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The prdm gene apoptix antagonizes programmed cell death in tribolium

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THE PRDM GENE APOPTIX ANTAGONIZES PROGRAMMED CELL DEATH IN *TRIBOLIUM*

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ant: antenna

Apox: Apoptix

APF: after pupal formation

app: appendage

BCIP: 5-bromo-4-chloro-3-indoly phosphate

BSA: bovine serum albumin

cns: central nervous system

dapaf1: Drosophila caspase-activating protein

DIG: digitoxigenin

dcp1: death caspase1

debcl: Drosophila death executioner Bcl2 homologue

death related CED3/Nedd2-like: dredd

death executioner caspase related to Apopain/Yama: decay

dsRNA: double stranded RNA

DABCO: 1, 4-diazabicyclo [2.2.2] octane

drice: Drosophila/Ice

EGFP: enhanced green fluorescent protein

EGF: epidermal growth factor

h: hours

hid: head involution defective

PCD: programmed cell death

PBT: phosphate buffered saline plus Tween-20

RT-PCR: reverse transcription polymerase chain reaction

GA-1: Georgia-1

IAP: Inhibitor of Apoptosis Protein

KD: knockdown

Nc: Nedd2-like caspase

N: notch

man: mandible

NBT: nitro-blue tetrazolium chloride

Ola: optic lobe anlage

PBS: phosphate buffered saline

pro: protocerebrum

RNAi: RNA interference

rpr: reaper

sto: stomodeum

vis: visual anlage

wg: *wingless*

WT: wildtype

1. Introduction:

1.1. Tribolium castaneum as a model organism

The red flour beetle, *Tribolium castaneum*, is not only a major pest species but also a powerful organism to study insect development (Altincicek, Knorr et al. 2008). Being a holometabolous insect of the order Coleoptera, *Tribolium* separated from *Drosophila* about 250 million years ago (Brown, Denell et al. 2003; Savard, Tautz et al. 2006). *Tribolium* has many advantages for experimental work in the laboratory. It is easy to culture at a wide range of temperature and relative humidity (Sokoloff, 1972). The generation time is about 8 weeks and life expectancy is approximately 3 years. Genetic research in *Tribolium* was initiated by the study of spontaneous mutations and genetic linkage maps (Sokoloff, 1972). Subsequently, comparative analyses of *Tribolium* and *Drosophila* segmentation genes were carried out, initially focusing on the homeotic gene complex (Beeman, Stuart et al. 1989; Beeman, Stuart et al. 1993; Brown, Denell et al. 2003). Eventually, the sequencing of the *Tribolium* genome identified 7579 genes as ancient, 1462 genes as insect-specific and 129 orthologous gene groups as present in *Tribolium* and humans but lost in *Drosophila* (Richards, Gibbs et al. 2008).

Currently, major efforts are being invested in determining the function of these genes, in particular those which appear to be novel. One especially important method is the lack-of-function analysis by gene knockdown (KD), which is efficiently initiated in Tribolium by systemic RNA interference (RNAi) in embryo (Brown, Mahaffey et al. 1999), the larva (Tomoyasu and Denell 2004) or the adult stage (Bucher, Scholten et al. 2002). Other molecular

genetic tools available include genetic maps, expressed sequence tag collections and cDNA libraries (Lorenzen, Doyungan et al. 2005; Wang, Wang et al. 2007).

1.2. Development of the larval and adult eyes in Tribolium

Like *Drosophila*, *Tribolium* has visual organs which are specific for the larval stage, the larval eyes or stemmata, and visual organs that are specific for the adult, the compound eyes (Liu and Friedrich 2004). The *Tribolium* larva has a eucephalic head with a bilateral pair of larval eye clusters, which are located at the lateral side of the head. A single lateral eye cluster consists of two pigmented cell groups which comprise approximately 25 photoreceptor cells forming a single stemma (Liu and Friedrich 2004).

The adult eye of *Tribolium* develops in the lateral ectoderm of the larval head capsule during late larval development and in the pupa (Fig. 1).The gena is a rim like lateral head cuticle which protrudes deep into the anterior margin of the eye. During this process, the gena protrudes into the anterior midline of the differentiating retina, resulting in the bilobed shape of the adult eye. A single compound eye of *Tribolium* contains on average close to 95 ommatidia. Each of these comprises eight photoreceptor cells, four cone cells and 12 pigment cells (Friedrich et.al, 1996). The progressive differentiation of the photoreceptors begins 6 h after pupa formation (APF) (Fig. 1a). It is visible to external inspection due to the pigment granules, which accumulate in the photoreceptor cells (Yang, Zarinkamar et al. 2009). At 48 h APF, the differentiating retina is characterized by regular array of pigmented cells which eventually lead to homogeneous pigmentation of adult eye (Fig. 1b-1d) (Friedrich et. al, 1996) (Yang, Zarinkamar et al. 2009).

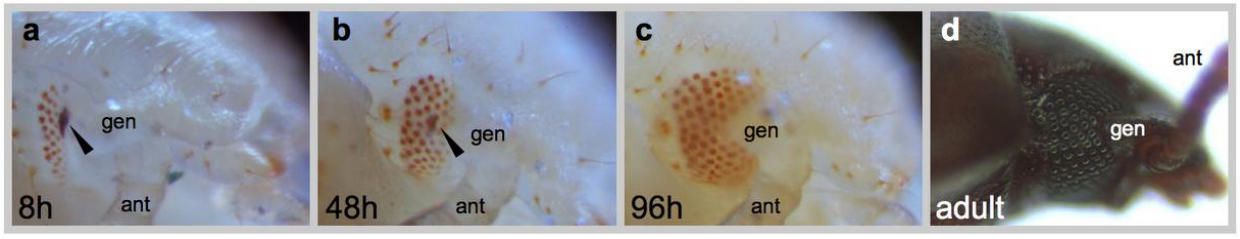

Fig. 1: Adult eye development in *Tribolium*. (a-c) Lateral view of pupal head at (a) 8 h, (b) 48 h and (c) 96 h after pupal formation. (d) Lateral view of adult head. Anterior to right. Arrowhead points at the retracted larval eye, which is still visible at (a) 8 h and (b) 48 h after pupal formation. Abbreviations: ant = antenna, gen = gena.

1.3. Molecular regulation of programmed cell death

Programmed cell death (PCD), which is is divided into three classes: apoptosis, autophagy and non-lysosomal cell death (Lee and Baehrecke 2000), plays many important roles in animal development and tissue homeostasis (Jacobson, Weil et al. 1997). PCD is important for the final shaping of structures. One example is the removal of cells during the formation of digits in vertebrates (Milligan, Prevette et al. 1995). Other examples concern the control of cell number and the elimination of abnormal and harmful cells (Jacobson, Weil et al. 1997). Genetic studies in *C. elegans* provided the first evidence of apoptosis and led to the identification of genes involved during this process (Ellis and Horvitz 1986). In the past 15 years, extensive progress has also been made in identifying and characterizing both conserved and novel PCD-controlling genes in *Drosophila* (Fig. 2). Caspases are cysteine-dependent aspartate-specific proteases that are highly conserved. There are two types of caspases: initiator caspases and effector caspases. Initiator caspases are activated by binding to adaptor protein and in turn activate effector caspases through proteolytic cleavage. The active effector caspases then degrade intracellular proteins which lead to cell death.

So far, five caspases have been identified in *Drosophila: death caspase1 (dcp1*), *Drosophila/Ice* (*drice*), *death related CED3/Nedd2-like protein* (*dredd*), *Nedd2 like caspase* (*Nc*) and *death executioner caspase related to Apopain/Yama (decay*). Three additional caspases are predicted based on genomic sequence (Fraser and Evan 1997; Inohara, Koseki et al. 1997; Song, McCall et al. 1997; Chen, Rodriguez et al. 1998; Dorstyn, Colussi et al. 1999; Dorstyn, Read et al. 1999). *Nc* and *DREDD/DCP2* are initiator caspases involved in upstream apoptotic signaling pathways and therefore resemble human initiator caspase 8 and 9 (Chen, Rodriguez et al. 1998) (Dorstyn, Read et al. 1999). DECAY, DCP1 and DrICE are activated by initiator caspases and

act downstream as effector caspases, similar to human caspase 3 and 7 (Dorstyn, Colussi et al. 1999)(Inohara, Koseki et al. 1997; Song, McCall et al. 1997).

