THESIS BOOK

Investigation of the cellular functions of Drosophila p53

Zsuzsanna Újfaludi

Supervisors:
Dr. Éva Bálint
Dr. Imre Miklós Boros

University of Szeged
Faculty of Sciences and Informatics
Department of Biochemistry and Molecular Biology

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Introduction

The p53 tumor suppressor gene and its protein product have became the objects of intense study since their discovery in 1979, primarily because more than half of the human tumors contain mutations of this gene. In Li-Fraumeni syndrome, mutations of p53 occurring in the germ line lead to tumor formation in young ages with high familiar frequency. Various types of stress factors, which cause oncogenic alterations and DNA damage, including activated oncogenes, hypoxia, ionizing- and UV irradiation, rapidly activate human p53 which in turn induces or represses the transcription of its target genes as a transcription factor. Through regulation of its targets p53 acts as a "guard" of genome integrity and it plays crucial role in determining the final fate of damaged cells. Mutations of p53 result in the accumulation of DNA damages and unlimited proliferation of the injured cells. Detailed analysis of the functions of p53 and identification of its target genes may lead to a better understanding of tumor formation and development of cancer therapies.

In normal developing cells p53 is a short-lived protein, which is maintained at low, often undetectable levels. Degradation by continuous polyubiquitination of p53 is ensured through an autoregulatory feedback loop, which is based on the interaction of p53 and the Mdm2 ubiquitin ligase. Upon DNA damage, p53 is phosphorylated by Chk1 (checkpoint kinase 1) and Chk2 (checkpoint kinase 2) kinases leading to the rapid elevation of p53 level and its activation as transcription regulator. Similarly, activated oncogenes, such as Myc, Ras or E2F1, are able to stabilize p53. Activated p53 is transported to the nucleus where it functions as a transcription factor inducing or repressing its target genes, activates cell cycle block and accompanying DNA repair or apoptosis. In the case of UV caused transcription blockade, apoptosis also can be induced by p53 in a transcription independent pathway through permeabilization of the mitochondrial outer membrane. Via interactions with a number of transcriptional co-activators and co-repressors: i) p53 is able to modify the expression of its target genes, ii) p53 induces DNA repair by influencing the global acetylation of chromatin, iii) the transactivation function of p53 is modulated by itself. The effect of p53 on the transcription of its target genes

can also be regulated by interactions between p53 and other transcriptional factors and co-factors.

Genome analysis studies have shown that two third of the genes implicated in human cancers have counterparts in fruit fly. Getting more detailed information about cellular pathways in which p53 is involved is important for the better understanding of tumor formation and developing cancer therapies. After completion of the Drosophila genome projects the *Drosophila melanogaster* homologue of p53 was identified based on its sequence similarity to the human counterpart. The *Drosophila melanogaster* p53 (Dmp53) protein possesses the characteristic protein domains of human p53. The two homologue proteins share the highest similarity (25%) in the central DNA-binding domain, while the amino acid conservation of the transactivation and oligomerization domains, located in the amino and carboxy terminus respectively, is lower. In spite of the relatively low sequence homology, biochemically Dmp53 acts in a similar way than its human counterpart. Dmp53 is able to recognize and initiate transcription from the consensus DNA-binding site of human p53 in several reporter assays. Based on the similarity between the two homologues, studies were performed to assess the role of Dmp53 in cell cycle arrest, apoptosis and DNA repair. The *in vivo* functions of Dmp53 were investigated through overexpression of wild type and dominant negative forms of the protein. The dominant negative forms of Dmp53 were generated by introducing point mutations in the DNA-binding domain of the protein which, like in mammals, are capable to block the function of the wild type form. Results of these studies also indicate that Drosophila p53, in contrast to human p53, has no effect on G1 cell cycle arrest in imaginal discs. The Drosophila homologue of Chk2 (dChk2) is capable to phosphorylate Dmp53 after ionizing radiation (IR) treatment. This modification has no influence on the cellular concentration of Dmp53 indicating that Dmp53 activity is regulated by a mechanism different from the human p53-Mdm2 autoregulatory feedback loop. If the cell is exposed to DNA damaging agents, such as IR, activated Dmp53 can induce apoptosis via the regulation of the transcription of several proapoptotic genes such as *reaper*, for example.

