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Grapes- and stonewall-related DNA damage responses in Drosophila melanogaster

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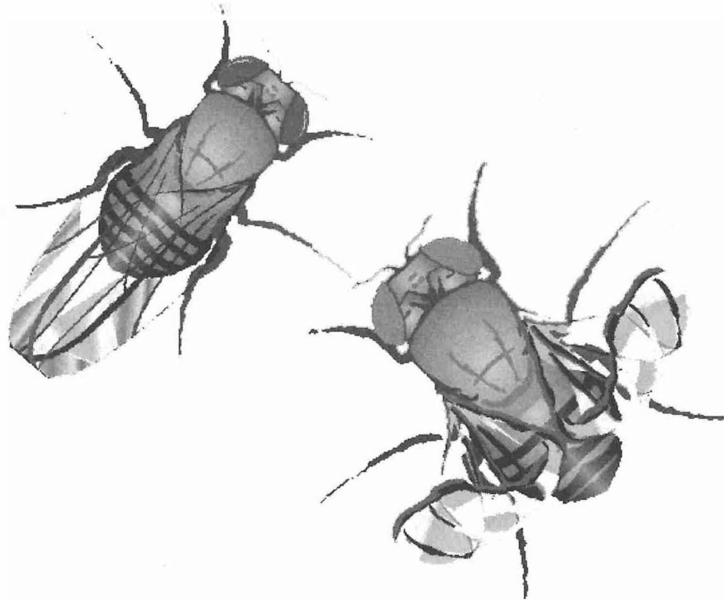
Chapter 6

Summarizing discussion



Introduction

Maintenance of genomic stability is of prime importance for dividing cells of a multicellular organism. Signal transduction pathways known as cell cycle checkpoints are well-established mechanisms that function in guarding genome stability before cells commit to either replicate or segregate their DNA. Events that eliminate a cell cycle checkpoint(s) severely affect the proliferating cell itself but might also have severe consequences for the multicellular organism when defects in the genetic material are given to the daughter cells during mitosis. Studies that focus on the working mechanism of cell cycle checkpoints have been done predominantly in the unicellular model organism yeast and in cell lines from mammalian origin. Relatively little is known about what the consequences are of impaired cell cycle checkpoints and DNA repair pathways for multicellular organisms. *Drosophila*, a multicellular model organism with powerful genetic tools, is highly suitable to get more insight in stress responses at the organismal level. In this thesis, the function of several *Drosophila* genes required to survive genotoxic stress are described and below, these results will be discussed.

The Grp/DChk1-dependent pathway

Grp/DChk1 expression in larvae is required to survive treatment with the DNA synthesis inhibitor and DNA damaging agent HU [21,22]. In *grp/Dchk1* mutants treated with HU, metamorphosis but not larval growth is impaired (**chapter 4**) suggesting that Grp/DChk1 is required to continue mitotic replication that occurs in larval imaginal discs. Our data are in agreement with a previous report in which it was demonstrated that *grp/Dchk1* is required for cell cycle arrest in imaginal discs in the presence of HU [17]. Using *Drosophila* S2 cells, we demonstrate (**chapter 2**) that Grp/DChk1 becomes phosphorylated in response to HU and IR and this phosphorylation depends on the presence of Mei-41/DATR. Downstream targets of Grp/DChk1 in these DNA damage response pathways are String (*Drosophila* homolog of Cdc25) and Cdc2. Activation of Grp/DChk1-dependent pathways is required to induce cell cycle arrest in response to HU and IR, demonstrating that the function of Grp/DChk1 in imaginal discs is comparable to the function of Grp/DChk1 in S2 cells. When Grp/DChk1-dependent checkpoint activation fails, cells enter mitosis in the presence of incompletely replicated or damaged DNA and undergo mitotic catastrophe. The characteristics of this mitotic catastrophe are comparable with those observed in *Drosophila* embryos [22,23] and in human cell lines [14]. We also found that Dmnk/DChk2 is dispensable for HU- and IR-induced G_2/M cell cycle arrest in S2 cells (**chapter 2**). Taken together, our results demonstrate in detail how DNA damage and DNA replication checkpoint pathways are regulated in *Drosophila* (Figure 6.1). We demonstrate that both DNA damage and DNA replication defects mediate G_2/M cell cycle arrest in *Drosophila* via the same regulatory pathway including Mei-41/DATR, Grp/DChk1, $Cdc25^{Stg}$ and Cdc2 (Figure 6.1B, depicted in bold). This means that in *Drosophila* the DNA damage response