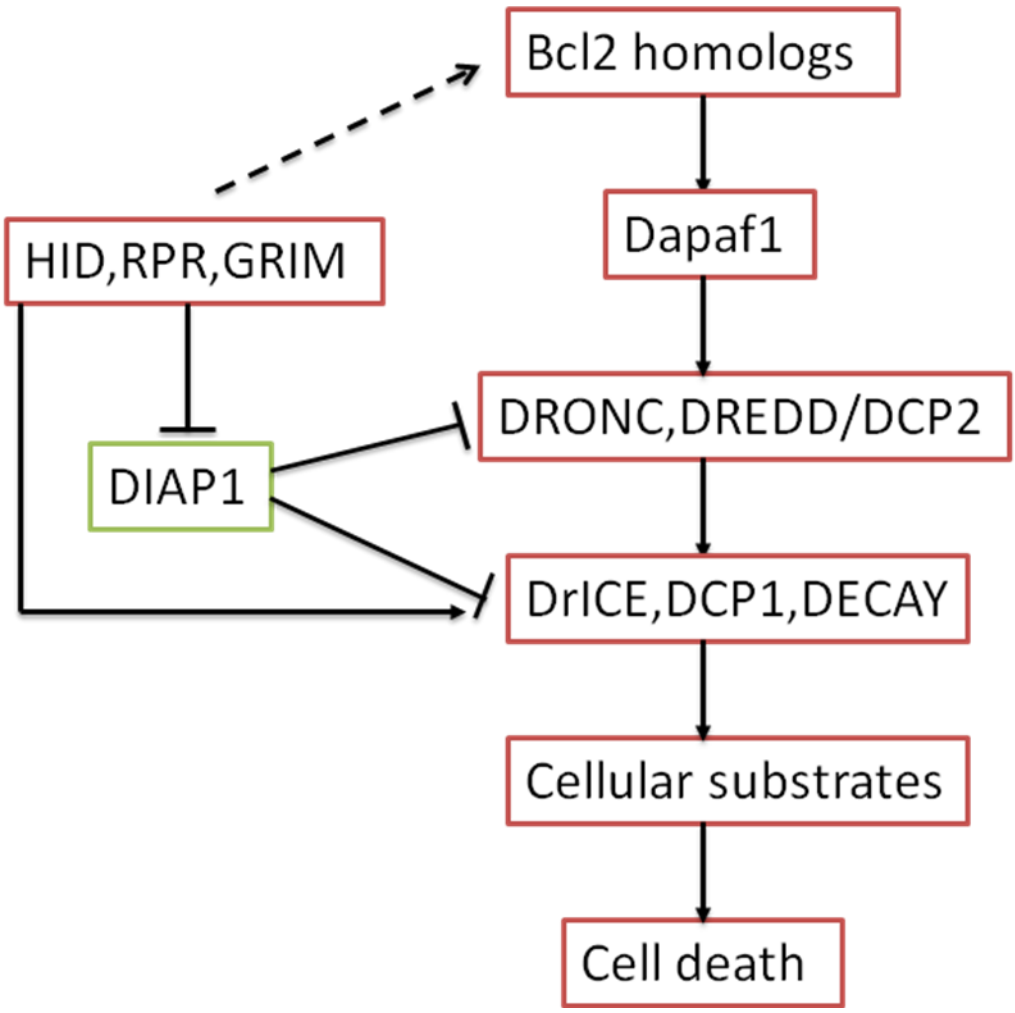

Fig.2: Pathway of programmed cell death in *Drosophila.* Red boxes indicate the proteins which activate cell death. The green box indicates proteins which suppress cell death. Dashed arrow indicates activation or suppression

Mammalian Apaf1 and *C.elegans* CED-4 activate pro-caspases in similar and conserved manner (Zou, Henzel et al. 1997). The *Drosophila* caspase-activating protein Dapaf1 encodes two isoforms generated by alternative splicing - Dapaf1L (mammalian Apaf1 like) and Dapaf1S (*C.elegans* CED-4 like) (Kanuka, Sawamoto et al. 1999). They possess a WD domain, which interacts with cytochrome-c and this interaction is involved in caspase activation (Li, Nijhawan et al. 1997).

Two orthologs of the Bcl2/CED-9 protein family have been identified in *Drosophila*: *death executioner Bcl-2 homologue (debcl)* and *buffy* (Colussi, Quinn et al. 2000; Igaki, Kanuka et al. 2000; Zhang, Huang et al. 2000)(Quinn, Coombe et al. 2003). *Debcl* is similar to the pro-apoptotic Bcl-2 family members which function in both caspase-dependent and-independent manners. *Buffy* is a Bcl-2-like anti-apoptotic protein (Quinn, Coombe et al. 2003).

Three Inhibitor of Apoptosis Protein (IAP) genes have been identified in *Drosophila*. This includes DIAP1, DIAP2, and Deterin (Hay, Wassarman et al. 1995; Jones, Jones et al. 2000).

In contrast to the above conserved proteins, the *reaper* (*rpr), head involution defective (hid)* and *grim* genes encode proteins that are novel regulators of cell death in *Drosophila*. These genes exhibit no notable sequence similarity to known mammalian genes. Ectopic activation of any of these three genes is sufficient to ablate the adult compound eyes by activating apoptosis (Grether, Abrams et al. 1995; Chen, Nordstrom et al. 1996; White, Tahaoglu et al. 1996).

1.4. The role of programmed cell death during Drosophila compound eye development

Each adult compound eye of *Drosophila* contains about 800 ommatidia (Baker 2001). There are two processes during which programmed death occurs in the *Drosophila* eye. The first

one is the removal of surplus cells across the eye. The second concerns the patterning of the eye margin (Fig. 3).

The final size of the eye and cell number depends on the balance between cell proliferation and cell death in combination with cell fate specification (Baker 2001). In *Drosophila*, these processes are coordinated during cell differentiation, triggered by the passage of morphogenetic furrow (MF) from posterior to the anterior across the eye disc (Fig. 3a). Posterior to the MF, photoreceptor cells differentiate in regularly spaced clusters during the late larval stages (Baker and Yu 2001). Once all the ommatidium cells have been specified and differentiated one day after pupation more apoptosis occurs to remove interommatidial cells around each ommatidium (Miller and Cagan 1998). The Epidermal Growth Factor (EGF) signaling pathway yields survival signals to nearby survivor cells that differentiate as secondary and tertiary pigment cells and sensory bristle cell types (Spencer, Powell et al. 1998; Baker and Yu 2001; Yu, Yoo et al. 2002). Notch (N) activity is required for cell death but is inhibited by the cone or primary pigment cells (Cagan and Ready 1989). N signaling is speculated to activate various caspases (Cagan and Ready 1989; Wolff and Ready 1991; Rusconi, Hays et al. 2000).

During mid pupation at 28-32 h APF in *Drosophila*, the signaling factor gene *wingless (wg)* is expressed in the edge of ommatidia and activates *grim*, *hid* and *reaper* (Fig. 3b), which leads to the elimination of approximately 80-100 ommatidia at the perimeter of the eye (Lin, Rogulja et al. 2004). This second round of cell death is responsible for proper patterning of the eye margin.

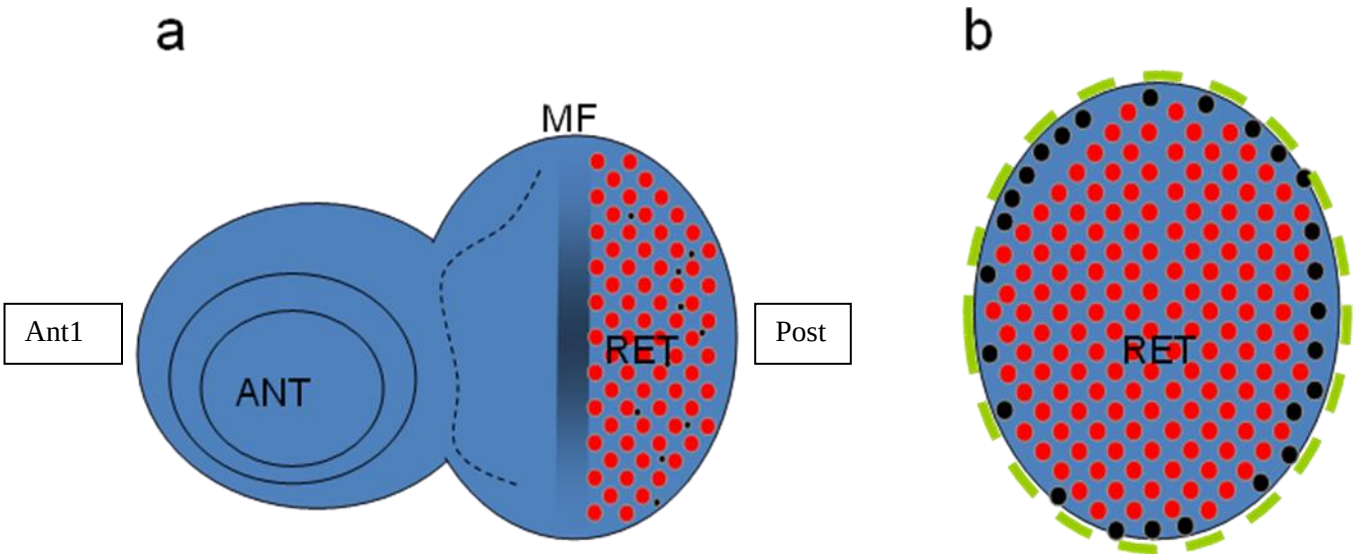

Fig 3. Programmed cell death in the developing adult eye of *Drosophila*. (a) Late 3rd instar eye disc, when surplus inter-ommatidial cells indicated as black dots are removed by programmed cell death. (b) Retina in 28-32 h old pupa. Ommatidia labeled black experience programmed cell death elicited by Wg signaling due to the expression of the Wg ligand along the periphery of the retina (green dashed line).Abbreviations: Ant1-Anterior, Ant-antenna, MF-morphogenetic furrow, RET-Retina

1.5. The Tribolium Prdm gene Apoptix

In an ongoing screen for genes which are differentially expressed in the developing adult eye of *Tribolium,* the Friedrich lab identified the zinc finger transcription factor gene TC011610 as a putative eye regulatory gene. Based on preliminary evidence that TC011610 is essential for photoreceptor cell survival, the gene has been named Apoptix (Apox) to reflect its impact on the regulation of programmed cell death in the developing *Tribolium* eye. Homolog searches revealed that Apox is highly conserved in arthropods including the mosquito *Aedes aegypti* (XP_001650281), the honeybee *Apis mellifera* (XP_003249388), the pea aphid *Acyrthosiphon pisum* (XP_001952629) and the predatory mite species *Metaseiulus occidentalis* (XP_003742843) (Fig. 3). However, no homologs are present in *Drosophila* suggesting gene loss during dipteran evolution. Apox is also conserved in the lancelet *Branchiostoma floridae* (XP_002597122), while no homologs could be detected in vertebrate genomes including humans, mouse or zebrafish.