The overproduction of Dmp53 itself is able to induce programmed cell death. Dmp53 stimulates the expression of certain DNA repair genes, *Ku70* and *Ku80*, under X-ray radiation, suggesting the role of Dmp53 in the regulation of DNA repair.

Aims

1. Investigation the role of tumor suppressor p53 in the maintenance of genome stability, regulation of cell cycle and apoptosis using Dmp53 as a model

2. Establishment of an experimental system for examination the effect of different genotoxic agents on Dmp53

3. Investigation, what is the effect of the interactions between Dmp53 and a dADA2a containing histone acetyltransferase complex (ATAC) and other proteins on the functions of Dmp53

Methods

- DNA damage induction using UVC and IR in Drosophila larvae
- Determination of half lethal doses of Drosophila larvae for UVC
- Total RNA isolation
- Firs strand cDNA synthesis
- Quantitative real time PCR
- Transcriptome analysis by DNA microarray method
- Transcription factor binding site analysis using bioinformatical approach
- Recombinant DNA techniques: polymerase chain reaction (PCR), restriction digestion, ligation, DNA purification
- Examination of protein-protein interactions by yeast two hybrid system
- Detection of apoptosis in Drosophila larval tissues by acridine orange
- Protein overproduction in Drosophila using heat shock driver

Results

Dmp53 null mutants are more sensitive to UVC than wild type

Dmp53 is necessary for the proper response to IR, since *Dmp53* deficient flies are radiation sensitive and show genome instability. Investigating the response of *Dmp53* mutants to UVC radiation, high sensitivity was detected. The half-lethal dose of UVC for *Dmp53* null mutant larvae is only about one-fifth of that of wild type. UVC radiation causes failure in DNA repair and injures the ommatidal structure of the developing retina of *Dmp53* deficient larvae as a consequence of predominant apoptosis. In concert with observation with the human counterpart, lack of Dmp53 may provoke incomplete activation of DNA repair leading the accumulation of DNA damages. The decreased viability of *Dmp53* null mutants possibly results from the decrease of DNA repair processes.

The expression of several pro-apoptotic genes show different Dmp53-dependent alterations upon distinct genotoxic agents

Since Dmp53 acts as a transcription regulator, similarly to its mammalian homologue, functions of Dmp53 are well tractable by investigating the transcriptional alterations of its target genes upon DNA damages. Several genes are known to play a role in apoptosis, however their Dmp53-dependence has not been studied yet. I studied the Dmp53-dependence of the expression of *hid*, *Ark* and *reaper* pro-apoptotic genes using different DNA damaging treatments as UVC or ionizing radiation. Each of the examined pro-apoptotic genes showed alterations in expression in a Dmp53-dependent manner. Nevertheless, I observed differences in the Dmp53-dependent activation of *hid*, *Ark* and *reaper* upon distinct genotoxic agents. Expression of *hid* was induced upon both UVC and IR treatment suggesting a general role of *hid*

in cellular responses to various DNA damages. *Ark* and *reaper* were differentially activated by different genotoxic agents reflecting a more specified role of these genes in apoptotic responses.

Dmp53 induces the expression of several genes upon UVC irradiation

In order to examine further the Dmp53-dependent cellular responses, I performed a genome wide study for identification of more Dmp53 potential target genes. On a microarray analysis 71 genes showed Dmp53-dependent expression alteration upon UVC treatment. The transcription of 55 genes was up-regulated and 16 genes showed repression. For confirmation of these results an independent method, QPCR was used. I chose genes which are possible participants of the cellular responses regulated by Dmp53, such as apoptosis and DNA repair, for the QPCR validation. I detected Dmp53-dependent upregulation of 10 genes chosen for further studies, in separate biological samples using an independent experimental approach. I considered these genes as putative specific target genes of Dmp53.

