CHAPTER 2.

Identification of novel *Drosophila melanogaster* loci that cause mutagen sensitivity and female sterility

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Identification of novel *Drosophila* loci that respond to genotoxic stress

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CHAPTER 2

Forward genetic screen

*Drosophila* lines carrying mutations in various cell cycle checkpoint or DNA repair genes are frequently hypersensitive to DNA damaging agents and are female sterile. Although many independent screens have been carried out to identify either female sterile mutations or mutagen sensitive mutations, limited screens have been performed using both characteristics as selection criteria, and these screens only uncovered a few new genes. Because only limited screens have been performed using the combination mutagen sensitivity and female sterility as selection criteria, it is likely that these screens were not saturated and many candidate genes were missed.

Taking in consideration female sterility and mutagen sensitivity as phenotypes we employed a forward genetic screen to identify novel genes involved in the response to genotoxic stress during *Drosophila* development. Thereto a library of 117 single P element insertion lines was generated (by M. Ritsema and therefore initially referred to as MR1-117) by P element mediated transposon mutagenesis and this collection was expanded with female sterile P element collections kindly provided by A. Ephrussi (EMBL, Germany) and S. Hawley (Stowers Institute, USA). We used transposon mutagenesis because of two properties. The first is that creating large scale mutant libraries and mutation mapping of transposons is relatively easy. Secondly, transposons have a preference for non-coding regions to insert in and therefore usually induce hypomorphic phenotypes, which allows to analyze and study essential genes. However, this latter property also makes it frequently difficult to analyze the effect of the P element insertion.

To identify which mutant lines displayed sensitivity to DNA stress, flies heterozygous for the P element were crossed (day 1-3) and larvae from the F1 generation were exposed to DNA damaging agents on day 5 (2-4 day old larvae) (Fig. 1A). Sensitivity was determined by scoring the ratio between heterozygous:homozygous adult survivors in the F1 generation (day 14-21). An increase in the ratio heterozygous to homozygous survivors was considered to result from a P element insertion event at an essential locus, that caused a genetic defect associated with increased sensitivity to genotoxic stress during larval development. Mutant flies were screened for sensitivity to hydroxyurea (HU) and ionizing radiation (IR). HU blocks DNA replication, causes oxidative damage to the DNA, and can induce hypermethylation of the DNA. Although HU can cause DNA damage and DNA hypermethylation, this compound is generally used to induce a S-phase arrest and to identify cell cycle checkpoint mutants. In addition we also used γ-radiation to induce DNA damage. IR causes multiple types of damage including base damage, crosslinks and single- and double-stranded DNA breaks (DSBs). IR also induces a cell cycle arrest required to repair the damaged DNA.

The larval stadium was chosen because, during this developmental stage the larval imaginal discs are rapidly proliferating and extremely vulnerable to DNA damage as clearly demonstrated for the *grp/dChk1* and *mei-41/dATR* mutants, which were used as control lines during the screening procedure (Fig. 1A). The *mei-41/dATR* and *grp/dChk1* genes mediate the DNA damage dependent G2/M checkpoint in response to IR and HU. In the absence of these two checkpoint genes cells do not arrest their cell cycle progression required to repair the DNA damage inflicted by administration of HU and IR. As a result *mei-41/dATR* and *grp/dChk1* mutant cells enter mitosis in the presence of incompletely repaired/replicated DNA which result in mitotic catastrophe and eventually cell death. The expected ratio for untreated *mei-41/dATR* (X chromosome) mutants would be 1:2, while the expected ration for *grp/dChk1* (2nd chromosome)
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Mutants would be 2:1. The ratio hetero:homo in untreated *mei-41/dATR* flies was 0.65 ± 0.09 SEM (n=30) and after 20 Gy the ratio was 10.68 ± 2.85 (n=17). When *grp* mutants were fed with a 50 mM HU solution the ratio between hetero:homo was 47.05 ± 8.23 (n=14), while the ratio in the control group (H$_2$O only) was 2.30 ± 0.12 (n=63), proving that the screening procedure used can be applied to determine sensitivity to HU and IR and demonstrating that indeed *mei-41/dATR* and *grp/dChk1* mutants can be used as positive controls during the screening procedure.