The multiple alignment of Apox homologs and domain analysis revealed the presence of a PR type SET domain followed by a string of five C2H2 zinc finger motifs (Fig. 4). This combination characterizes Apox as member of the Prdm gene family of transcriptional regulators (Keller and Maniatis 1991). However, Apox appears to represent a novel subfamily which has so far not yet been report or functionally characterized in any species. In combination, these data characterize Apox as a new member of the Prdm gene family, which is ancient but was lost independently in *Drosophila* and vertebrates.

```
Bflo_Apox   --MESCILIPPE--F-------AV--GPSHVG--------------DQTNFLEMSISVWS
Aphis_Apox  --METCVLIPKELSLQAAPVANVQRSNNNRHH-------------A------EPEITVMT
Tcas_Apox   --METCVLIPQEFSL-------CVRGPAPRRT-------------A------EPEVSVWS
Aaeg_Apox   --MKICEQI-----------------------------------------------------
Amel_Apox   --METCVLIPKEFSL-------AL--ANDRSNYKGLFKNDSGSLVT------DVRISVWS
Mocc_Apox   MTMEICVLIPQE--L-------IL--GSSSRD-------------------ESVLSVKA

Bflo_Apox   ARTLRPGAVFLPDQGRVVLDRLEVYTYLKKDDVRHEFGNYDDIQVVD-DRKVRHCNWVRF
Aphis_Apox  NVNVPRGTLIYPFQGTVRMDKLDVFSFLDDTDIRHRFGCYDQVTEVN-RLRVRYCNWVRF
Tcas_Apox   NTSIAQGSLCYPFQGTIRIDKLDVYGQLDDDDIRHRFGCYDEISGSG-SRRVRHCNWVRF
Aaeg_Apox   --HIV-------------------------IRHRFGLYDEITTAN-GRRVRHCNWIRF
Amel_Apox   NVLIPAGTLIYPFQGSIRFDKIDLYSLLDDNDIRREYGCYDEVFFSNYSLNKRQCNWIRF
Mocc_Apox   NQRFARSKRLLPFQGTVRTDKVETCDRLDDDDIRNRLGCYEEIVAVD-NKKIKYCNWVRF

Bflo_Apox   LRSADK-EED-VSLVGYREKDRVYFKVVKLVPPNTELLAAFHRDQEATDPAAEAAESQRL
Aphis_Apox  LKITQHHSEQHVNVLGTKIKGEPMYEVIKNIPANTELIVHYL-------PER----PEEI
Tcas_Apox   VRVASSFNPA-VNFVATKVRGEPVYEAVKPISPDTELLVYYL-------PER----PEEL
Aaeg_Apox   LRVSETYGPQ-VNVVCAKVKGEPIYEIVKPIPSHQELVVYYL-------PEG----PEEL
Amel_Apox   LRIVQSYDEQ-VNLIGTKVKGDPIFEVIKDVQPDTELVAWFL-------PTA----EQDF
Mocc_Apox   LRVVTSMTEE-VNLIATIAQGETIFESIVEIPPGGELVA-FLDRREGSPPSL----PPNF

Bflo_Apox   FLEFTAPLFA--A---LQQRLGERGSDSPSKRTSPDLSPGARAQ------AARSITLVTP
Aphis_Apox  FF-MPAVHYL--RNTLYRRTMDTILEDSP-----LDLSMSLLSRA-YGTSSASS-ASSPP
Tcas_Apox   FF-VR----M--RASLYRQTMDSILEDSP-----LDLSMSLLSRA-L--------SGSPP
Aaeg_Apox   FF-IR----M--RSQLYRQTMDSILEDSP-----LDLSTSLLSRVML--------PISPP
Amel_Apox   VF-IP--HDMCSRSALYRCTIDSILDESP-----LDLSMALLSH------HSSS-TISHP
Mocc_Apox   AL-HRSVNFM--Q---YRNGMGSILEENP-----LDLSQSLVA--------GSS-TRSPS

Bflo_Apox   PLEE-EERRALSGATGHLGHQTAQISH------SAK-PASPPFRPQTAHTAHPPTHTAHH
Aphis_Apox  SGLDTDERKSLSGE-----------SS------SAN-S----------------------
Tcas_Apox   S-AE-DERKSVSGD-----------SS------AAS-S----------------------
Aaeg_Apox   SGTE-DERKSVSGD-----------SSISISSGAST-S----------------------
Amel_Apox   YYEV-DEYESTSEE-----------SS------SSTLS----------------------
Mocc_Apox   SSPR-PEIMTPPSE-----------GL------TTF-P----------------------

Bflo_Apox   PTHTAHPPTLNSHPSTHK------AYPPT--------LTSHPLNLTTHPPTRTAHPPSHV
Aphis_Apox  -------------LSGDT---------ASLDHNVNMIVC----NTT--------------
Tcas_Apox   --------------QGDL---------PE--------AT----VPT--------------
Aaeg_Apox   --------------GGDLDTCSAI---PS--------VP----RSS--------------
Amel_Apox   -------------LTGDH---------LD--------CS----ILT--------------
Mocc_Apox   -------------PLGGL------AIPPT--------QR----SLG--------------

Bflo_Apox   SHSPTHTPHPPIHVTHPPTHAAHPHTHKTPLPQTHTPHPPTR----RRNGERTLLPCEVC
Aphis_Apox  ----------------------------------------PVKPRPFR--GERTLLPCEVC
Tcas_Apox   ----------------------------------------P-RARPR----ERTLLPCGVC
Aaeg_Apox   ----------------------------------------P-KARPSR--GERAMLPCEVC
Amel_Apox   ----------------------------------------TIK----R--GERVLLPCEVC
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Mocc_Apox   ----------------------------------------PKK----P--RERTLLPCEVC

Bflo_Apox   GKAFDRPSLLRRHMRTHTGEKPHACDVCGKAFSTSSSLNTHRRIHSGEKPHVCQVCGKRF
Aphis_Apox  RKAFDRPSLLKRHMRTHTGEKPHVCAVCNKGFSTSSSLNTHRRIHSGEKPHQCGVCGKRF
Tcas_Apox   NKAFDRPSLLKRHMRTHTGEKPHVCMVCGKGFSTSSSLNTHRRIHSGEKPHQCPVCLKRF
Aaeg_Apox   GKAFDRPSLLKRHMRTHTGEKPHVCGVCGKGFSTSSSLNTHVRIHSGEKPHQCQVCGKRF
Amel_Apox   GKSFDRPSLLKRHMRTHTGEKPHVCMVCNKGFSTSSSLNTHKRIHSGEKPHQCLVCGKKF
Mocc_Apox   GKAFDRPSLLRRHMRTHTGEKPHVCDVCGKGFSTSSSLNTHRRIHSGEKPHQCNVCGKRF

Bflo_Apox   TASSNLYYHRMTHMKDKPHKCTMCSKSFPTPGDLKSHMYVHNGSWPFKCDVCNRGFSKLT
Aphis_Apox  TASSNLYYHRMTHIKEKPHKCTLCAKSFPTPGDLKSHMYVHNGSWPFKCHICNRGFSKHT
Tcas_Apox   TASSNLYYHRMTHIKDKPHKCNLCSKSFPTPGDLRSHMYVHSGSWPFKCHICSRGFSKHT
Aaeg_Apox   TASSNLYYHRMTHIKDKPHKCSLCSKSFPTPGDLKSHMYVHNGSWPFKCHICSRGFSKQT
Amel_Apox   TASSNLYYHRMTHIKEKPHKCSQCSKSFPTPGDLKSHMYVHNGLWPFRCHICSRGFSKPT
Mocc_Apox   TASSNLYYHRMTHSKEKPHKCTLCSKSFPTPGDLKSHMYVHSGSWPYKCHICNRGFSKQT

Bflo_Apox   NLKNHMVLHSGEKKYECPLCNKRFALPCNLRTHLKTVCHQGQLPQQP---CARCGQ----
Aphis_Apox  NLKNHLFLHTGDKPHACELCQKKFALACNLRAHMKT--HESE-TQEE---CNKCGK----
