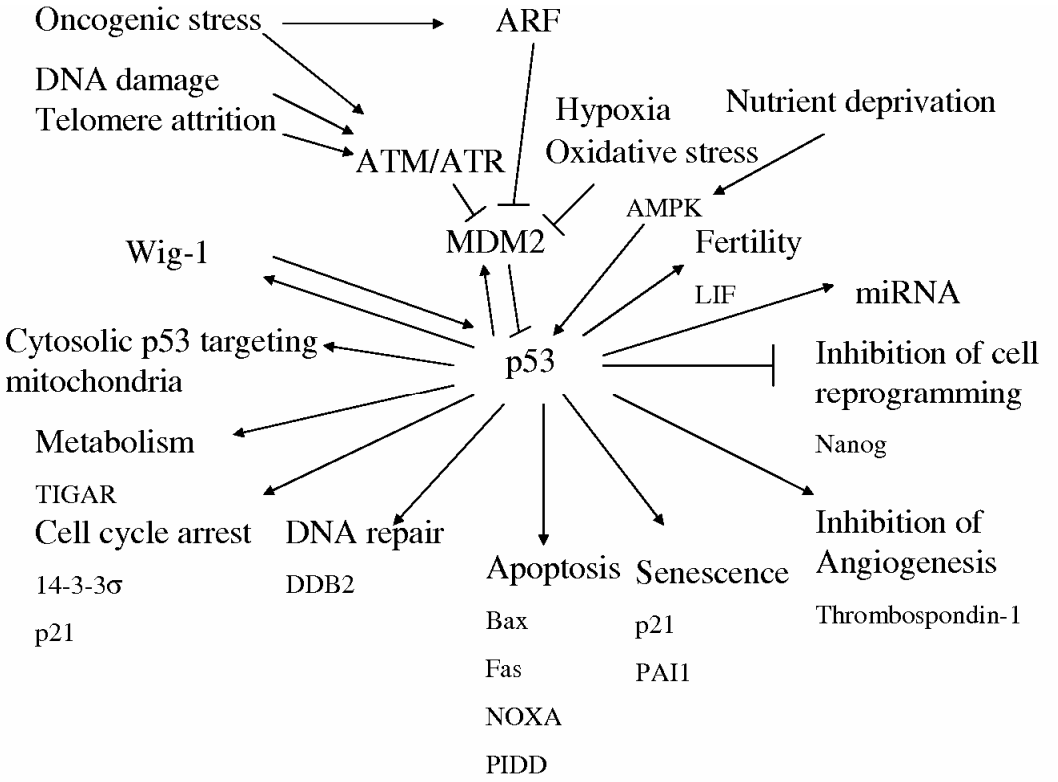

Figure 3. p53 signaling pathways.

The function of p53 family members p63 and p73

The full-length TAp63 and TAp73 proteins function as transcription factors similar to p53 and induce some p53 downstream targets, such as p21, MDM2, GADD45 and Bax (Kaghad et al. 1997; Lee and La Thangue 1999; Zhu *et al.,* 1998). However, this conclusion is drawn only based on exogenous overexpression studies. The activation of endogenous TAp63 so far is only observed in oocytes upon DNA damage stress (Suh *et al.,* 2006).

The ΔN isoforms of p63 and p73 that lack the N-terminal transactivation (TA) domain, can block the TA activity of the full-length proteins by forming complexes or by competing for DNA-binding sites (Grob *et al.,* 2001; Stiewe and Putzer 2002). Recent studies have also shown that ΔNp63 and ΔNp73 can activate transcription through an additional N-terminal domain and transcribe unique p63 and p73 targets genes: Rad51, BRCA2, mre11 and Rad50 involved in DNA repair (Ghioni *et al.,* 2002; Helton *et al.,* 2006; Lin *et al.,* 2009; Wolff *et al.,* 2009).