Using this forward screening approach we recovered 16 MR lines, 1 Hawley line (X. Yi & O.C.M. Sibon, submitted) and 1 Ephrussi line that displayed hypersensitivity to IR or HU, or both DNA damaging agents (Fig. 1B, Table 1). We only screened for potential loci on the 2nd and 3rd chromosome of *Drosophila*. According to Mendelian inheritance the expected ratio of heterozygous to homozygous individuals in all control groups should have a ratio of 2:1. However several mutant lines already displayed high/low ratios without exposure to DNA damaging agents, indicating that the P element insertions in these mutant lines caused viability defects.

Three of the identified mutant lines displayed hypersensitivity to IR, but not HU (MR7, MR80, MR103), while 11 mutants displayed hypersensitivity to HU only (E709, MR33, MR53, MR51, MR56, MR62, MR67, MR73, MR84, MR92, MR99) and the remaining 3 mutants (MR28, MR65, MR98) were hypersensitive to both HU and IR (Table 1). As these mutants could harbor a mutation in an essential locus involved in surviving genotoxic stress, the P element insertion sites of these fly lines were mapped by P element rescue analysis. The 5' and/or 3' genomic regions flanking the 8 bp insertion target of the P element were recovered, sequenced and used to BLAST against the *D. melanogaster* genome sequence to identify their insertion sites (Table 1). Of these novel mutants the E709, MR65 and MR67 mutant lines also showed female fertility defects. In addition, another novel mutant, *stonewall* (*stwl*), was isolated from the Hawley collection and also displayed sensitivity to DNA damaging agents and was found to be female sterile (X. Yi & O.C.M. Sibon, submitted). Besides the mutants presented in Table 1 several alleles of *grp/dChk1* were recovered from the Ephrussi collection using a similar screening approach confirming the validity of our screening procedure (O.C.M. Sibon, unpublished). Some of the identified mutants will be discussed below.

**Novel genes potentially required to survive genotoxic stress**

Because the combination mutagen sensitivity and female sterility is a characteristic of many mutant alleles of genes involved in checkpoint signaling and DNA damage repair, mutant lines E709 and MR65, which displayed sensitivity to DNA damaging agents and suffered from female fertility defects, were analyzed in more detail and are characterized in this thesis. The MR67 mutant also displayed a female sterile and DNA damage hypersensitive phenotype, and the transposon in this line was recovered from the 5'-UTR of the *oo18 RNA-binding protein* (*orb*) gene. Because *orb* is a well known gene required for normal oogenesis and the mutant did not display strong hypersensitivity to DNA damaging agents we did not further characterize this mutant.

Several mutant lines that displayed no fertility defects, but were hypersensitivity to DNA damaging agents were recovered from our screen and are therefore candidates for further investigation. MR lines 7, 33, 53, 51, 56, 62, 73, 80, 92, 98 and 99 did not display strong hypersensitivity to DNA damaging agents and P element mapping in these mutant lines uncovered several genes of unknown function or genes with functions unrelated to surviving
Figure 1. Identification of novel Drosophila loci that respond to DNA damage.

(A) Genetic screening procedure used to identify novel loci (on the 2nd and 3rd chromosome) that cause sensitivity to DNA stress during larval development. Heterozygous flies were crossed for 3 days. On day 5 larvae were exposed to DNA damaging agents and the ratio between heterozygous and homozygous adult survivors in the F1 generation was determined.

(B) DNA stress response of selected fly lines that displayed sensitivity to DNA damaging agents (Table 1). Larvae (2-4 day old) from heterozygous crosses were exposed to H2O, 20 Gy IR, 40-50 mM HU or left untreated. The ratio between heterozygous and homozygous survivors was determined 14-21 days after exposure. According to lethality of the balancer and assuming normal Mendelian inheritance takes place the expected ratio would be 2:1 (dashed line). Fly lines displaying an increase in the ratio are considered as ‘hypersensitive’ to the DNA damaging agent applied. mei-41 and grp/dChk1 are control lines for IR and HU hypersensitivity, respectively. Mutants were grouped according to DNA damage sensitivity: IR, IR & HU or HU hypersensitivity. Mutant lines MR67, E709 and MR65 displayed female fertility defects.