Tcas_Apox   NLKNHLFLHTGDKPHACDLCNKKFALACNLRAHMKT--HEGD-PQEE---CTRCGK----
Aaeg_Apox   NLKNHLFLHTGDKPHVCEVCNKSFALACNLKAHMKT--HEEG-AQDG-------------
Amel_Apox   NLKNHMLLHLG----------------------------------------RCSN----
Mocc_Apox   NLKNHIFLHTGDKPHICEICNKRFALACNLRAHLKT--HEEQ-TQLEKTQCPQCDKFPCE
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Fig. 4: Multiple protein sequence alignment of Apox orthologs. Green background: PR-domain signature residues based on Hohenauer and Moore (2012). Red background: Zinc finger region H_2C_2 motifs. Species abbreviations: Aaeg = *Aedes aegypti* (*X*P_001650281), Amel = *Apis mellifera* (XP_003249388), Aphis = *Acyrthosiphon pisum* (XP_001952629), Bflo = *Branchiostoma floridae (*XP_002597122), Mocc = *Metaseiulus occidentalis* (XP_003742843)

1.6. Research objectives

The objective of this project was to study and investigate the putative developmental role of Apox in *Tribolium,* with particular focus on the visual system. To this end, I started with examining the expression pattern of Apox in the embryo. As these experiments revealed that Apox was differentially expressed in the embryo, indicative of a pleiotropic developmental gene, I further investigated its role in the embryo by gene knockdown. The phenotypic consequences confirmed the requirement of Apox for the normal development of many developing structures in the embryo including the visual system.

To study the role of Apox in the development of the adult eye, I examined the expression pattern of Apox in the pupal head by whole mount *in situ* hybridization. These experiments revealed broad and dynamic expression of Apox in the differentiating retina. To elucidate the function of this gene during pupal development and adult eye development in *Tribolium,* I performed gene knockdown using the larval RNAi protocol. These experiments revealed that Apox is required for the survival of retinal tissue after onset of differentiation and also generally in tissues which experience high amounts of proliferation and differentiation during pupal development. To test whether these phenotypes were due to a role of Apox specifically in the regulation of PCD, I studied the effects of donwregulating the activity of initiator caspase genes on the Apox KD phenotype. These experiments produced unambiguous evidence that Apox represents a novel regulator of PCD in insect development.

2. Materials and Methods

2.1. Animals

The Banos-Ecuador wildtype (WT) strains of *Tribolium castaneum* or the inbred Georgia-I (GA-I) strain were used for cloning, KD experiments and *in situ* hybridization. Additional knockdown experiments were performed using pearl pBac (3XP3-EGFP) transgenic *Tribolium castaneum* (Horn and Wimmer 2000)(Lorenzen, Brown et al. 2002). All the stages of *Tribolium* were kept in constant darkness at 31^{o}C. Animals were maintained in 5% yeast and 0.5% fumagillin enriched whole wheat flour or unbleached white flour for egg collection.

2.2. Molecular Biology

An RT-PCR fragment of 750 bp of Apox was amplified using gene specific primers that included the highly conserved zinc finger region and the PR region (Supplementary table). Total RNA was extracted from 0-48 h pupal heads using the RNAqueosTM-R midi kit (Ambion). Template cDNA was generated by reverse transcription with the RetroscriptTM-R kit (Ambion). PCR amplification (Appendix D Primers) was carried out in an Eppendorf Mastercycler ep Gradient 5341. Both primary and secondary PCR reactions were performed with Taq polymerase (Fisher) using the same cycle conditions: (1) initial denaturation step: 2 mins at 95^{o}C, (2) denaturation: 30 sec at 94^{o}C, (3) annealing: 40 sec at 40^{o}C, (4) elongation: 60 sec at 72^{o}C. Amplification steps 2 to 4 were repeated for 35 cycles and final elongation was carried out for 2 mins at 72^{o}C. The amplified region was confirmed by gel electrophoresis and the PCR product was cloned into pGEM-T (Promega).

2.3. RNA interference

Double-stranded RNA of 750 bp was synthesized from the plasmid by bidirectional *in vitro* transcription using the MegaScript T7 transcription kit (Ambion). Larval and parental RNAi were performed following published protocols (Brown, Mahaffey et al. 1999; Bucher, Scholten et al. 2002; Tomoyasu and Denell 2004). Fifth instar stage larva were injected to induce gene knockdown in the pupae and adults. For parental RNAi the dsRNA was injected into the adult female instead of late female pupae. The adult females were anaesthetized using CO_2 and placed laterally on a slide with double stick tape. Once the dsRNA was injected, the animals were kept in whole wheat flour for 24 h for recovery.

2.4. Whole mount in situ hybridization