Although p63 and p73 are rarely mutated in human tumors (Strano *et al.,* 2001), inactivation of p63 or p73 contribute to tumor development in mice, and p63 and p73 can cooperate with p53 in tumor suppression (Flores *et al.,* 2005; Guo *et al.,* 2009). TAp63 knockout mice show enhanced genomic instability and premature aging as well as defects in maintaining skin proliferation and differentiation (Su *et al.,* 2009). Mice

with an isoform specific deletion of TAp73 show a high incidence of spontaneous tumors and increased sensitivity to carcinogens (Tomasini *et al.,* 2008). Methylation-induced silencing of the TAp73 promoter has been found in lymphoblastic leukemias and Burkitt lymphomas (Corn *et al.,* 1999; Kawano *et al.,* 1999). The ΔNp73 and TAp73 isoforms are co-upregulated in primary rhabdomyosarcomas and tumor-derived cell lines, as compared with normal muscle (Cam *et al.,* 2006). ΔNp73 isoforms are expressed at high levels in many different tumor types and are related to poor prognosis (Buhlmann *et al.,* 2008). Thus, accumulating evidence clearly indicates that both p63 and p73 may have a role in tumor development.

Mice null for p73 develop neurological and immunological defects (Nemajerova *et al.,* 2009; Wilhelm *et al.,* 2010). p63 null mice show craniofacial abnormalities, limb truncations, lack of prostate and a complete absence of an epidermis. The mice die soon after birth due to dehydration (Candi *et al.,* 2006; Mills *et al.,* 1999; Signoretti *et al.,* 2000; Yang *et al.,* 1999). Since both mice models die of young age, it is difficult to judge the role of p63 and p73 in tumor development. The ΔNp63α protein is highly expressed in basal layers of epidermis and TAp63α is only induced in differentiating cells, indicating the role in ΔNp63α in stem cell maintenance and role of TA63α in differentiation. However, Studies of TAp63 null mice also show that TAp63 is crucial for maintenance of the dermal and epidermal stem cell pool as it was shown for wound healing and hair growth (Su et al. 2009). The exact functions of p63 isoforms require further investigation.

Heterozygous mutations in p63 are associated with several autosomal-dominant developmental disorders in humans, including the EEC syndrome (ectrodactyly, ectodermal dysplasia, and cleft lip/palate) (Brunner *et al.,* 2002; Celli *et al.,* 1999). Interestingly, the amino acid substitutions in mutant p63 in EEC (204, 227, 279, 280, and 304) correspond to the hotspot mutations in p53.

Structure of Epidermis

A fertilized egg undergoes mitotic division, differentiation and eventually forms an embryo. During gastrulation and early embryo development, cells form three different germ layers: endoderm, mesoderm and ectorderm. Epidermis, stratified upper layer of skin, develops from a single layer of multipotent ectoderm cells through asymmetric cell divisions (Wilson and Hemmati-Brivanlou 1995). It is composed of basal, spinous, granular, transition and terminal differentiated layer (figure 4). Cells in the basal layer show the stem cell-like properties and proliferate to maintain a sufficient epidermal cell population. Cells from higher layers are more differentiated. The terminal differentiated upper layer is developed from cross-linked dead cell envelopes. Thus epidermis functions as a physical barrier between the body and the environment for prevention of dehydration and infection (Eckert *et al.,* 2005; Schoenwolf G.C. 2001).

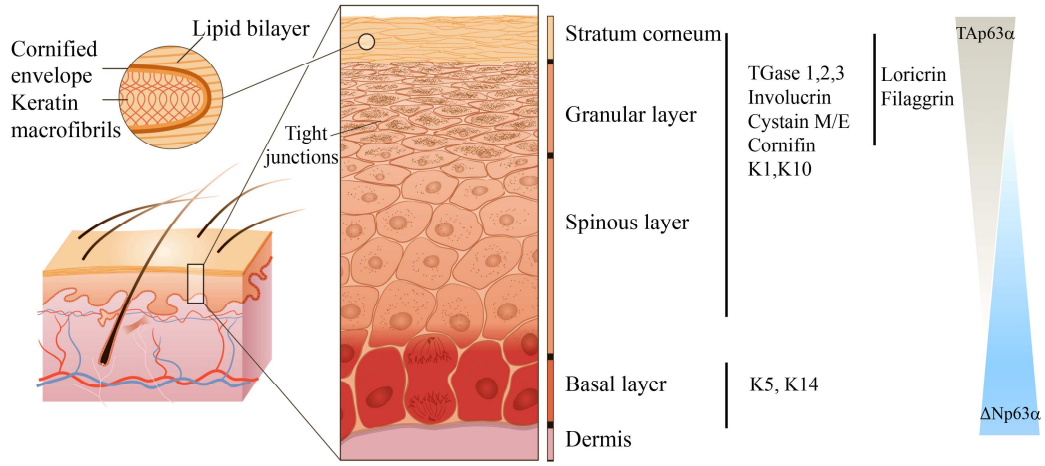

Figure 4. keratinocyte differentiation. Adapted and revised from Segre J.A., 2006, Journal of Clinical Investigation.