is comparable with the DNA damage response in fission yeast (see Figure 1.1A) and that in *Drosophila* the DNA replication response is comparable with the DNA replication response in humans (see Figure 1.1C). Dmnk/DChk2 is not involved in G_2/M cell cycle arrest, at least not in response to HU or IR (Figure 6.1B). It is possible that Dmnk/DChk2 is involved in maintaining rather than accomplishing a G_2/M cell cycle arrest or it maybe that Dmnk/DChk2 is involved in DNA damage responses induced by agents other than HU or induced by doses IR lower than 150Gy, as used in this thesis. In conclusion, our data presented in this thesis form a solid basis for how DNA damage and DNA replication cell cycle checkpoints are regulated in *Drosophila*. Henceforth, *Drosophila* can be utilised to provide further insight into G_2/M checkpoint regulation or to study other cell cycle checkpoints (G_1-S, intra S) and other DNA damaging or DNA replication interfering agents using a great diversity of assays (genetics, RNAi, life-recording etc.) in the whole organism (embryos, larvae, pupa) or in cultured cells.

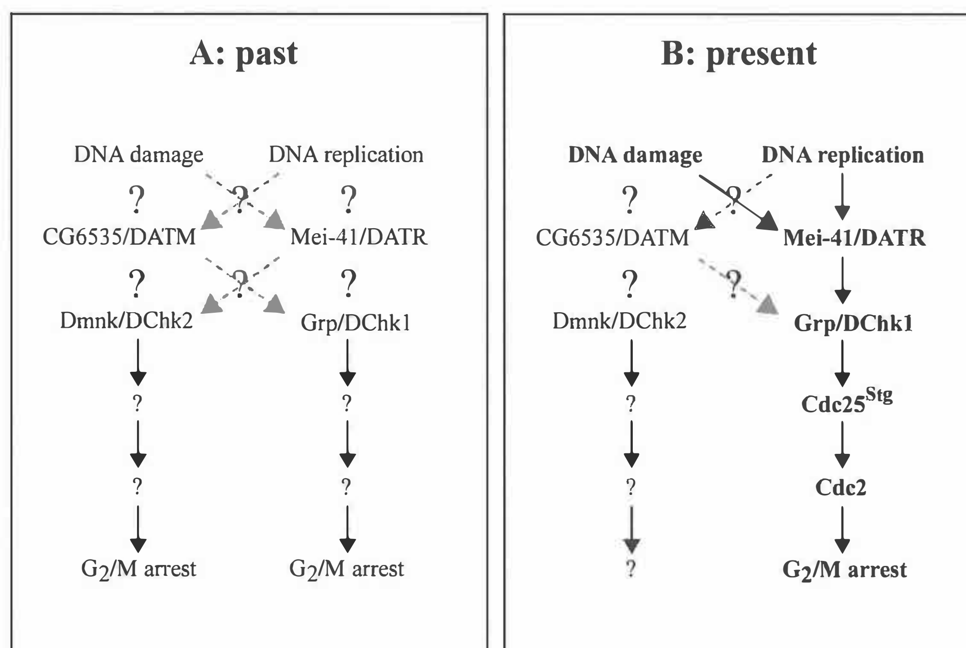


Figure 6.1. DNA damage and DNA replication checkpoints in *Drosophila*.
(A) Overview of what was known about DNA damage and DNA replication checkpoint pathways in *Drosophila* before the study described in this thesis was initiated.
(B) In bold, an overview is given of the results described in this thesis. Both the DNA damage and DNA replication checkpoints in *Drosophila* are regulated via the same pathway including Mei-41/DATR, Grp/DChk1, $Cdc25^{Stg}$ and Cdc2. Dmnk/DChk2 is not involved in these checkpoint pathways.