Whole mount *in situ* hybridization on embryo and postembryonic pupal tissue was performed with a digoxigenin-labeled RNA probe as published with the following modifications (Friedrich and Benzer 2000). For probe synthesis, template DNA was generated by PCR amplifications of a region resulting in a product of approximately 530 bp. The PCR product was purified using a MiniElute PCR purification kit (Qiagen) and used as a template to generate digoxigenin-labeled RNA probe (Roche). Postembryonic tissue was incubated with the probe at 55 C for 48 h following 48 h incubation with hybridization buffer. Antibody blocking and incubation was carried out for 2 h each at room temperature followed by three to four 30 minute washes with maleic acid buffer (+ 0.1% Tween20) and overnight wash.

2.5. Microscopy

Images of pupae and adults were taken with a Leica MZ16 A microscope coupled to a Leica DFC490 camera. Postembryonic tissue and embryos labeled by *in situ* hybridization were examined with a Zeiss Axioplan microscope and images were taken using a SPOT RT digital camera (Diagnostic instruments Inc.).

For confocal microscopy of adult eyes, the adult heads were dissected and fixed in 4% formaldehyde/PBT for 20 minutes and mounted ventrally. Confocal images were collected on a Leica TCS SP2 laser scanning confocal microscope and processed with the Leica Confocal software package.

3. Results

3. 1. Embryonic expression of Apox

To explore the role of Apox in embryonic development of *Tribolium,* I studied its expression by whole mount *in situ* hybridization on embryos. Earliest expression was seen in the early germ band elongation stage, mainly in the developing stomodeum and antennae (Fig. 5b). There was also incipient expression in the thoracic appendages (Fig.5a). In the late germ band elongation stage, there was strong expression in the visual anlage, mandible, appendages and the central nervous system (Fig. 5c and d). During germ band retraction, all the expression domains persisted. Apox was also expressed in the protocerebrum and the optic lobe anlage during early and late germ band retraction (Fig. 5e-h). These findings indicated that Apox had multiple expression domains in the developing head and body of *Tribolium* consistent with a function as developmental regulator.

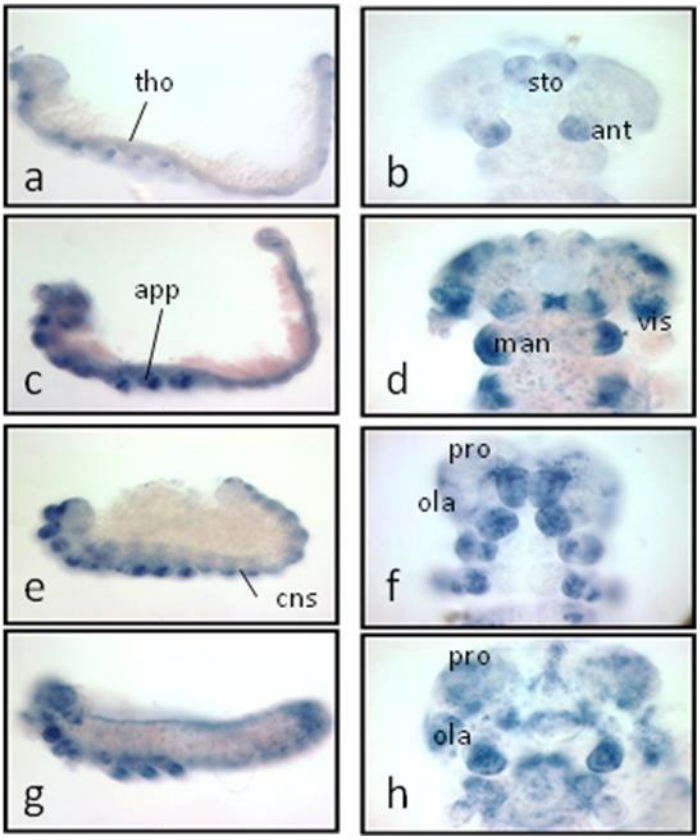

Fig 5: Embryonic expression of *Apox* in *Tribolium*. (a,c,e,g) Lateral views (anterior-left, ventral at top) and (b,d,f,h) ventral views (anterior at top) of *Tribolium* embryos stained by whole mount *in situ* hybridization for expression of Apox. (a,b) Early germ band elongation stage. (c,d) Late germ band elongation stage. (e,f) Early germ band retraction stage. (g,h) Late germ band retraction stage. Abbreviations: tho = thorax, sto = stomoteum, ant = antenna, app = appendage, man = mandible, vis = visual anlage, cns = central nervous system, pro = protocerebrum, ola = optic lobe anlage.

3.2. Embryonic knockdown of Apox

Given the differential expression in the embryo, I investigated the role of Apox during embryonic development by gene knockdown using the parental RNAi protocol (Bucher, Scholten et al. 2002). I injected Apox dsRNA at a concentration of 1 ug/ul and 2 ug/ul (Fig. 6). 5% of the first instar larvae resulting from dsRNA injection at 1 ug/ul concentration exhibited informative phenotypes. 23% of these affected animals had larval eyes displaced into the antenna (Fig. 6e). These animals also had deformed antenna. Some phenotypic larvae also showed defective mandible (Fig 6d). This phenotype corresponded to the expression of Apox in the mandible at the germ band retraction stage (Fig. 5d and f). Some larvae (11%) had no eyes or large eyes with three to four stemmata (Fig. 6b, c). 20% of the eggs from animals injected with 1 ug/ul concentration remained unhatched.

The percentage of affected larvae resulting from injection of Apox dsRNA at 2 ug/ul was higher (Table 1). 61% of the affected animals had large eyes compared to 11% with 1 ug/ul dsRNA concentration (Fig. 6 g). Also, additional abnormalities were detected at 2 ug/ul concentration. Some larvae hatched with apparent black pigment in the head, abdomen and thoracic segment and with disarranged stemmata suggesting cell death in these regions (Fig. 6d, f, i). 42% of the eggs remained unhatched revealing higher embryonic lethality compared to injection at 1 ug/ul (Table 1). The high lethality rate of the eggs collected from animals injected with 1 ug/ul and 2 ug/ul Apox dsRNA indicates that Apox plays an important role during normal embryonic development. No such phenotypes were observed in WT larvae. Taken together, these results revealed that Apox played important roles in the development of the larval head and potentially other regions consistent with its complex expression pattern. Moreover, the