Rescue of wild type p53 function in cancer therapy

Activation of wild type p53

MDM2 gene is amplified in some tumors which result in excessive degradation of wild type p53, mitigating its role as tumor suppressor and stress sensor. Several compounds were shown to stabilize p53 through disrupting the interaction of p53 and MDM2.

Nutlin, a low molecular weight compound, was identified based on detailed structural analysis of the p53-binding pocket in MDM2. It mimics three amino acid residues in p53 that fit in the pocket. Thus it prevents ubiquitination of p53 by MDM2 and impedes p53 degradation in the proteasome. The prolonged p53 protein half life results in upregulation of p53 target genes and triggers cell cycle arrest and apoptosis in wild type p53- expressing tumor cells (Vassilev 2004; Vassilev *et al.*, 2004).

RITA was identified by screening an NCI library for compounds inhibiting the proliferation of HCT116 cells expressing wild type p53. It binds to p53 N-terminus and inhibits its interaction with MDM2 and p300, thus stabilizing p53, which subsequently induces apoptosis in wild type p53 expressing cells, but does not affect p53 null or HDF cells *in vitro* and *in vivo* (Issaeva *et al.*, 2004); (Grinkevich *et al.*, 2009).

Tenovin-1 was identified in a screening of chemical library for compounds which engage transcription transactivation of wild type p53 in tumor cells. Tenovin-1 and its more water-soluble analog tenovin-6 keep p53 acetylated by inhibiting deacetyleses and thus activate p53 transcription. Interestingly, tenovins inhibit p53 null tumor cell proliferation by inhibiting the protein deacetylating activity of SirT1 and SirT2 (Lain *et al.*, 2008).

Reactivation of mutant p53

Since p53 is essential for maintaining genome stability and is frequently mutated and accumulated in cancer cells, restoring wild type function to mutant p53 is a promising strategy to target cancer cells.

The p53 antibody PAB421 that recognizes a C-terminal epitope and peptide 46, corresponding to p53 residue 361-382, were shown to stimulate p53 DNA binding and restore its pro-apoptotic function (Hupp *et al.,* 1995; Niewolik *et al.,* 1995; Selivanova *et al.,* 1997). CDB3, derived from the p53 binding protein ASPP, binds the p53 DNA binding domain, stabilizes it, and enhances p53 transcription activity (Friedler *et al.,* 2002). However, the exact molecular mechanism involved is still not clear.

Some chemical chaperones, such as polyols (glycerol) and methylamines, are found to stabilize wild type p53 conformation in mutant p53 *in vitro* (Brown *et al.,* 1997; Ohnishi *et al.,* 1999; Ohnishi *et al.,* 2002; Welch and Brown 1996). Thus applying chemical chaperones is also a possible approach, however, this process requires high concentrations, which limits the application of chemical chaperons in the clinic.

Much effort has also been put on screening for small molecular weight compounds that can restore wild type conformation to mutant p53.

CP-31398 was identified by Foster *et al*. in a chemical library screening, aiming at stabilizing the active conformation of the p53 DNA binding domain. CP-31398 can stabilize wild type p53 conformation in both wild type and mutant p53 cells *in vitro* and *in vivo*, induce p53 target genes e.g. PUMA, Bax and trigger capase-3 activation and apoptosis (Foster *et al.,* 1999; Luu *et al.,* 2002; Tang *et al.,* 2007). However, some p53 independent cell death was also observed during the treatment (Rippin *et al.,* 2002; Wischhusen *et al.,* 2003).

The maleimide molecule, MIRA-1, was identified by screening an NCI library of low molecular weight compounds. It suppresses cell proliferation and triggers apoptosis in a mutant p53-dependent manner. Due to the presence in its structure of a maleimide group, MIRA-1 reacts rapidly with free thiol and amino groups. However, this compound is toxic to mice and therefore not suitable for further preclinical development (Bykov *et al.,* 2005).