The function of Stonewall during larval development in the presence of HU

The above-mentioned opportunities to use *Drosophila* as a model organism encouraged us to search for modifiers (enhancers or repressors) of *grp/Dchk1* in response to genotoxic stress. Making use of *Drosophila* genetics, a screen was started to identify genes that, when mutated, were able to enhance the HU sensitive phenotype of *grp/Dchk1* mutant larvae. Using this screen, we identified *stonewall* (*stwl*) as a dominant enhancer of *grp/Dchk1* (**chapter 3**). Mutants heterozygous for both *grp/Dchk1* and *stwl* are more sensitive than mutants heterozygous for *grp/Dchk1* alone or for *stwl* alone, demonstrating that *grp/Dchk1* and *stwl* genetically interact. Homozygous *stwl* mutant larvae are, like homozygous *grp/Dchk1* mutant larvae, hypersensitive to HU and MMS, demonstrating that Stwl, like Grp/DChk1 is required to survive genotoxic stress. Homozygous *stwl* mutant larvae are not sensitive to treatment with IR, EMS or paraquat (**chapter 3**). The reason why *stwl* mutant larvae are sensitive to HU and MMS might be explained by the fact that both HU and MMS (but not IR, EMS or paraquat) are able to cause hypermethylation of DNA. Unlike *grp/Dchk1* mutant larvae, *stwl* mutant larvae show impaired growth in the presence of HU, indicating that Stwl is required to continue endoreplication after HU treatment (**chapter 4**). The above-summarized observations showing the differences in response to HU of *stwl* mutant larvae and *grp/Dchk1* mutant larvae are schematically given in figure 6.2.

Endoreplication is a specific cell cycle in which rounds of DNA replication occur without cell division and is observed in *Drosophila* but also in plants and mammals [5,16]. Based on our results, it is most likely that Stwl functions in different larval structures/tissues and thus in different survival pathways compared to Grp/DChk1. Now it will be of interest to investigate the function of Stwl in endoreplicating tissue in more detail. It is possible that Stwl is involved in repair of DNA damage induced by HU in endoreplicating cells or it may be that Stwl is required to restart DNA synthesis after stalled replication by HU. Currently, it is largely unknown which genes are involved in DNA repair or cell cycle regulation in endoreplicating cells and future experiments may resolve whether *stwl* is a candidate gene. The observation that *stwl* and *grp/Dchk1* genetically interact with each other but do not function in the same larval tissue was unexpected. However, these data demonstrate the power of genetic screens in *Drosophila* since in our case, *stwl* could never be identified using a yeast-two-hybrid screen, an immunoprecipitations approach or a cellular screen using cultured cells. Our results demonstrate that *Drosophila* can be used as a valuable tool to investigate complex survival pathways in response to DNA damaging agents in a multicellular organism and to investigate the function of several genes in these response pathways.