pigmentation phenotypes resulting from injection of Apox dsRNA at 2 ug/ul indicated a possible role in cell survival.

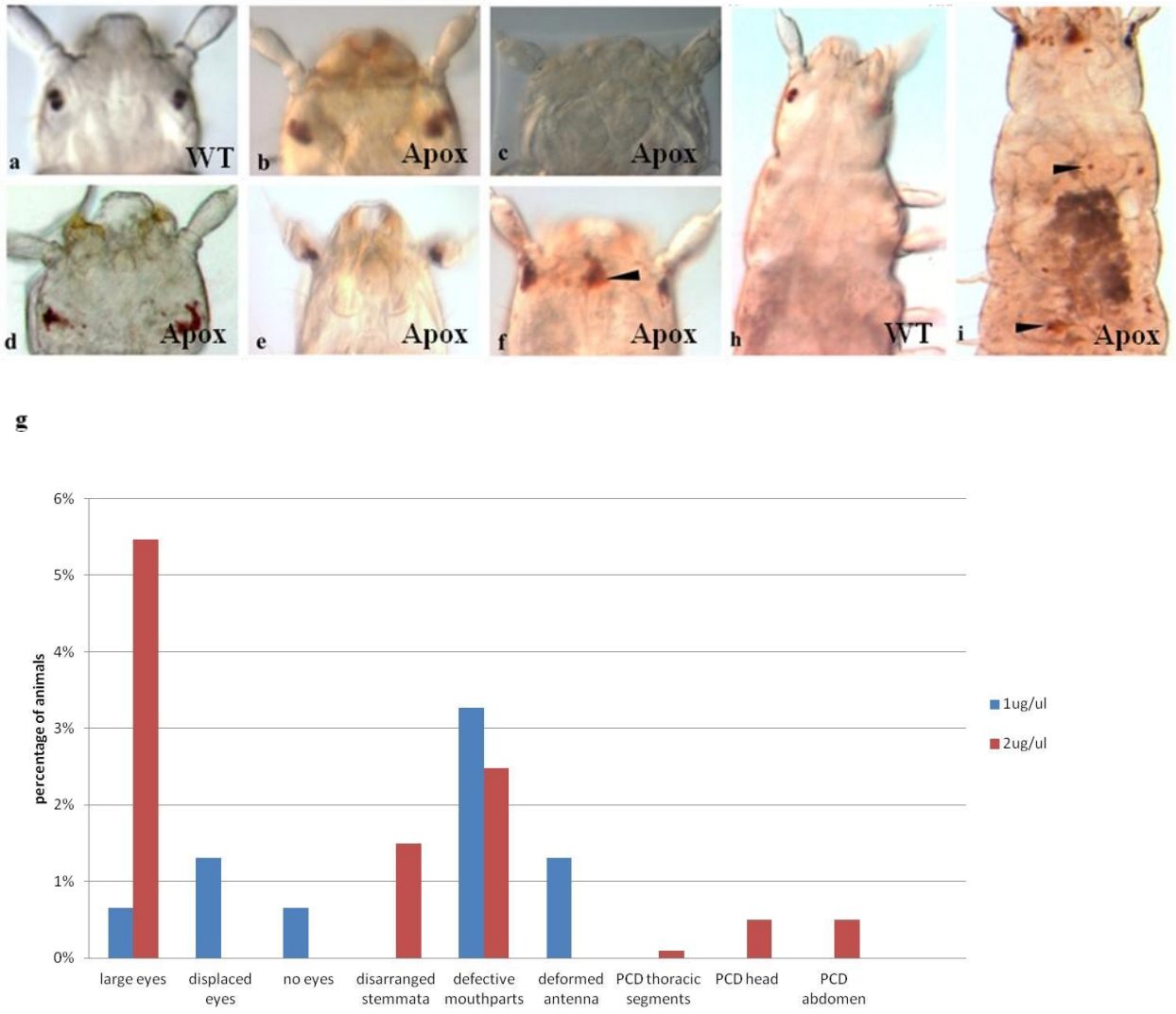

Fig. 6: Effect of Apox knockdown in *Tribolium* larvae. (a-f) Dorsal view of first instar larval head. (a) Wildtype. (b-f) Apox knockdown phenotypes showing examples with (b) bilaterally enlarged eyes, (c) eyes missing, (d) disarranged stemmata and reduced mandibles, (e) displaced eyes and deformed antenna, (f) cell death in the head (arrow head). (g) Quantitative analysis of phenotype frequency in 1 ug/ul and 2 ug/ul Apox dsRNA knockdown experiments. Y–axis: percentage of phenotypic animals, X-axis: phenotypes. (h and i) Upper body of first instar larvae. (h) Wild type. (i) Apox knockdown phenotype with darkened tissue spots in thoracic and abdominal segments (arrow heads).

Summary of embryonic KD of Apox				
	1ug/ul	2ug/ul	% 1ug/ul	% 2ug/ul
Number of eggs harvested	382	345		
Number of larvae hatched	305	201	79.84%	58.26%
Number of egg unhatched	77	144	20.15%	41.73%
Number of normal larvae	288	183	94.42%	91.04%
Number of phenotypic larvae	17	18	5.57%	8.95%

Table 1: Summary of embryonic KD of Apox

3.3. Postembryonic expression of Apox

To explore the role of Apox in the development of the *Tribolium* adult eye, I studied the expression pattern in the late larval and pupal head by whole mount *in situ* hybridization. In the early pupal stage at 24 h APF, expression of Apox began in the developing retina (Fig. 7a). At the same stage, low expression was seen in the antenna but no expression in the mouthparts. Segmental expression in the antenna became prominent in the late pupal stages. At 48 h APF, Apox transcript continued to be detectable at low levels in the developing retina. A sharp decrease of expression was notable in the peripheral margin of the eye (Fig. 7b). However Apox was also expressed strongly in the adjacent head epidermis and the gena. Taken together, these patterns suggested that Apox gene might be specifically involved in the development of adult eye, antenna and gena.

3.4. Effect of postembryonic Apox knockdown on pupal development

To investigate the role of Apox during pupal development in *Tribolium,* I initiated knockdown by injecting 1 ug/ul of Apox dsRNA into pre-metamorphic fifth instar larvae, which were actively moving and feeding. 66% of 112 injected animals died during subsequent larval

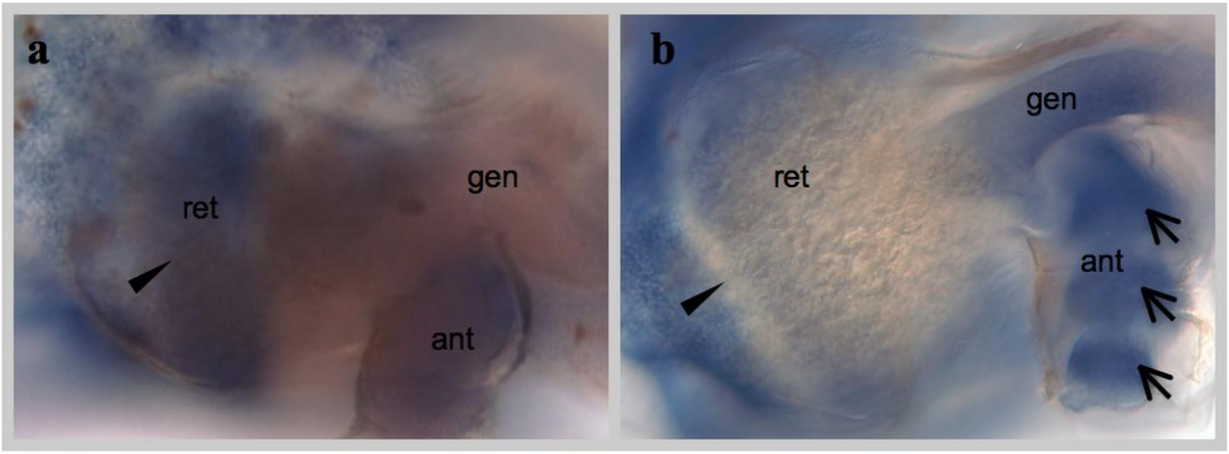

Fig. 7: Postembryonic expression of Apox in *Tribolium*. (a) Lateral (anterior to right) view of 24 h old pupa. Arrowhead indicates expression in the developing retina. (b) 48 h old pupa. Arrowhead indicates sharp decrease of expression in the eye periphery. Arrows indicate segmental expression in the antenna. Abbreviations: ant = antenna, gen = gena, ret = retina.

development (Table 2). This compared to 4% of 50 animals control-injected with EGFP dsRNA. Further, 71% of the 38 pupae resulting from Apox dsRNA injection died during pupal development compared to 4% in the EGFP dsRNA control-injected animals (Table 2). The increased lethality of Apox KD larvae and pupae indicated a strong impact of *Tribolium* Apox on pupal development.

Consistent with this, many of the Apox KD pupae exhibited blackening peripheral tissue areas, suggestive of necrosis or apoptosis (Fig. 8). The spatial distribution of these areas varied within and between areas. However, the survey of 38 phenotypic Apox dsRNA injected animals identified a reproducible range of tissues that were prone to develop necrotic or apoptotic areas. This included the wing appendages (13%) (Fig.8b, 8g), frontal head epidermis (24%) (Fig.8c, 8i), walking appendages (11%) (Fig.8d, 8h) and the terminal abdomen (37%) (Fig.8e, 8j). All pupae which developed local apoptosis or necrosis failed to complete metamorphosis. No such phenotype was seen in the EGFP dsRNA control-injected animals.

3.5. Effect of postembryonic knockdown of Apox on pupal eye development

Close inspection of the developing eye, revealed evidence of mild to severe patterning defects during early development of adult eye in Apox KD animals (Fig. 9). In 26% of the 38 pupae, the developing photoreceptor clusters formed an irregular pattern compared to WT animals (Fig. 9b). In 37% of 38 pupae, the area of the differentiating retina was strongly reduced and many developing ommatidia appeared to be dislocated into the gena and the antenna (Fig. 9c). All of these Apox KD phenotypic pupae failed to hatch into adults. No such phenotype was seen in the EGFP dsRNA control-injected animals.

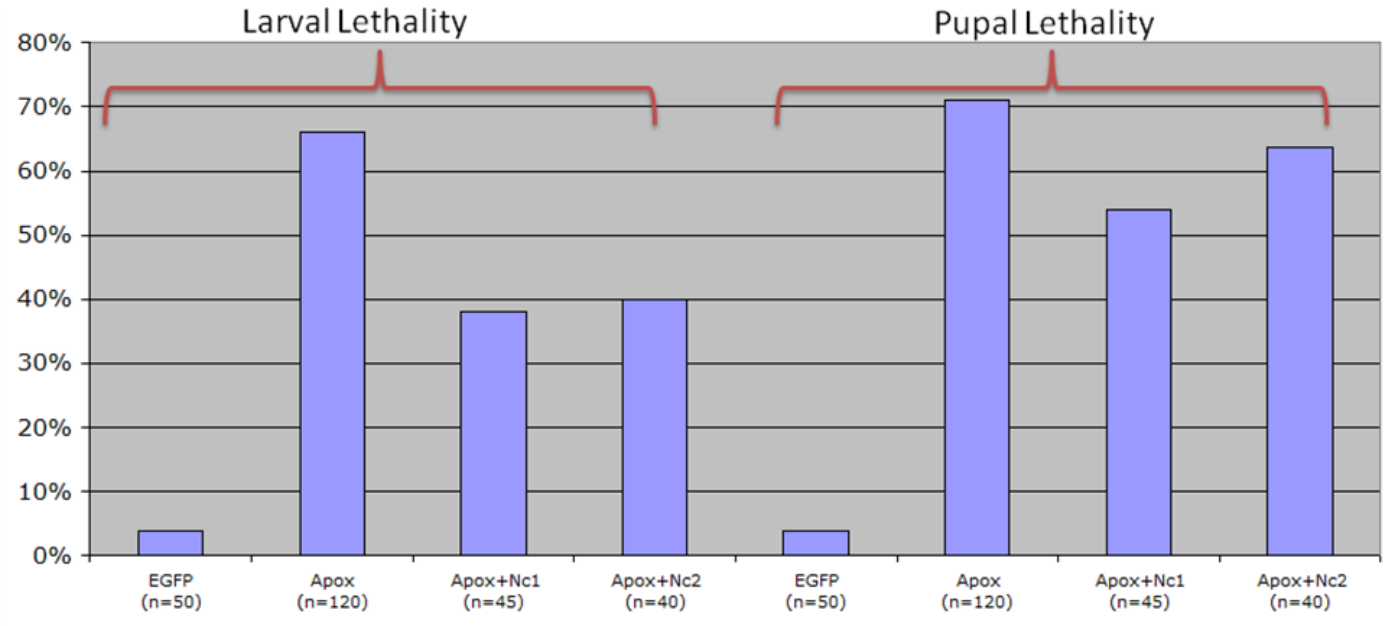