PRIMA-1 was identified by screening the same chemical library. It inhibits cell proliferation preferentially in mutant p53-expressing cells (Bykov *et al.,* 2002a; Bykov *et al.,* 2002b). Later on, we discovered that PRIMA and its more potent analog PRIMA-1^{MET}/APR-246 are converted to methylene quinuclidinone (MQ), a reactive electrophil with Michael acceptor activity. We have shown that it binds covalently to thiol groups in the mutant p53 DNA binding domain. Our results suggest that such modification is responsible for a change in p53 conformation towards wild-type and for the restoration of wild type p53 function and induction of massive apoptosis in mutant p53-expressing cells (Lambert *et al., ;* Lambert *et al.,* 2009; Shen *et al.,* 2008; Zache *et al.,* 2008b).

STIMA-1, which has some structural similarities to CP-31398, was identified from a small library of molecules in a screening for mutant p53-dependent growth suppression in osteosarcoma cells. STIMA-1 has a structural characteristics of a Michael acceptor and thus participates in reactions of nucleophilic addition. As a potent electrophil, STIMA-1 is prone to react with several potential targets in cells, such as free thiol groups. Similar activity was also described for CP-31398, MIRA-1, as well as MQ, the

conversion product of PRIMA-1 and PRIMA-1^{MET}/APR-246. However, *in vivo* treatment with STIMA-1 is limited due to its poor solubility (Zache *et al.*, 2008a).

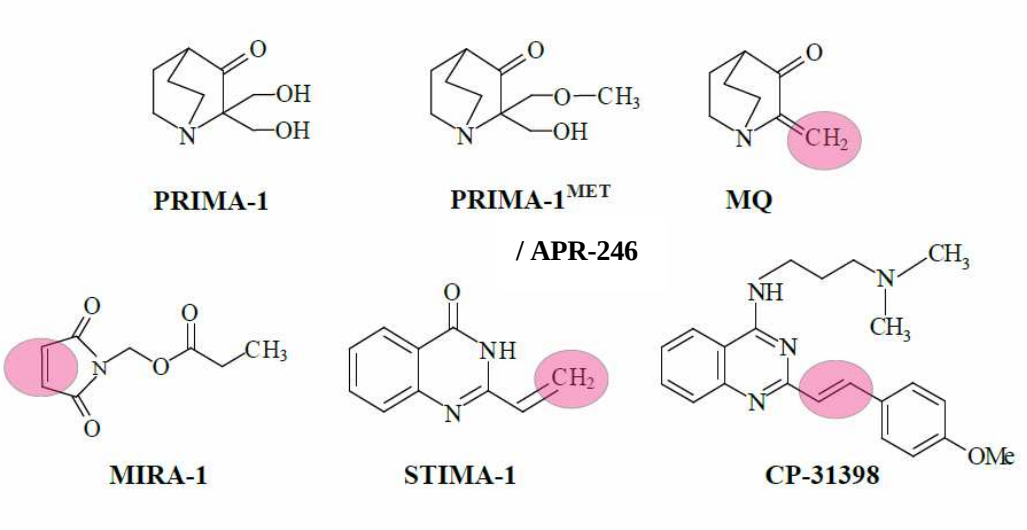

Figure 5. Chemical structure of mutant p53 reactivating compounds.

AIMS OF THE THESIS

The general aim of this thesis is to investigate the mechanisms responsible for reactivation of mutant p53 by PRIMA-1^{MET}/APR-246 and STIMA-1, and examine the effect of PRIMA-1^{MET}/APR-246 on mutant forms of the p53 family members p63 and p73. The study may open new therapeutic applications for PRIMA-1^{MET}/APR-246.

Specific aims

Paper I

To elucidate apoptotic signaling pathways activated in mutant p53-expressing tumor cells after PRIMA-1^{MET} treatment.

Paper II

To characterize STIMA-1, a novel mutant p53 targeting compound

Paper III

To investigate whether PRIMA-1^{MET}/APR-246 can rescue isoforms of mutant p53 family members p63 and p73, that share high structural homology with p53.

Paper IV

To investigate whether PRIMA-1^{MET}/APR-246 can restore wild type function to endogenous mutant p63α expressed in EEC syndrome patients.

RESULTS AND DISCUSSION

Paper I

PRIMA-1^{MET} induces mitochondrial apoptosis via activation of caspase-2.

