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PhD THESIS

Genetic analysis of the TPR subunits of the APC in *Drosophila melanogaster*

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Introduction

Progression through the cell cycle in all eukaryotes is governed by the sequential degradation of different cell cycle regulatory proteins mediated by the ubiquitination pathway. In this process an ubiquitin chain is covalently attached to the target proteins in a series of step-wise reactions leading to its recognition and degradation by a large cytosolic protease complex named as 26S proteasome. First the ubiquitin-activating enzyme (E1) forms a thiol ester bond between its own active-site cysteine and the carboxy-terminal glycine of ubiquitin. Second, the activated ubiquitin is transferred to the ubiquitin-conjugating enzyme (E2). Finally, the ubiquitin molecule is coupled to the substrate protein in a process mediated by an ubiquitin-protein ligase enzyme (E3). The key component of this proteolytic system is the E3 ubiquitin-protein ligase which provides both platform and substrate specificity of the ubiquitination process. Two different ubiquitin-ligases play important roles in the cell cycle regulation: the SCF (Skp1/Cullin/F-box) and the anaphase-promoting complex or APC. The SCF complex controls principally the G1/S and G2/M transitions by inducing the degradation of different regulatory proteins at these phases of the cell cycle. The APC is active during mitosis and G1 phases of the cell cycle in which, by mediating securing and mitotic cyclin degradation, it triggers the metaphase to anaphase transition and the exit from mitosis. It is a large, 1.5 MDa protein complex containing at least 13 stably associated core subunits in yeast. Orthologues of the yeast APC subunits could be identified in higher eukaryotes as well indicating that the structure and the function of APC are evolutionarily conserved. Despite the essential role of APC in regulating mitosis, little is known about the function of most of its individual subunits.

There are four structurally related proteins within the vertebrate APC with not yet precisely understood functions. All of them contain nine to ten copies of the tetratrico-peptide repeat (TPR) motifs in tandem arrays. The TPRs are repeats of 34 amino acid structural motifs with a consensus sequence restricted only to eight residues. Though, there is no invariant residue even within the consensus sequence, these positions are conserved in terms of size, hidrophobicity and spacing of the amino acid residues. TPR motifs are present in functionally divergent proteins and thought to mediate protein-protein interactions and the assembly of multiprotein complexes. The TPR subunits in budding yeast are coded by the *Cdc27, Cdc16* and *Cdc23* genes that are all essential with uniform loss of function mitotic arrest phenotypes.

Specific aims

The aim of our work was to functionally analyze the TPR subunits of the APC in *Drosophila melanogaster.* The TPR subunits constitute half of the total mass of APC. We hope that our effort will contribute to better understanding of the function of individual APC subunits and the complexity of the most complicated ubiquitin-protein ligase. My research plan contained the following steps:

1. Identification of the *Apc6*, *Apc7* and *Apc8* genes using bioinformatics methods.
2. Establishment of the *Apc6*-, *Apc7*- and *Apc8*-specific transgenic RNS interference lines.

3. Induction of the RNS interference followed by genetic, cell biological and cytological analysis of the induced phenotype.
4. Analysis of protein interactions with affinity chromatography.

Methods

- Recombinant DNA technology
- Polymerase Chain Reaction (PCR)
- Inverse PCR
- Semiquantitative reverse transcription coupled PCR (RT-PCR)
- DNA sequencing
- P-element remobilization
- P-element transformation
- Gel filtration chromatography
- Western blot
- Neuroblast preparation and cytological characterization

Results

Orthologues of the yeast TPR subunits have been identified in *Drosophila.* These subunits are the Apc3 (also known as Cdc27 or Mákos; hereafter referred to as Apc3), Apc6 (also known as Cdc16; hereafter referred to as Apc6) and Apc8 (also known as Cdc23; hereafter referred to as Apc8). Characterization of *Apc3* mutants indicated that this subunit is

essential for development, and perturbation of its function results in mitotic cyclin accumulation and metaphase-like arrest.

In my PhD thesis, I focused on the genetic and biochemical characterization of the other TPR subunits of the Drosophila APC. First I established that in addition to the yeast TPR orthologues, a gene is present in the *Drosophila* genome that codes for a protein closely related to the APC7 subunit of the vertebrate anaphase promoting complex. This gene, designated as *CG14444*, was identified by the *Drosophila* genome project (BDGP) as a gene coding for a putative TPR protein. The primary sequence of this protein appears to be conserved from plants to humans, including the number, location and distribution of its TPR motifs. The TPR motifs in the *Drosophila* Apc7 protein are more related to the TPR motifs of the human APC7 subunit than to the motifs of other *Drosophila* TPR proteins, suggesting functional correspondence. These data support the nomination of the CG14444 protein as the *Drosophila* Apc7 homologue, and its gene was further characterized together with the other TPR genes.