<table>
<thead>
<tr>
<th>Fly line</th>
<th>Affected genomic region (GadFly)</th>
<th>Function</th>
<th>Target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR67</td>
<td>5'-UTR Orb (CG10868)</td>
<td>RNA binding/U-rich cytoplasmic, polyA binding</td>
<td>CTGCAACC (3'-5')</td>
</tr>
<tr>
<td>E709</td>
<td>5'-UTR Trap18 (OS1H31) (CG1057)</td>
<td>transcription mediator component</td>
<td>GTCGAGAT (5'-3')</td>
</tr>
<tr>
<td>MR65</td>
<td>5'-UTR (CG5629)</td>
<td>4' phosphorylantioxycteine synthase</td>
<td>GTCGAGAT (5'-3')</td>
</tr>
<tr>
<td>MR7</td>
<td>2L 33E5-33E9 intron Bun (CG541)</td>
<td>Rfpol II transcription factor</td>
<td>CCGAGAC (5'-3')</td>
</tr>
<tr>
<td>MR80</td>
<td>5'-UTR CG9533</td>
<td>UDP-N-acetylglucosamine pyrophosphorylase</td>
<td>ATCCGAAT (5'-3')</td>
</tr>
<tr>
<td>MR103</td>
<td>5'-UTR of Prl1 (CG4993)</td>
<td>prenylated Tyr phosphatase, Tyr/Ser/Tyr phosphatase</td>
<td>AGCCCTCT (5'-3')</td>
</tr>
<tr>
<td>MR981</td>
<td>3L 70C11-70C12 intron CG9007</td>
<td>unknown</td>
<td>TTCTGCT (5'-3')</td>
</tr>
<tr>
<td>MR281</td>
<td>3R 91E4-91F1 intron CG1020</td>
<td>Ser/Thr kinase</td>
<td>TCCGACGT (5'-3')</td>
</tr>
<tr>
<td>MR62</td>
<td>3R 87C6-87C7 second exon of Men (CG10120)</td>
<td>malate dehydrogenase</td>
<td>CCCACC (5'-3')</td>
</tr>
<tr>
<td>MR33/MRS3</td>
<td>3L 6789-67810 intergenic region (CG11024, CG3048)</td>
<td>unknown</td>
<td>GCAAGGC (5'-3')</td>
</tr>
<tr>
<td>MR99</td>
<td>3L 65E7-65E7 intergenic region (CG8627 &amp; CG8624)</td>
<td>unknown</td>
<td>GCTAAAGT (5'-3')</td>
</tr>
<tr>
<td>MR92</td>
<td>3L 64A7 intron CG13908</td>
<td>unknown</td>
<td>GCCCACG (5'-3')</td>
</tr>
<tr>
<td>MR731</td>
<td>3L 75E1-75E1 intron CG6817</td>
<td>heme binding/catalase/Ca2+- dependent cell adhesion</td>
<td>GCCCGAG (5'-3')</td>
</tr>
<tr>
<td>MR51</td>
<td>3R 85C3-85C4 intron (CG11033)</td>
<td>DNA binding (transcription factor)</td>
<td>CTCCGAT (5'-3')</td>
</tr>
<tr>
<td>MR84</td>
<td>3L 80A1-80A1 intron CG13928</td>
<td>unknown</td>
<td>CACCGG (5'-3')</td>
</tr>
<tr>
<td>MR88</td>
<td>2L 61B2-61B2 intron (CG13874)</td>
<td>malate &amp; glycerol kinase</td>
<td>CCGCAGC (5'-3')</td>
</tr>
</tbody>
</table>

In silico analysis of single P element insertion lines that displayed sensitivity for hydroxyurea (HU) and/or ionising radiation (IR). Genomic regions flanking the P element were recovered, sequenced and used to search the genome database for their localization. The response to HU and IR is indicated: (-) not sensitive, (-) marginally sensitive, (+) sensitive, (++) highly sensitive. The 8bp target sequence used by the P element is indicated, 5' to 3' (between brackets) relative to its orientation in the genome as determined by sequence analysis. The P element is known to integrate preferably in UTRs, introns and intergenic regions (ref. 17).