We previously showed that the low-molecular-weight compound PRIMA-1^{MET}/APR-246 reactivates mutant p53, induces apoptosis in human tumor cells and inhibits tumor xenograft growth *in vivo*.

In this paper, we investigated the role of rescued mutant p53 by PRIMA-1^{MET}/APR-246 in the activation of apoptotic signaling pathways. We first observed that PRIMA-1^{MET}/APR-246 induced mutant p53-dependent loss of mitochondrial membrane potential ($\Delta\psi m$), accumulation of sub-G1 cell population and activation of pan-caspases in a dose- and time-dependent manner in three cell lines expressing endogenous mutant p53 (H1299-His 175, Saos-2-His 273 and SKOV-His 175). These results indicate induction of apoptosis. $\Delta\psi m$ and cell survival were not much affected in H1299, Saos-2 and SKOV –TA, p53 null cells after PRIMA-1^{MET}/APR-246 treatment.

To further study the involvement of mitochondria in PRIMA-1^{MET}/APR-246 induced apoptosis, we examined the status of cytochrome *c* in PRIMA-1^{MET}/APR-246-treated H1299 and H1299-His175 cells using immunofluorescence staining. We found that treatment with PRIMA-1^{MET}/APR-246 triggered release of cytochrome *c* from mitochondria to the cytoplasm in mutant p53 expressing cells. At later time points, these cells displayed formation of pyknotic nuclei indicative of apoptosis. Furthermore, treatment with PRIMA-1^{MET}/APR-246 in mutant p53 expressing cells induced expression of wild type p53 target genes Bax and PUMA, that are know to collaborate and target mitochondrial membrane, leading to drop of $\Delta\psi m$.

In order to determine the specific capases involved in mitochondria-mediated apoptosis induced by PRIMA-1^{MET}/APR-246, we assessed activation of caspase-2, -3, -9 and -8 by using the fluorogenic caspase substrates. We found that PRIMA-1^{MET}/APR-246 treatment triggered early activation of caspase-2, followed by activation of caspase-9 and eventually activation of caspase-3. The inhibition or silencing of caspase-2 can block activation of caspase-9 and caspase-3, and attenuates cell death within certain time points and concentrations of PRIMA-1^{MET}/APR-246. Interestingly, loss of $\Delta\psi m$ induced by treatment with PRIMA-1^{MET}/APR-246 couldn't be rescued by inhibition or silencing of caspase-2, indicating an existence of alternative upstream signals involved in targeting mitochondria. We also observed an induction of full-length PIDD protein and its cleavage fragment PIDD-CC in PRIMA-1^{MET}/APR-246-treated H1299-His175 cells. PIDD is a known p53 target gene and PIDD-CC is involved in a formation of PIDDosome complex, which activates caspase-2.

We also tested the combination of both PUMA siRNA and caspase-2 inhibitor in PRIMA-1^{MET}/APR-246-treated H1299-His175 cells. That did not result in any additional protection from cell death.

In summary, we have investigated the mechanism of PRIMA-1^{MET}/APR-246-induced apoptosis with focus on the mitochondrial and death receptor pathways. We showed that PRIMA-1^{MET}/APR-246 induces mutant p53-dependent mitochondria-mediated apoptosis through early activation of caspase-2, followed by $\Delta\psi m$ loss, cytochrome c release, further activation of downstream caspase-9 and -3, and apoptosis.

Mutant p53-dependent induction of PUMA and Bax by PRIMA-1^{MET}/APR-246 forms a parallel signaling pathway converging on mitochondria. Inhibiting activation of caspase-2 or knocking down PUMA simultaneously did not enhance inhibition of $\Delta\psi m$ loss and apoptosis, as compared to knocking down only one of them, suggesting that they function in the same pathway and other mutant p53-dependent pathways may be activated by PRIMA-1^{MET}/APR-246.

Paper II

Mutant p53 targeting by the low molecular weight compound STIMA-1.

It has been demonstrated that compounds with structural resemblance to CP-31398 possess activity against tumor cells. Therefore we synthesized a number of 2-styrylquinazolin-4-(3H)-one-related molecules and performed a cell-based screening to check the effect on mutant p53-dependent growth suppression. STIMA-1 was identified as the most potent molecule. It has Michael acceptor activity and can potentially react with thiol groups in cellular proteins. Here we aimed at a detailed characterization of STIMA-1 with regard to its ability to target mutant p53.