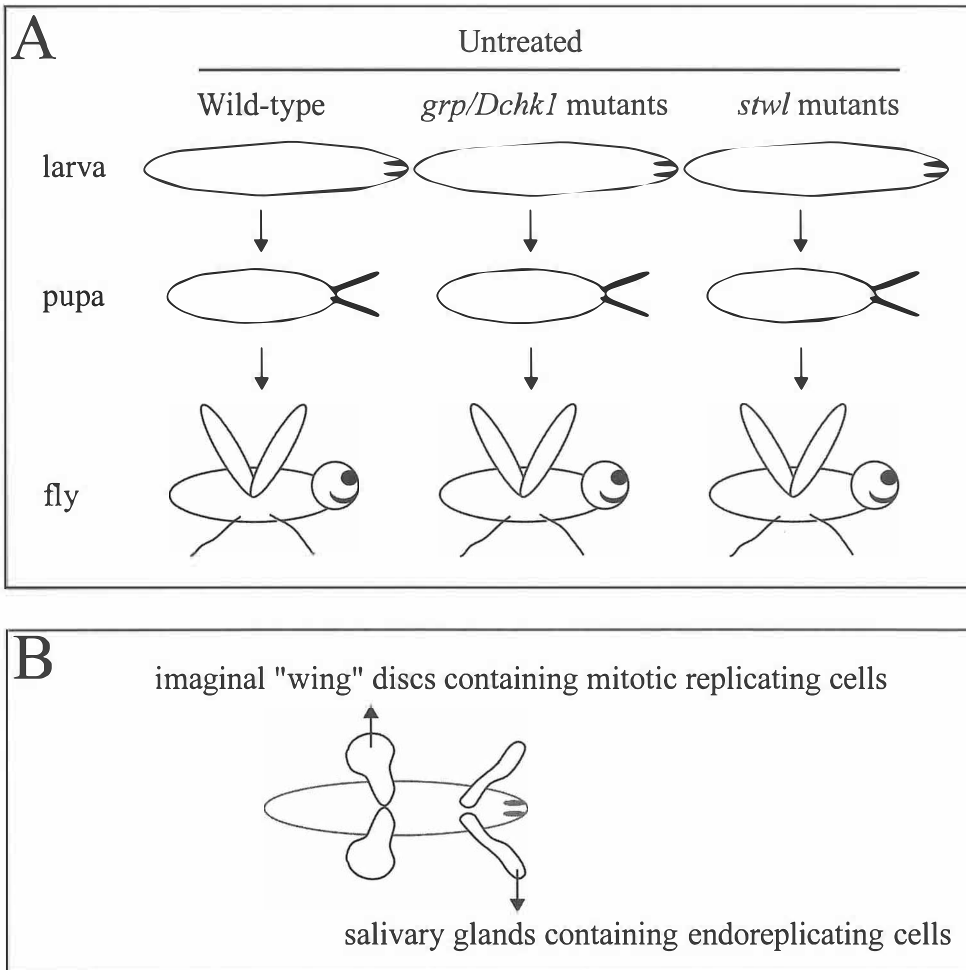
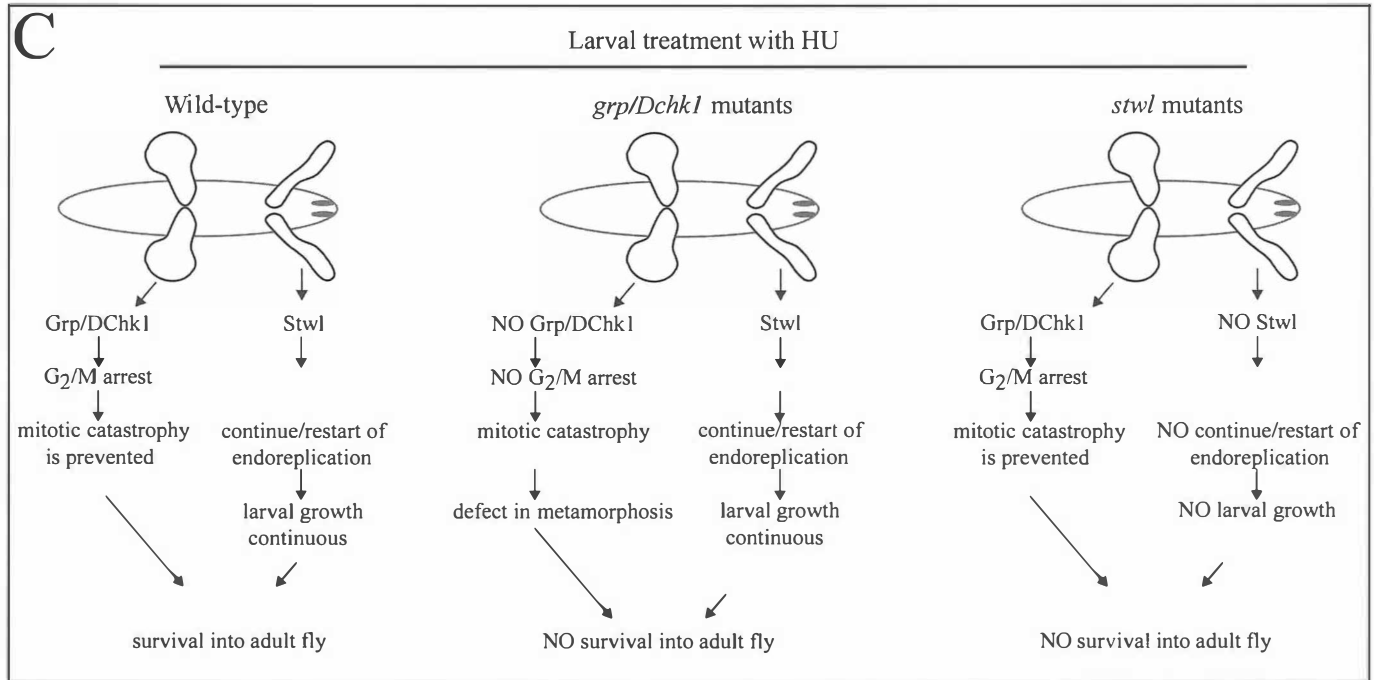