Table 1. Mapping Results of Single P Element Insertion Lines

In Table 1, the results are grouped into the following categories:

- **DNA stress response of selected fly lines**
- **Function**
- **Target sequence**

The values in the table provide information about the DNA stress response of selected fly lines and their functions, along with the target sequences for each fly line.

The table includes columns for fly line identification, sterility status (IR, IR & HU, HU) and the ratio hetero:homo.

The data is used to identify novel loci that respond to DNA damage, providing insights into the mechanisms and genetic factors involved in DNA repair and response to stress in Drosophila.
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DNA damage (Table 1). Therefore these mutant lines will not be discussed further and were not selected for characterization in this thesis. Mutant lines MR28, MR84 and MR103 did not display fertility defects, but in contrast with the other mutant lines that did not display female fertility defects, these mutants were extremely sensitive to DNA damaging agents and characterization of the affected genes might provide new insights in the survival after DNA stress. Although, we mapped the P element insertion sites in these latter 3 mutants we did not attempt to confirm phenotypic linkage with the P element.

The MR84 mutant was hypersensitive to HU and the P element was recovered from the 5’ untranslated region (5’-UTR) of CG11238, which encodes a protein of unknown function (Table 1). The open reading frame (ORF) encoded by the CG11238 gene displays sequence homology with a human rootletin like protein (gi:113413563). Although this human protein is also uncharacterized, rootletin is required for proper chromosome segregation and centrosome cohesion \(^32\). Thus CG11238 might be a novel gene required for centrosome function during cell division, however in depth characterization of this mutant is required to substantiate this hypothesis.

Mutant lines MR103 and MR28 were extremely sensitive to IR and the P elements in these two mutant lines were recovered from the 5’-UTRs of genes encoding a protein phosphatase and kinase respectively (Table 1), suggesting that these genes might be involved in signal transduction cascades required to survive radiation-induced DNA damage. The *Drosophila center divider (cdi/TSKI)* gene, affected in MR28, encodes a kinase that is involved in cytoskeleton remodeling and morphogenesis \(^33,35\). Currently the role of *cdi/TSKI* during signaling in response to DNA stress is unknown and further characterization of the MR28 mutant may reveal a novel link between DSB signaling responses and the cytoskeleton.

The gene affected in the MR103 mutant encodes the *Drosophila* homolog of *phosphatase of regenerating liver (dPRL)*. PRL is a protein-tyrosine phosphatase that is characterized by a C-terminal CAAX motif, a signal for farnesylation/prenylation. The human genome encodes three *PRL* genes (*PRL1-3*), while *Drosophila* has only a single *PRL* gene. dPRL is closely related to all the human PRL proteins as determined by sequence/structural comparison (not shown). The dPRL contains a protein-tyrosine phosphatase (PTPase) active consensus (HCXXGXXR) referred to as P loop \(^36\), the general acid loop and the Cys49 and Cys104 which can form an intramolecular disulfide bond \(^37\). Therefore, it is likely that the dPRL protein fulfils similar functions as those observed for other PRL proteins. Mammalian PRLs are involved in cell proliferation, migration, invasion and metastasis \(^38-44\). Consistent with an oncogenic role of the PRL proteins overexpression of PRL proteins increased cell proliferation by stimulating progression from G1 into S phase possibly due to the down regulation of the cyclin dependent kinase inhibitor p21\(^{Cip1/Waf1}\) (ref. 43). Similarly, overexpression of PRL-3 promoted cell proliferation and invasion, which coincided with a downregulation of Csk and an upregulation of the Src kinase \(^44\). During cell division the PRL-1 protein localized to centrosomes and the mitotic spindle, while in interphase cells the protein localized to the membrane of the endoplasmic reticulum in a farnesylation-dependent manner \(^45\). Removal of the farnesylation signal resulted in abnormal mitotic bridges and lagging chromosomes. Because cells expressing mutant PRL-1 arrested in G2/M after treatment with the microtubule destabilizing drug nocodazole and cells initiated mitosis after the nocodazole block, PRL-1 does not interfere with the spindle checkpoint itself but rather with the spindle dynamics \(^45\). The function of the PRL proteins during signaling in response to DNA damage remains unknown. However, preliminary analysis of the MR103
mutant suggest that dPRL might be required for exit from the G2/M checkpoint in response to IR (not shown). Exit from the cell cycle checkpoints in response to DNA damaging agents is far from understood and further characterization of the MR103 mutant might reveal new insights in how cells restart cell cycle progression after DNA damage repair.