We asked whether STIMA-1 can form adducts with cysteines. We first incubated STIMA-1 with the cysteine analog N-acetylcysteine (NAC) and detected formation of adducts. The cell growth suppression induced by STIMA-1 could be blocked by preincubation with NAC. Compared to STIMA-1, the growth suppression effect by the structurally related compound CP-31398 was only partially blocked by NAC.

We observed a reduced number of free thiol groups in recombinant mutant p53 protein after incubation with STIMA-1 using a maleimide binding assay. The number free thiol groups also reduced in STIMA-1 treated mutant p53-expressing cells.

We also found that STIMA-1 upregulates p53 target genes, suppresses cell growth, and triggers caspase activation and cell death in a mutant p53-dependent manner, in contrast to cisplatin and CP-31398. Tumor cells expressing mutant p53 are more sensitive to STIMA-1 than wt p53-expressing tumor cells, p53 null tumor cells, or human diploid fibroblasts. Switching off mutant p53 expression in tumor cells by doxycyclin made the cells more resistant to STIMA-1 treatment, confirming that the effect of STIMA-1 is mutant p53-dependent. Finally we showed that mutant p53 DNA binding is stimulated after incubation with STIMA-1.

In conclusion, we have identified STIMA-1, a low molecular weight compound and Michael acceptor that targets mutant p53 expressing tumor cells and restores suppression of cell growth and apoptosis activities to mutant p53. This rases the possibility that thiol group modification plays a role in mutant p53 reactivation and might give us a lead to develop mutant p53 targeting anti-cancer drugs.

Paper III

PRIMA-1^{MET}/APR-246 targets mutant forms of p53 family members p63 and p73.

In this paper, we investigated whether PRIMA-1^{MET}/APR-246 can restore wild type function to mutant p63 and p73 and trigger apoptosis in tumor cells. We first tested the effect of PRIMA-1^{MET}/APR-246 on different isoforms of temperature sensitive p63 and p73 in H1299 cells. We found upregulation of p63/ p73 target genes p21 and PUMA at both mRNA and protein level in p63γ and p73β-expressing cells. In contrast, p73α expressing cells were not very sensitive to PRIMA-1^{MET}/APR-246 treatment. We also found that the treatment leads to supression of cell proliferation, activation of caspases and increased cell death in mutant p63γ and p73β-expressing cells, but has less effect on TAp73α.

We next generated Saos-2 cells stably expressing Tet-inducible TAp63γ-R204W or TAp63γ-R304W. We observed that PRIMA-1^{MET}/APR-246 treatment triggered cell growth supression in cells expressing TAp63γ-R204W or TAp63γ-R304W. Moreover, treatment with PRIMA-1^{MET}/APR-246 induced apoptosis (induction of subG1 population and active caspases) in TAp63γ-R304W expressing cells. This effect was more pronounced in the presence than in the absence of doxycycline for the TAp63γ-R304W cells. For the TAp63γ-R204W-expressing cells, we observed a significant G1 cell cycle arrest and a decreased S-phase fraction, indicating that this mutant promotes cell cycle arrest in response to PRIMA-1^{MET}/APR-246 treatment.

We previously found that PRIMA-1^{MET}/APR-246 treatment causes a nucleolar accumulation of mutant p53 along with PML. Here we also examined whether PRIMA-1^{MET}/APR-246 affects the subcellular localization of mutant p63. Treatment with PRIMA-1^{MET}/APR-246 induced a redistribution of both p63 mutants partially to PML-NBs and nucleoli.

To examine the DNA binding of mutant p63, we incubated nuclear lysate of PRIMA-1^{MET}/APR-246 treated TAp63γ-R204W and TAp63γ-R304W cells with p53 consensus binding oligo nucleotides. We observed an increased binding of p63 to DNA in PRIMA-1^{MET}/APR-246-treated cells. In addition, we also found that p63 downstream targets p21, Noxa and keratin 14 were induced in PRIMA-1^{MET}/APR-246 treated TAp63γ-R204W and TAp63γ-R304W cells. The induction of keratin 14 protein expression after treatment with PRIMA-1^{MET}/APR-246 was only observed at PRIMA-1^{MET}/APR-246 concentrations that did not induce apoptosis.