Figure 6.2: Schematic overview of *grp/Dchk1* and *stwl* mutant development in the absence and presence of HU.
(A) Mutant larvae of *grp/Dchk1* and *stwl* develop like wild-type larvae into pupae, undergo metamorphosis, and develop into adult flies.
(B) In larvae, different tissues can be distinguished, containing different cell cycle types. In imaginal wing discs, the cells replicate in mitotic cycles (G_1-S-G_2-M) whereas in salivary glands, the cells replicate in endocycles (G-S).
(C, next page) In the presence of HU, both Grp/DChk1 and Stwl are required for the development into adult flies. However, Grp/DChk1 and Stwl are required in different larval tissues. In mitotic replicating cells, Grp/DChk1 protects against mitotic catastrophe and therefore enables metamorphosis. In endoreplicating cells, Stwl is required to continue and/or restart endoreplication, which is required for larval growth.


C
Larval treatment with HU
Wild-type
grp/Dchk1 mutants
stwl mutants
Grp/DChk1
Stwl
G2/M arrest
mitotic catastrophy is prevented
continue/restart of endoreplication
larval growth continuous
survival into adult fly
NO Grp/DChk1
Stwl
NO G2/M arrest
mitotic catastrophy
continue/restart of endoreplication
defect in metamorphosis
larval growth continuous
NO survival into adult fly
Grp/DChk1
NO Stwl
G2/M arrest
mitotic catastrophy is prevented
NO continue/restart of endoreplication
NO larval growth
NO survival into adult fly

The function of Stonewall in *Drosophila* S2 cells in the presence of HU

In addition to investigate the function of Stwl during larval development we studied a possible function of Stwl in the cellular response to HU using *Drosophila* S2 cells. We observed that (unlike Grp/DChk1) Stwl is not required for G_2/M checkpoint regulation in response to HU (**chapter 3**) but is required to maintain chromosome integrity during mitosis after short HU treatments. In S2 cells, Stwl is associated with heterochromatin (**chapter 3**) and by using a reporter gene assay in mammalian cells, it was demonstrated that Stwl functions as an HDAC-independent transcription repressor (**chapter 5**). All together, these data (as summarized in Figure 6.3) reveal that Grp/DChk1 and Stwl most likely do not function in the same biochemical pathway in response to impaired DNA integrity in cultured S2 cells. In response to short HU treatment, Stwl-depleted cells (but not Grp/DChk1-depleted cells) undergo mitotic catastrophe (see Figure 3.7B), which is comparable to the mitotic catastrophe observed in Grp/DChk1-depleted cells in response to long HU treatment (see Figures 2.6D and 2.7B). Based on these results we hypothesise that when DNA is modified (and maybe also damaged) by HU, this modification does not provoke G_2/M checkpoint activation followed by cell cycle arrest in Stwl-depleted cells. However, in the absence of Stwl, these DNA modifications cause disintegration of chromosomes during mitosis.