**Phenotypic confirmation and linkage: The dMED31 locus is affected in E709**

Transposons have a preference for non-coding regions to insert in and therefore usually induce hypomorphic phenotypes\(^\text{17}\). Although this property of P elements can be useful to study the function of essential genes, interpretation of the function of a gene based on a hypomorph phenotype is sometimes complicated. Because of this and in order to draw firm conclusions based on P element induced phenotype additional control experiments are required. As mentioned previously, mutant lines E709 and MR65 were, based on their DNA damage hypersensitive and female fertile phenotypes, selected for further characterization and additional experiments were performed to confirm phenotypic linkage. In the E709 mutant the P element was recovered from the 5'-UTR of CG1057 (Table 1), which encodes the transcriptional Mediator component MED31 (allele \(dMED31^1\)) (CHAPTER 3). As a first approach towards confirming phenotypic linkage we used PCR mapping using a P element specific primer and a genomic primer located in close proximity of the transposon to check whether the identified location of the transposon was correctly mapped. PCR mapping confirmed that the identified P element location was inside the 5'-UTR of \(dMED31\) in the E709 mutant line (not shown).

Next, we used the identified polythene segments that contain the \(dMED31\) locus (3R 82D1) to search for deficiency chromosomes (Df) spanning the \(dMED31\) locus. In case the DNA damage sensitivity and female fertility are linked to the P element insertion inside the \(dMED31\) gene, then transheterozygous mutants carrying both the Df chromosome and the \(dMED31^1\) allele, should also display sensitivity to HU and should be female sterile. In short, the Df chromosomes should not complement (rescue) the E709 phenotype. However, when we placed \(dMED31^1\) over \(Df(3R)110,ru'thi'si'kni'^i-4^m'^i-1p'e'ca'l\) (82C4;82F3-7) and \(Df(3R)Z1,ry^*\) (82A5-6;82E4), transheterozygous larvae were not hypersensitive to HU (not shown), suggesting that sensitivity to DNA damage is not linked to the P element insertion in E709. Nevertheless, transheterozygous females displayed fertility defects, indicating that the P element insertion does affect female fertility. To restore the original \(dMED31\) gene in the E709 mutant we removed the P element using the \(\Delta2-3\) transposase\(^\text{46}\). From this allelic series we recovered a P element excision line, allele \(dMED31^2\), in which the \(dMED31\) gene was fully restored (not shown). Transheterozygous \(dMED31^{1/2}\) females were fertile, but remained hypersensitive to HU (not shown), further demonstrating that the P element insertion in the E709 mutant is not linked to DNA damage sensitivity, while female fertility is linked to the P element insertion. This implies that the E709 mutant carries an additional mutation that causes hypersensitivity to HU. Although it might be interesting to identify this additional locus, we did not attempt to map this mutation.