We conclude that PRIMA-1^{MET}/APR-246 can restore the wild type function to mutant p63 and p73, that share high sequence homology of DNA-binding domains with p53. Treatment with PRIMA-1^{MET}/APR-246 triggered cell growth suppression and apoptosis in mutant p73 or p63 expressing cells. Thus, our analysis demonstrates significant differences among mutant p63 and p73 isoforms with respect to their ability to confer an apoptotic response to PRIMA-1^{MET}/APR-246.

Paper IV

PRIMA-1 ^{MET} targets endogenous mutant p63 and enhances keratinocyte differentiation

We have shown in paper III that PRIMA-1^{MET}/APR-246 can restore pro-apoptotic activity to mutant isoforms of TAp63γ in human tumor cells. However, we wished to test the effect of PRIMA-1^{MET}/APR-246 on mutant p63 in a more physiological setting. ΔNp63α, which shares same DBD as TAp63γ is found dominately expressed in the basal layer of epidermis and it plays an important role in stem cell maintanance and differentiation. The mutations in p63α are associated with a number of human developmental defect syndromes. Here we address the question whether PRIMA-1^{MET}/APR-246 could reactivate endogenous mutant p63α in human keratinocytes derived from patients with the EEC syndrome and whether it can stimulate keratinocyte differentiation.

We first discovered that PRIMA-1^{MET}/APR-246 promoted differentiation in both p63α R204W and R304W expressing keratinocytes. Cells formed tighter cell-cell contacts, changed to flattened or elongated cell morphology and a three-dimensional organization with stratified cell layers in a manner similar to differentiated wt p63 expressing cells. The untreated mutant p63 expressing keratinocytes only form mono layer in differentiation culture. After treatment, the differentiation morphology in p63 R204W expressing cells is not as pronounced as p63 R304W expressing keratinocytes. It is plausible that p63 R304W mutant is easier to be rescued by PRIMA-1^{MET}/APR-246 than p63 R204W mutant. However, we can not exclude that inter-individual variation in the response to PRIMA-1^{MET}/APR-246 and a limited number of patients have affected our results.

We then observed that K1, K10, Involucrin and TGS, which are involved in classical epidermal differentiation are upregulated in mutant p63-expressing cells upon PRIMA-1^{MET}/APR-246 treatment. All these observations suggest restoration of keratinocytes differentiation. Claudin-1, a ΔNp63α target gene is also induced by PRIMA-1^{MET}/APR-246. This suggests that the transcription transactivation activity of ΔNp63α is restored by PRIMA-1^{MET}/APR-246.

Our preliminary data showed that PRIMA-1^{MET}/APR-246 also promoted cell proliferation. Flores' papers showed that TAp63 is essential for maintenance of epidermal and dermal precursors. The hair follicle-associated dermal and epidermal cells from TAp63 null mice undergo senescence. Therefore it is also plausible that PRIMA-1^{MET}/APR-246 treatment may rescue ability of mutant TAp63 to maintain cell proliferation. Thus the increased cell density will also promote differentiation machinery (Figure 6).

Thus we demonstrate that PRIMA-1^{MET}/APR-246 is not only targeting exogenous mutant p63 in a tumor cell background and promote mutant p63-dependent cell death by apoptosis, but it also rescuing endogenous mutant p63 in a physiological context where functional p63 is essential for normal differentiation. Our study raises the

possibility for improved treatment of EEC syndrome patients by mutant p63 rescue in the future.

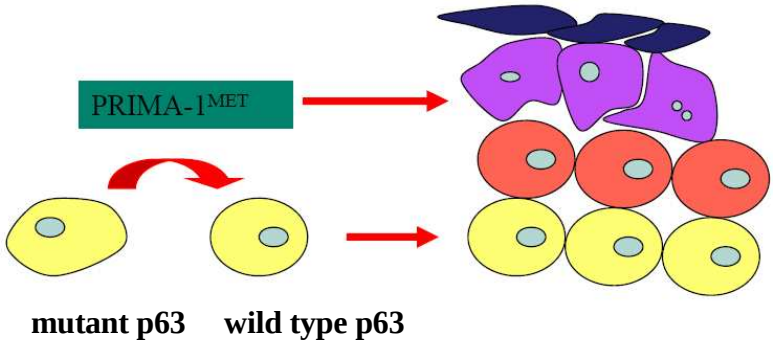

Figure 6. Model for enhanced differentiation in mutant p63 expressing keratinocytes by PRIMA-1^{MET}/APR-246.