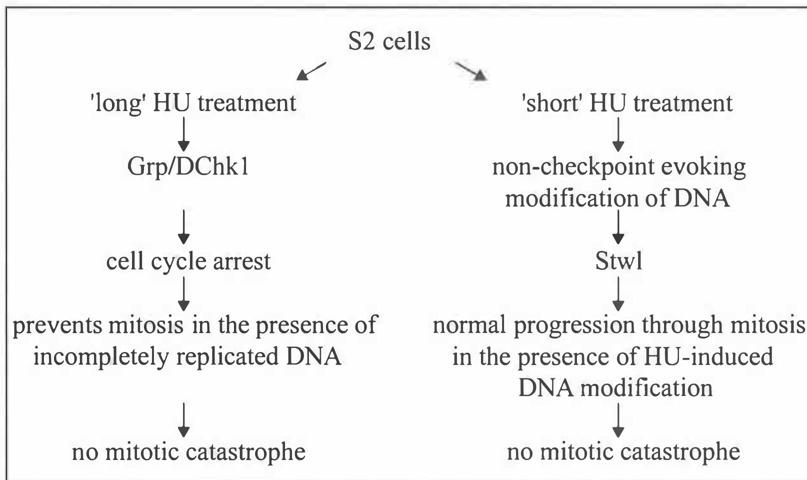


Figure 6.3: Grp/DChk1- and Stwl-dependent DNA damage response pathways in *Drosophila* S2 cells.

Grp/DChk1 is required for checkpoint activation and the prevention of mitotic catastrophe in response to long (15 hours) HU treatment of S2 cells. In response to short (6 hours) HU treatment, Stwl is required to prevent mitotic catastrophe after induction of non-checkpoint evoking DNA modifications in the preceding G_2.

Below, possible mechanisms that might explain how Stwl is involved in surviving modified (and/or damaged) DNA will be discussed based on the presence of conserved domains and based on similarity between phenotypes. These proposed mechanisms explain at least the mitotic catastrophe as observed in Stwl-depleted S2 cells but may also be involved in continuing (or restart) endoreplication in larval tissue in the presence of HU.
In this thesis we demonstrate that Stwl functions as a transcriptional repressor and that Stwl is a heterochromatin-associated protein. Previously, is has been reported that the methylpurine-DNA glycosylase (MPG) is (like Stwl) a transcriptional repressor and that its function is (like Stwl) independent of histone deacetylaces (HDAC) [24]. MPG forms a complex with the methylated DNA-binding domain protein 1 (MBD1) and MPG-mediated transcriptional repression is enhanced by MBD1 [24]. MPG knockout mice and MBD1-depleted cells are (like *stwl* mutants) sensitive to MMS [11,24] which suggest that the MBD1-MPG complex is involved in the repair of base lesions induced by MMS. Altogether, these results indicate that the MBD1-MPG complex links transcriptional repression and DNA repair and it is possible that Stwl acts in a MBD1-MPG-like manner. It will be interesting to investigate whether Stwl acts in a complex with DNA glycosylases, such as *Drosophila* Oggl [9] and S3 [8] and/or methylated DNA binding proteins and whether this whole complex is required to survive DNA damage.
Regardless, whether Stwl functions in a MGP-MBD1-like complex, it is most likely that Stwl exerts its function via chromatin remodelling. As mentioned earlier (**chapter 5**) Stwl contains a MADF domain (aa 11-97) and a BESS domain (aa 602-641). MADF shows similarity with the DNA binding domain of Myb-related proteins and is therefore a member of the SANT family. BESS stand for the names of three proteins that originally defined the domain: Boundary element associated factor 32, Su(var)3-7 and Stonewall and seems to have a primary role in protein-protein interactions. The BESS motif is found in 19 *Drosophila* proteins (known and/or predicted) and 14 of these show the same architecture as present in Stwl (a MADF domain near the NH_2-terminus and a BESS domain near the COOH-terminus) including the transcription factors Adf-1 [12] and Dip3 (Dorsal-interacting protein 3) [2;7]. The observed transcriptional repression effect of Stwl could be modulated by its subnuclear compartmentalization (Stwl localizes to the heterochromatin) trough protein-protein interaction, possibly mediated via BESS. Analysis of Adf-1 revealed that not only MADF but also the BESS domain might be distantly related to the SANT domain [7] based on its presence of a SANT-like helix-turn-helix (HTH) domain which is in agreement with the observation that the BESS domain in Dip3 is required and sufficient to bind itself and to bind Dorsal [2]. The SANT domain is a highly conserved motif which is involved in chromatin remodelling based on its similarity to the DNA binding domain of Myb-related proteins, based on its role in several chromatin remodelling enzyme complexes [1] and based on its binding capacity to post-translationally modified histone tails [3,4]. In humans, a functional role for the SANT domain in chromatin remodelling has also been