Table 2: Lethality chart of combinatorial and single KD of Apox, Nc1 and Nc2

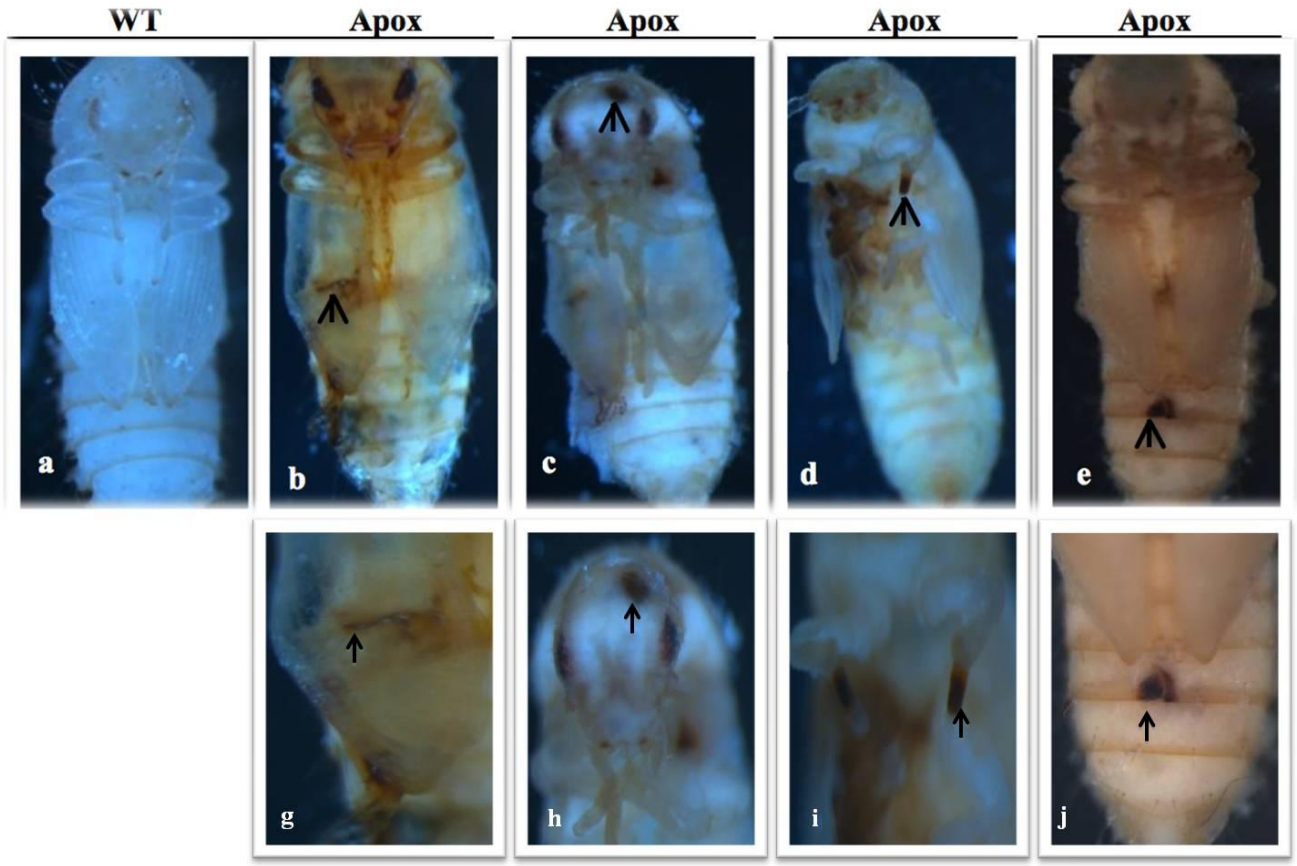

Fig. 8: Effect of Apox knockdown on pupal body development in *Tribolium* .Ventral view of 48 h pupae. (a-e) Overall body view of the pupae at lower magnification (30X) a) WT (b-e) Apox KD pupae. (f-j) Higher magnification (65X) (g) wing in Apox KD pupa (h) head in Apox KD pupa (h) appendages in Apox KD pupae (j) abdomen in Apox KD pupa. Arrow heads indicate blackening of tissue in Apox KD pupae

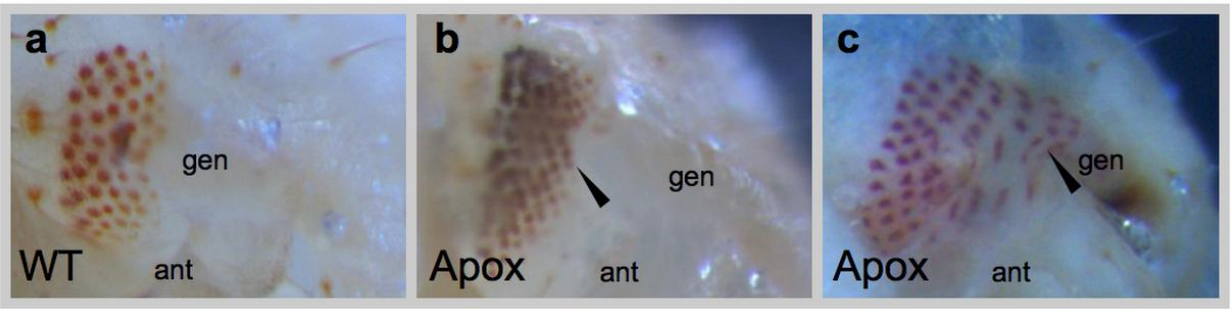

Fig. 9: Effect of Apox knockdown on *Tribolium* pupal eye development. (a-c) Lateral view (anterior to right) of the 48 h pupal head. (a) WT. (b) Densely packed ommatidia irregularly arranged in the retina of Apox KD pupa (arrowhead). (c) Ommatidial dislocation into the gena in Apox KD pupa (Arrowhead).

3.6. Effect of Apox knockdown on adult eye morphology

Based on external morphology, three out of the 11 Apox KD adult animals exhibited an eye phenotype (Fig. 10). These three phenotypic adults had unusual clear unpigmented ommatidia in the center of the eye (Fig. 10b). Moreover, these adults were unable to completely shed the pupal cuticle (not shown). Further inspection revealed that these individuals were also characterized by significant shortening of the antenna due to the lack of proximal segments (not shown). These antennal defects were also detected in five additional individuals, which hatched normally from the pupa. The eye size is measured by counting the number of ommatidia per eye in adult animals. Comparing average eye size in the affected Apox KD individuals with EGFP dsRNA control-injected animals revealed a mild but significant reduction of eye size in the Apox KD specimens (Fig. 10e). The latter were characterized by an average of 87.2 (+/-3.1) ommatidia compared to 95.4 (+/-4.5) in the control.

3.7. Effect of Apox knockdown on adult photoreceptor development

To determine if the clear ommatidia in the phenotypic Apox adult animals were due to cell death or loss of pigmentation I injected Apox dsRNA into the transgenic strain 3XP3-EGFP (Horn and Wimmer 2000; Lorenzen, Brown et al. 2002). In untreated animals of this strain, the photoreceptor cells are labeled by strong expression of EGFP (Fig. 11e). Similar to the GA1 Apox KD animals, 27% of the Apox KD 3XP3-EGFP animals exhibited cuticle shedding defects (Fig. 11b). Investigation with confocal microscopy revealed that the latter lacked above background GFP signal in the retina in contrast to the strong expression of EGFP in the adult photoreceptors of the 3XP3-EGFP strain (Fig. 11e and f). This finding revealed the absence of photoreceptor cells in Apox KD animals despite the formation of lens structures.

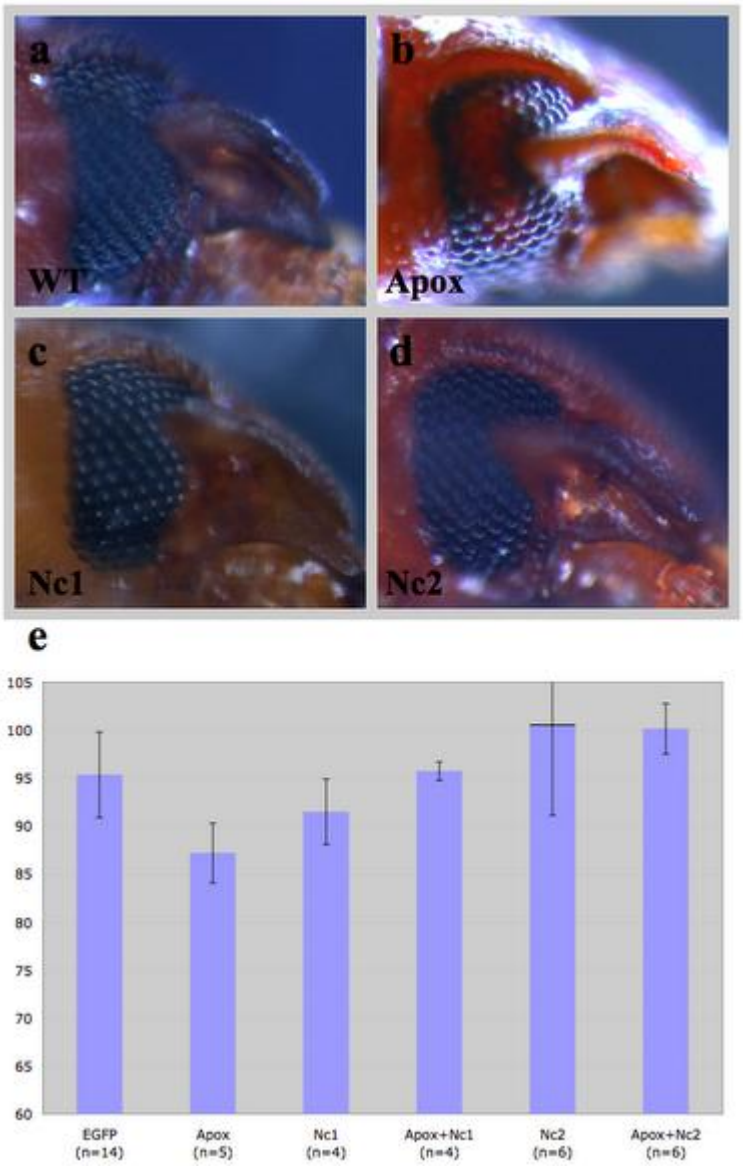

Fig. 10: Effect of Apox knockdown on adult eye morphology of *Tribolium* (a-d) Lateral view of adult head in (a) WT and (b) Apox knockdown phenotypic animal. (c) Nc1 knockdown animal. (d) Nc2 knockdown phenotypic animal. (e) Quantitative eye size comparison. Y-axis is number of ommatidia. X-axis- dsRNA injected.

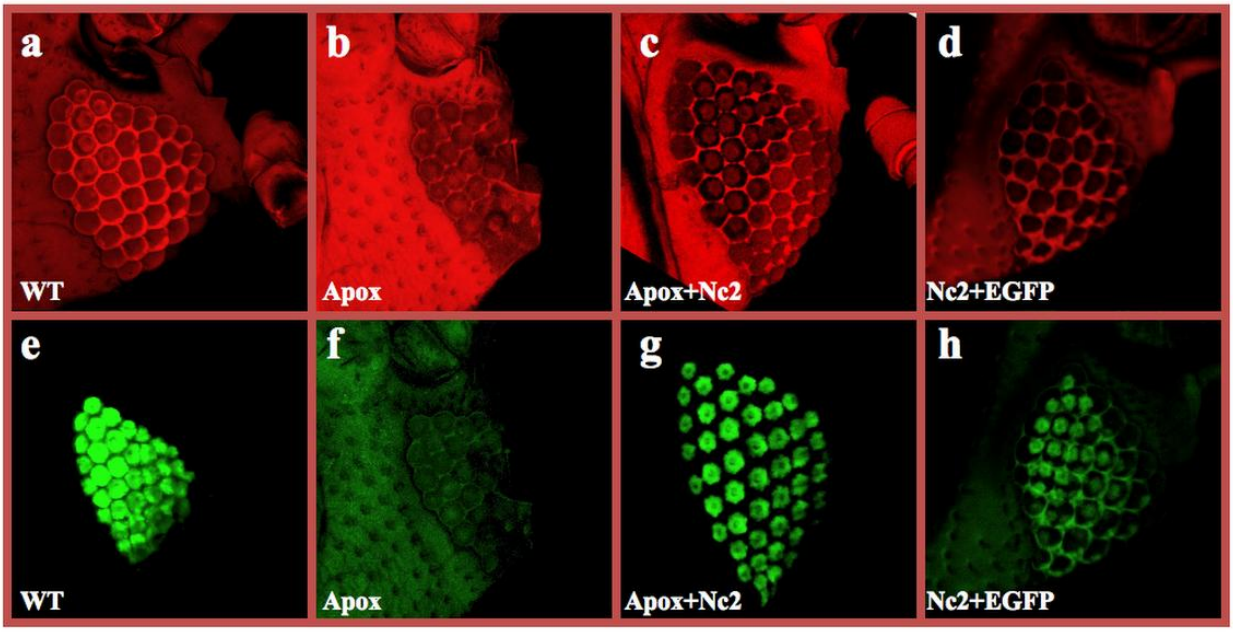

Fig. 11: Effect of Apox knockdown on adult photoreceptor survival in *Tribolium* (a-h) Laser scanning confocal image of the ventral adult eye in (a,e) the 3XP3 transgenic strain and (b-d, f-h) knockdown specimens. (a-d) Auto-florescence from the head cuticle. (e-h) GFP signal from photoreceptor cells. Note that in (f) there is no detectable GFP signal from the photoreceptor cells above background and in (h) sensitivity has been increased above background for detection of GFP signal from photoreceptor cells. (a, e) WT. (b, f) Apox KD. (c, g) Apox+Nc2 KD. (d, h) Nc2+EGFP KD.

To investigate whether the photoreceptors underwent cell death after the differentiation of the cone cells, which form the facet lenses during *Tribolium* eye development, three Apox KD 3XP3-EGFP pupae were tracked for live GFP signal in the photoreceptor cells every 24 hours following injection (Fig. 12). At 72 h APF, the GFP signal was weak in the photoreceptor cells (Fig. 12g). At 120 h APF, it had completely disappeared in all three individuals (Fig. 12h).

Taken together, the KD analysis results revealed that Apox was essential for photoreceptor survival after completion of cone cell differentiation and facet formation during normal eye development.

3.8. Effect of initiator caspase knockdown on Tribolium adult eye morphology

The pupal phenotypes of Apox KD animals suggested that Apox might be involved in blocking PCD during normal *Tribolium* development. If true, the Apox KD-elicited triggering of PCD should be mitigated or blocked by simultaneous KD-mediated downregulation of PCD. To be able to test for this possibility, I first examined the effect of downregulating PCD in *Tribolium*. This was attempted by knocking down the *Tribolium* orthologs of the *Drosophila* initiator caspase Nc (Dorstyn, Colussi et al. 1999).