CONCLUSIONS

Paper I
PRIMA-1^{MET} induces mitochondrial apoptosis via activation of caspase-2

- PRIMA-1^{MET}/APR-246 triggers apoptosis in a mutant p53 dependent manner.
- PRIMA-1^{MET}/APR-246 induces caspase 2 activation and loss of $\Delta\psi m$ in several tumor cell lines expressing different hot spot mutant p53.
- PRIMA-1^{MET}/APR-246 treatment rescues wild type function to mutant p53 and induces p53 target genes: PIDD, Bax and PUMA.
- BH3 family members (Bax and PUMA), along with active caspase 2 are essential upstream signaling for triggering mitochondrial apoptosis pathway.
- Release of cytochrome c from mitochondria leads to activation of caspase-9, followed by activation of caspase-3 and finally cell death.
- Inhibition or silencing caspase-2 rescues cell death in mutant p53 expressing cells treated with PRIMA-1^{MET}/APR-246.

Paper II
Mutant p53 targeting by the low molecular weight compound STIMA-1

- STIMA-1 induces mutant p53-dependent growth suppression and apoptosis in Saos-2-His273 and H1299-His175 cells.
- Mutant p53 expressing cells are more sensitive to STIMA-1 than wild type p53 expressing or p53 null cells.
- STIMA-1 binds to thiol groups in both recombinant mutant p53 and mutant p53 expressing cells.
- Pre-incubation with N-acetylcysteine blocks cell growth inhibition effect of STIMA-1, but not CP-31398.
- STIMA-1 improves DNA binding ability of mutant p53 and induces p53 target genes PUMA, p21 and Bax.

Paper III
PRIMA-1^{MET}/APR-246 targets mutant forms of p53 family members p63 and p73

- PRIMA-1^{MET}/APR-246 treatment inhibits cells growth and induces apoptosis in H1299 cells expressing tsp63γ and tsp73β, but not H1299 cells expressing tsp73α.

- PRIMA-1^{MET}/APR-246 treatment induces p53/ p63 target gene p21 in H1299 cells expressing tsp63γ and tsp73β.

- PRIMA-1^{MET}/APR-246 inhibits cell proliferation in Saos-2 cells expressing TAp63γ R304W or TAp63γ R204W.

- The induction of TAp63γ R304W or TAp63γ R204W sensitizes cells for apoptosis or G1 cell cycle arrest after PRIMA-1^{MET}/APR-246 treatment.

- PRIMA-1^{MET}/APR-246 improves DNA binding capacity of TAp63γ R304W and TAp63γ R204W.

- The mRNA and protein level of Noxa and p21 are induced in mutant p63 expressing cells after PRIMA-1^{MET}/APR-246 treatment.

- Epidermis basal layer marker Keratin 14 is induced in PRIMA-1^{MET}/APR-246 treated TAp63γ R204W cells.

Paper IV
PRIMA-1^{MET} targets endogenous mutant p63 and enhances keratinocyte differentiation

- PRIMA-1^{MET}/APR-246 improves cells stratification in endogenous mutant p63α expressing keratinocytes.

- PRIMA-1^{MET}/APR-246 treatment induces expression of differentiation markers (K1, K10, CYS_ME, involucrin and Transglutaminase) at mRNA and protein level in mutant p63 expressing cells.

- p63 target gene Claudin-1 is upregulated in mutant p63 expressing cells after PRIMA-1^{MET}/APR-246 treatment.

SUMMARY

We showed that the low molecular weight compound PRIMA-1^{MET}/APR-246 reactivate mutant p53 family members (p53, p63 and p73), that share high homology especially in the DNA binding domain. PRIMA-1^{MET}/APR-246 stimulated DNA binding of mutant p53 and p63, restored the pro-apoptotic activities to mutant p53 family members and triggered apoptosis in cultured tumor cells. Furthermore, PRIMA-1^{MET}/APR-246 reactivated mutant p63α promotes differentiation of human keratinocytes derived from patients carrying endogenous mutant p63. These results indicate that PRIMA-1^{MET}/APR-246 interacts with the homologous structures in mutant p53 family proteins and thus restores common functional defects.

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