demonstrated [10]. The human protein MI-ER1 (human mesoderm induction-early response 1) represses transcription of its own promoter when MI-ER1 binds via its SANT domain to the Sp1 protein. It may be possible that Stwl is involved in proper chromatin remodelling and this in turn is required for adequate DNA damage repair or maintenance of chromatin structure in response to short HU treatment.

The last possible working mechanism of Stwl we discuss here is based on the presence of a Daxx-related domain. Examination of the Stwl ORF at the NCBI conserved domain classification program showed the presence of the pfam (protein family) 03344.10-related domain, which links Stwl to the Daxx (death domain-associated protein) family. The Daxx-related domain of Stwl (aa 181-310) is found in between the two nuclear localization signals (aa 163-180 and aa 311-328), and was predicted earlier as a highly acidic domain [6]. Daxx is a multifunctional protein which localizes mainly in the nucleus, specifically in the promyelocytic leukaemia protein nuclear bodies (PML-NBs) [15]. Originally, Daxx was identified as a Fas-binding protein by yeast-two-hybrid screening functioning as an enhancer of Fas-mediated apoptosis through c-Jun N-terminal kinase (JNK) activation [25]. In contrast to this pro-apoptotic function, in Daxx knockout mice apoptosis was increased during embryonic development [19]. To date, the exact function of Daxx in apoptotic signalling remains still unclear. Daxx possesses also transcriptional repressor activities by inhibiting several sequence-specific transcription factors, such as Pax3, Pax5 and ETS1 via direct protein-protein interactions [13,18]. Daxx interacts specifically with the DNA methyltransferase 1 (DNMT1)-associated protein (DMAP1), which is a major enzyme that maintains mammalian DNA methylation. [20]. DNA methyltransferases function in direct repair, a mammalian DNA repair mechanism that is yet unknown in *Drosophila*. The presence of the Daxx-related domain might link Stwl to the presence of a DNA methyltransferase-like protein complex in *Drosophila* and therefore to the identification of direct repair in *Drosophila*. Future experiments are required to test whether Stwl is indeed a chromatin remodelling protein and whether Stwl-mediated chromatin remodelling is required to facilitate DNA repair and to prevent mitotic catastrophe.

In summary, this thesis describes the unexpected link between Grp/DChk1 and Stwl. Grp/DChk1 is a well-conserved G_2/M checkpoint regulator and Stwl is a heterochromatin associated transcription repressor and is involved in maintenance of chromosome integrity during mitosis and therefore involved in surviving HU in endoreplicating cells. Although *grp/Dchk1* and *stwl* genetically interact with each other, both genes are required to survive genotoxic stress via different biochemical pathways. Our results underscore the complex survival responses of organisms, a complexity that can be understood in future in more detail with the use of multicellular and genetic modifiable organisms like *Drosophila*.

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