Transgenic flies knocked down for the TPR genes *Apc6*, *Apc7* and *Apc8,* by RNA interference were established to investigate their function. Whole-body expression of subunit specific dsRNA efficiently silenced these genes resulting in only residual mRNA concentrations. *Apc6* and *Apc8* silencing did not significantly affect survival of embryos and early larvae; however it did induce developmental delay in later stages and caused different pupal lethality. While the $Apc8^{RNAi}$ pupae died at the end of the prepupal metamorphosis in the P4(ii) (moving bubble) stage, the $Apc6^{RNAi}$ animals developed further and died at the first part of the phanerocephalic pupal (malpighian tubules migrating) stage of P5(i). The *Apc8* specific RNAi effect was also more pronounced both in terms of larval lethality and formation of melanotic tumors. However, both $Apc6^{RNAi}$ and $Apc8^{RNAi}$

animals died earlier than the ones homozygous for the *mks*^{1} allele of the *Apc3* gene. Cytological examination showed that these animals had elevated level of apoptosis, high mitotic index and delayed or blocked mitosis in a prometaphase- metaphase-like state with overcondensed chromosomes. In many mitotic cells of *Apc6* and *Apc8* RNAi induced third instar larvae the chromosomes showed up as dot-like structures instead of the characteristic rod-like wild-type chromosomes, and frequently they appeared either scattered all over the cells or congressed at the metaphase plate. The chromosome overcondensation indicated that the cells had been delayed or arrested in mitosis, as chromosomes continued condensation during that time. Accordingly, the proportion of cells in mitosis was significantly higher, leading to more than twice as high mitotic index (MI) as in the wild-type. Most of the mitotic cells were in a prometaphase - metaphase-like state, and at the same time, the number of cells in ana- and telophases stayed relatively low. This is reflected in the two- and threefold higher metaphase - anaphase ratios (M:A) in the *Apc6* and *Apc8* RNAi preparations respectively, and suggested that loss of function of these subunits leads to metaphase-like delay or arrest. A proportion of *Apc6* and *Apc8* RNAi cells were polyploid, most frequently with highly overcondensed tetraploid or less frequently octaploid or higher ploidy chromosome complements. In addition to metaphase, irregular chromosome behavior could be detected in anaphase as well, which includes lagging chromosomes and chromosome bridges.

One of the main functions of APC is to aid the degradation of mitotic cyclins. The *Apc6* and *Apc8* RNAi arrested neuroblasts contained elevated levels of Cyclin B, but surprisingly, Cyclin A appeared to be degraded normally. It was interesting that the *de facto* metaphase arrest in *Apc6*^{RNAi} and *Apc8*^{RNAi} cells with overcondensed chromosomes aligned

precisely at the metaphase plate correlated with the ability of these cells to degrade Cyclin A. These observations suggest that Cyclin A degradation is required in *Drosophila* for proper chromosome alignment at metaphase.

Contrary to the loss of function phenotype of *Apc6* and *Apc8* genes, the apparent loss of *Apc7* function did not lead to the abnormalities. Instead, the *Apc7* knocked down animals were viable and fertile, though they displayed mild chromosome segregation defects and anaphase delay. In order to support the nonessential function of the *Apc7* gene, a null mutant allele was isolated by imprecise excision of a nearby P element. The remobilization generated a deletion which removed about two-third of the *Apc7* gene, including the functionally important eight tandem TPR repeat sequences. The phenotype of the *Apc7* null mutant allele was indistinguishable from that of the $Apc7^{RNAi}$ lines. We could not detect abnormal changes in level and localization of either cyclin A or cyclin B in mitotic cells and their turnover appeared similar to that in wild type. To see whether Apc7 is a genuine component of the *Drosophila* APC, we examined physical interaction between Apc7 and universal APC subunits Apc3 and Apc8 by co-transfection of S2 cells and affinity chromatography of cell extracts. At least a fraction of Apc7 was clearly associated with the APC in *Drosophila*, but perhaps not as core subunit. In addition to this, the *Apc7* gene showed synergistic genetic interaction with *Apc8* that together with the phenotypic data, suggests a limited functional role for Apc7 subunit within the APC complex in *Drosophila*.

The diverse phenotypic features of the TPR mutants appear to be inconsistent with one of the models of the APC based on the TPR subunits forming a central scaffold-like structure to mediate APC assembly. One would expect such a scaffold to become unstable or collapse when its components are removed one by one or in double mutant combinations,

leading to inactive APC, but we could not detect that in our RNAi lines. Instead, all single and double TPR^{RNAi} lines possessed slightly smaller than normal larval brains and imaginal discs that indicate reasonable mitotic activity and from that fact the existence of an APC with at least residual functions. Therefore, our data better fit an alternative model of APC that is centered on the largest subunit, the Apc1/Shtd, as the scaffold forming component of the complex. In this model, the Apc1/Shtd provides a platform, to which all the other subunits bind by forming functional subcomplexes. Taken all data together, our view is that the TPR subunits may form such functional subcomplexes of the APC that could bind to the Apc1/Shtd scaffold and most likely be involved in activator and substrate binding.

Summary

The main results of my work are the following:

- We have identified and characterized the TPR subunits of the *Drosophila* APC.
- Silencing of the *Apc6* and *Apc8* expression by RNA interference results in developmental arrest that is accompanied with the induction of metaphase arrest in mitotic cells, apoptotic cell death and elevated levels of Ciklin B.
- We have demonstrated that an orthologue of the human Apc7 subunit exists in *Drosophila.*
- The *Drosophila Apc7* gene showed synergistic genetic interaction with the *Apc8* gene.
- The *Drosophila* Apc7 protein co-purifies with the Apc3 and Apc8 subunits.
- The *Drosophila* Apc7 protein is not essential component of the APC.
- We were able to demonstrate that the APC contains at least two Apc8 subunits in *Drosophila*.

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