Dmp53 activates distinct sets of target genes upon UVC and IR treatment

Since I found that the pro-apoptotic gene *hid* was induced by both UVC and IR, while *reaper* and *Ark* were upregulated by only one of these DNA damaging stimuli in Dmp53-dependent manner, I decided to investigate whether the potential Dmp53 target genes newly identified upon UVC induction could be induced by IR as well. Based on the detected expression alteration of these genes upon UVC and IR treatment two groups of Dmp53 target genes were identified. The expression of the members of the first group, *Ark*, *tou*, *ftz-f1*, *ebi* and *CG11982*, was up-regulated in Dmp53-dependent manner only after UVC irradiation suggesting a specific role of these genes in the UVC induced Dmp53 regulated cellular responses. The second group includes

hid, *rho*, *ballchen*, *Grip75*, *l(1)dd4*, *CG8319* and *CG5620*, are possible participants of general cellular mechanisms activated by various DNA damages. These genes were significantly activated upon both IR and UVC in Dmp53-dependent manner. These results further support the existence of general and specific target genes of Dmp53 that activated upon different genotoxic stimuli suggesting.

Dmp53 may activate UVC-taget genes through non-consensus binding sites

Dmp53 has been shown in reporter assay to bind to human p53 binding elements and transactivate promoters harboring such elements *in vitro* and also *in vivo*. The consensus binding site of Dmp53 is still unknown. Dmp53 possibly regulates the newly identified UVC-target genes through specific Dmp53 binding elements. In order to find potential Dmp53-binding sites, I analyzed promoter regions of the putative Dmp53 target genes using bioinformatic approach. Symmetrical, mammalian p53 consensus binding site-like elements were absent in these promoters. Since the induction of these genes was detected soon after the UVC treatment it is unlikely that these identified genes are indirect targets of Dmp53, regulated by another transcription factor activated early directly by Dmp53. It is possible therefore, that Dmp53 activates UVC-target genes through non-consensus binding sites.

Drosophila homologue of Daxx, DLP, suppresses the functions of Dmp53 and effects *Ark* mRNA level

Mammalian death domain associated factor 6 (Daxx) is one of the regulators of apoptotic response, which, by interacting with other regulators modulates transcription and signal transduction pathways. Similarly to their human counterparts, Dmp53 and Drosophila Daxx (DLP, Daxx like protein)

interact directly. Several groups have reported on the interaction of the human homologues, however the consequences of this relationship are unclear and reports on it are contradictory. In order to investigate the interaction of the two Drosophila homologue, first the radiosensitivity of *DLP* mutants was studied. In contrast to *Dmp53* mutants, *DLP* deficient flies do not show radiosensitive phenotype indicating that DLP does not have a key role in the activation of Dmp53-dependent pathways after high dose of ionizing radiation. This assumption is confirmed by the finding that the lack of DLP has no effect on the ionizing radiation induced Dmp53 dependent activation and the basic transcription of the *reaper* proapoptotic gene. In contrast with *reaper*, the basic transcription level of another proapoptotic gene, *Ark*, is influenced by DLP since the expression level of *Ark* was reduced in *DLP* mutants and elevated in *DLP* overexpressing flies. In genetic interaction experiments, in parallel with the reduction of *DLP* level the level of apoptosis triggered by the overexpression of Dmp53 was elevated indicating a suppressor affect of DLP on Dmp53 regulated apoptotic response.