Because there were no antibodies available to analyze \(dMED31\) protein levels, we performed PCR analysis and rapid amplification of cDNA ends (RACE) to clone the 5'-UTR of the \(dMED31\) mRNA from E709 mutants. Using this approach we found that transcription of \(dMED31\) initiated from the P element indicating that mRNA production was hampered in this mutant. Finally, we created a transgenic fly carrying a full length genomic fragment of the \(dMED31\) gene (\(P[dMED31]\)) and this transgene rescued the \(dMED31^1\) female sterile phenotype, proving that female sterility was due the P element insertion in E709. The female sterile phenotype of E709 is described in detail in CHAPTER 3.
Phenotypic confirmation and linkage: The dPPCS locus is affected in MR65

The transposon in the MR65 line landed in the 5’-UTR of CG5629 (Table 1), which encodes a CoA biosynthesis enzyme, (R)-4’-phospho-\(N\)-pantothenoylcysteine synthetase (allele dPPCS\(^1\)) (CHAPTER 4). For phenotypic confirmation of the MR65 mutant we followed a similar strategy as for the E709 mutant. PCR mapping confirmed the P element insertion site (not shown), while complementation analysis demonstrated that when dPPCS\(^1\) was placed over Df(3R)Dl-KX23, e\(^{91C7-D03;92A5-08}\) and Df(3R)Dl-BX12, ss\(^{-}e^r-o\) (91F1-2;92D3-6) transheterozygous flies still displayed hypersensitivity to IR and were female sterile, suggesting that the P element insertion indeed caused hypersensitivity to IR and induced female fertility defects. In contrast with the E709 mutant, we were unable to restore the original dPPCS gene in MR65 mutants by P element excision (approximately 250 independent lines were analyzed). P element excision usually occurs imprecisely and using this property of the transposon we were able to create a knockout and lethal allele of the dPPCS gene (allele dPPCS\(^{33}\)). Since transheterozygous dPPCS\(^{1/33}\) females were sterile, displayed sensitivity to IR and dPPCS\(^{1/33}\) flies generally displayed a more severe phenotype it is likely that the mutation is located in the dPPCS locus and is responsible for the observed phenotypes. Moreover, RACE analysis of MR65 mutants revealed that the 5’-UTR of the dPPCS mRNA is truncated by approximately 250 bp, demonstrating that dPPCS mRNA production was also disrupted in the MR65 line. We also raised antibodies against the dPPCS protein and Western blot analysis revealed that dPPCS protein levels were mildly reduced in dPPCS\(^{1/1}\) mutants and further reduced in dPPCS\(^{1/33}\) mutants, proving that dPPCS protein levels are affect in the MR65 line. Finally, to conclusively prove that the dPPCS gene was affected in MR65 we created a transgenic fly that ectopically expressed a FLAG tagged dPPCS cDNA (P[dPPCS]) under control of an ubiquitin promotor. The entire dPPCS locus is approximately 10-11 kb, which makes it difficult to create a full length genomic transgenic fly and therefore we used an ectopically expressed dPPCS cDNA. Insertion of P[dPPCS] back into the dPPCS mutant (partly) rescued all the phenotypes of dPPCS\(^{1/1}\), confirming that all phenotypic characteristics were due to a mutation in the dPPCS locus in the MR65 line. The mutant phenotype of MR65 is further described in CHAPTERS 4 to 6.

Concluding remarks

We established that a mutation in the dMED31 locus in the E709 line results in fertility defects (CHAPTER 3) and we also demonstrated that the dPPCS locus is affected in MR65 and that a mutation in this locus affects female fertility and causes hypersensitivity to DNA damaging agents (CHAPTER4-6). The DNA damage sensitivity of the E709 line is not due to a mutation in the dMED31 locus and is likely the result of an additional mutation at a thus far unknown locus. Therefore, although genetic screens are a powerful tool to identify novel genes, additional analysis is required to identify false positives. Nonetheless, our screening procedure uncovered several novel genes required to survive genotoxic stress and also identified potential candidate genes that can be analyzed in the future. Because we only performed a relatively small forward genetic screen, screening the Drosophila genome for additional mutations that cause both female fertility defects and hypersensitivity to DNA damaging agents might uncover more unexpected players in DNA stress responses.
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