dADA2a Drosophila transcriptional adapter effects the functions of Dmp53

The ADA2 transcriptional adapter containing complexes take part in gene specific transcription activation through interactions with sequence specific transcription factors in yeast and human. It has already shown that the ADA2/ADA3/GCN5 containing adapter complex is required for the human p53-dependent gene activation and apoptosis. The Drosophila ADA2 proteins, dADA2a and dADA2b, are also involved in transcription activation as members of the GCN5, histone acetyltransferase (HAT) containing adaptor complexes. There are several evidences for the existence of relationship between Dmp53 and dADA2b containing SAGA complex. It has already been demonstrated that the dADA2b/SAGA complex participates in the apoptotic pathway induced by Dmp53 which do not include *reaper*. The physical interaction

between Dmp53 and dADA2b, but not dADA2a has also been shown by *in vitro* pull-down studies. dADA2a is a member of the so-called Drosophila ADA2a containing – ATAC – complex together with dGCN5 and dADA3 proteins. My aim was to investigate the effect of the dADA2a/ATAC complex on the Dmp53 mediated apoptosis. In the lack of dADA2a, similarly to the Dmp53 mutants, the proper induction of *reaper* failed, suggesting that Dmp53 and the dADA2a containing ATAC are required for the proper expression regulation of *reaper*. It is possible that the insufficient activation of *reaper* effects the apoptotic response in *dAda2a* mutant animals. In acridine orange staining the apoptotic response was significantly suppressed in imaginal discs of *dAda2a* mutants compared to *w1118* control animals after high dose of IR. This finding suggests that the high dose ionizing irradiation-mediated Dmp53 dependent apoptotic pathway that includes the activation of *reaper* requires the contribution of the dADA2a/ATAC complex.

Summary

Based on my results my conclusion are:

1. The proper functions of Dmp53 are necessary for the survival upon UVC caused DNA damage.
2. The expression of *Ark*, *hid* and *reaper* pro-apoptotic genes show different Dmp53-dependent alterations upon distinct genotoxic agents.
3. On a microarray analysis 71 genes showed Dmp53-dependent expression alteration upon UVC treatment. In the case of 10 genes chosen for further studies the Dmp53-dependent upregulation was proved in separate biological samples using an independent experimental approach. These genes were considered as putative specific target genes of Dmp53.
4. Based on my results and other researcher's data, three groups of potential Dmp53 target genes can be identified:

- the expression of the members of the first group, *Ark*, *tou*, *ftz-f1*, *ebi* and *CG11982*, was up-regulated in Dmp53-dependent manner only aftr UVC irradiation
- the second group includes *hid*, *rho*, *ballchen*, *Grip75*, *l(1)dd4*, *CG8319* and *CG5620*, are possible participants of general cellular mechanisms activated by various DNA damages since these genes were significantly activated upon both IR and UVC in Dmp53-dependent manner
- the third group represents genes induced by IR only, including genes such as *reaper*, *sickle*, *Eiger*, *Ku70* and *Ku80*.

5. A bioinformatical analysis showed that symmetrical, mammalian p53 consensus binding site-like elements are not present in the promoter regions of the putative Dmp53 target genes.

6. Genetic interaction experiments suggest that the Drosophila homologue of Daxx, DLP, suppresses the Dmp53 regulated apoptotic response.

7. Radiosensitivity studies demonstrated that DLP does not have a key role in the activation of Dmp53-dependent pathways after high dose of ionizing radiation. It was also confirmed by the finding that the lack of DLP has no effect on the ionizing radiation induced Dmp53 dependent activation and the basic transcription of the *reaper* proapoptotic gene. In contrast with *reaper*, the basic transcription level of another proapoptotic gene, *Ark*, is influenced by DLP since the expression level of *Ark*.

8. The high dose ionizing irradiation-mediated Dmp53 dependent apoptotic pathway that includes the activation of *reaper* requires the contribution of the dADA2a/ATAC complex.

Based on my results I suggest that the *Drosophila melanogaster* is a suitable model for examination the functions of p53 protein. More detailed studies on the cellular functions of Dmp53 may serve valuable informations for a better understanding of p53 regulated processes.

Publications included in the thesis

1. **Ujfaludi Z**, Boros IM, Bálint E. *Different sets of genes are activated by p53 upon UV or ionizing radiation in Drosophila melanogester.*
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