The *Tribolium* genome contains two orthologs of Nc: Nc1 (Gene id: 189241132) and Nc2 (Gene id: 282165746). dsRNAs were prepared for both genes and injected into active last instar larvae to elicit postembryonic KD. These experiments resulted in 60% viable adults in the Nc1 KD and 55% viable adults in the Nc2 KD experiments (Table 2). To probe for phenotypic evidence of PCD block as a consequence of Nc1 and Nc2 KD, I investigated average eye size (Fig. 10e). On average adults, hatching from the larvae injected with Nc1 dsRNA had eye size slightly smaller (91.5+/-3.4) than control injected animals (94.5 +/- 4.5). However, adults

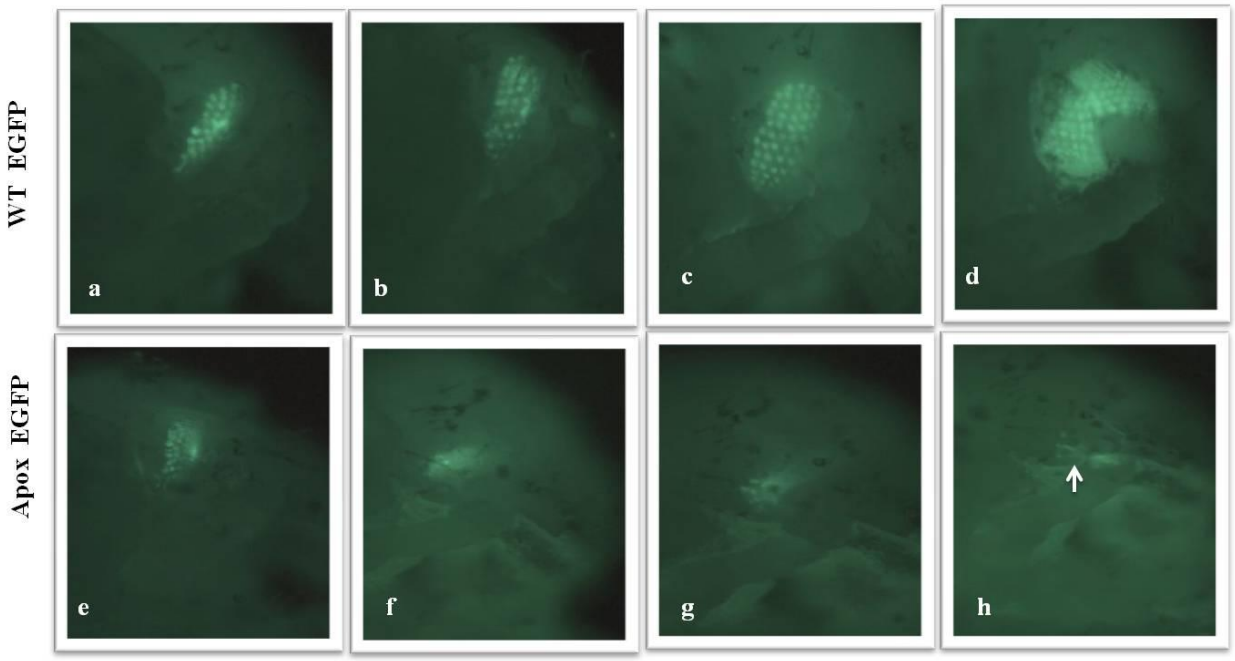

Fig. 12: Photoreceptor cell death in Apox KD animals. Live GFP tracking in (a-d) WT 3XP3-EGFP transgenic pupae and (e-h) Apox KD 3XP3-EGFP pupae at (a, e) 24 h APF, (b, f) 48 h APF, (c, g) 72 h APF and (d, h) 120 h APF). Arrow indicates complete disappearance of GFP signal.

resulting from Nc2 dsRNA injection had 5% higher number of ommatidia (101.5+/-2.6) than control injected animals. The significantly enlarged eyes of Nc2 KD animals were consistent with the predicted effects of increased cell survival as a consequence of PCD block.

3.9. Effect of combinatorial knockdown of Apox and initiator caspases on postembryonic development

To test for the possible PCD-specific regulatory function of Apox, I performed double KD experiments employing 1 ug/ul concentration of dsRNA per gene. If the downregulation of Apox caused cell death specifically by PCD, the simultaneous downregulation of the PCD initiator caspases Nc1 or Nc2 was expected to relieve or completely abolish the Apox KD mediated increase in cell death.

38% of 45 animals coinjected with Apox and Nc1 dsRNA died during larval development. Of these, 54% pupae died during pupal development. 36% of animals coinjected with Apox and Nc2 dsRNA died as larvae. Of these, 64% pupae resulting from Apox+Nc2 KD died during pupal development. In both cases, the survival of double knockdown in *Tribolium* larvae and pupae was notably higher compared to Apox single KD (Table 2).

Apox+Nc1 KD (n=10) and Apox+Nc2 KD (n=18) were examined for the presence of the blackening tissue areas characteristic of Apox single KD animals. None of these examined double KD pupae exhibited detectable darkening tissue areas (not shown). Second, no evidence of ommatidial disarrangement or displacement could be detected in contrast to the single Apox KD animals (not shown). The apparent rescue of the Apox KD phenotype by simultaneous downregulation of either of Nc1 or Nc2 was consistent with the hypothesis that Apox functioned specifically by antagonizing PCD during normal development.

An alternative explanation for the lack of pupal tissue degeneration in the double KD animals was that double injection of dsRNA resulted in the reduced efficiency of each single dsRNA species by limiting the amounts of RNAi machinery proteins for each of the two gene specific dsRNAs. To control for indirect dosage effects, I repeated the experiment co-injecting EGFP dsRNA at 1 ug/ul in the single KD experiments targeting Apox or Nc2 to normalize the total amount of injected dsRNA in single and double KD experiments.

45% of the animals injected with Apox+EGFP hatched into pupae. Of these 36% hatched into adults, whereas with injection of Apox alone 34% of animals hatched into pupae and 29% hatched into adults (not shown). The survival of Apox+EGFP KD animals was thus mildly higher than animals injected with Apox alone, consistent with the prediction that the coinjection of EGFP dsRNA compensates for the RNAi protein machinery limitation effect in double KD experiments. Further consistent with this, no evidence of apoptosis could be detected in mouthparts or walking appendages (Fig. 13A). Importantly, however, the injection of Apox dsRNA together with EGFP dsRNA at 1 ug/ul still resulted in pupae with blackening tissues in the head area (20%), the abdomen (20%) and the wing appendages (6%) (Fig. 13B). Overall, these findings corroborated the conclusion that the rescue of the Apox KD phenotype in the animals coinjected with Nc1 dsRNA or Nc2 dsRNA was due to the simultaneous downregulation of initiator caspase activity.

3.10. Effect of combinatorial knockdown of Apox and initiator caspases on adult eye morphology

Adult animals resulting from the injections with both Apox+Nc1 dsRNA or Apox+Nc2 dsRNA were further analyzed for the patterning defects and evidence of photoreceptor cell death

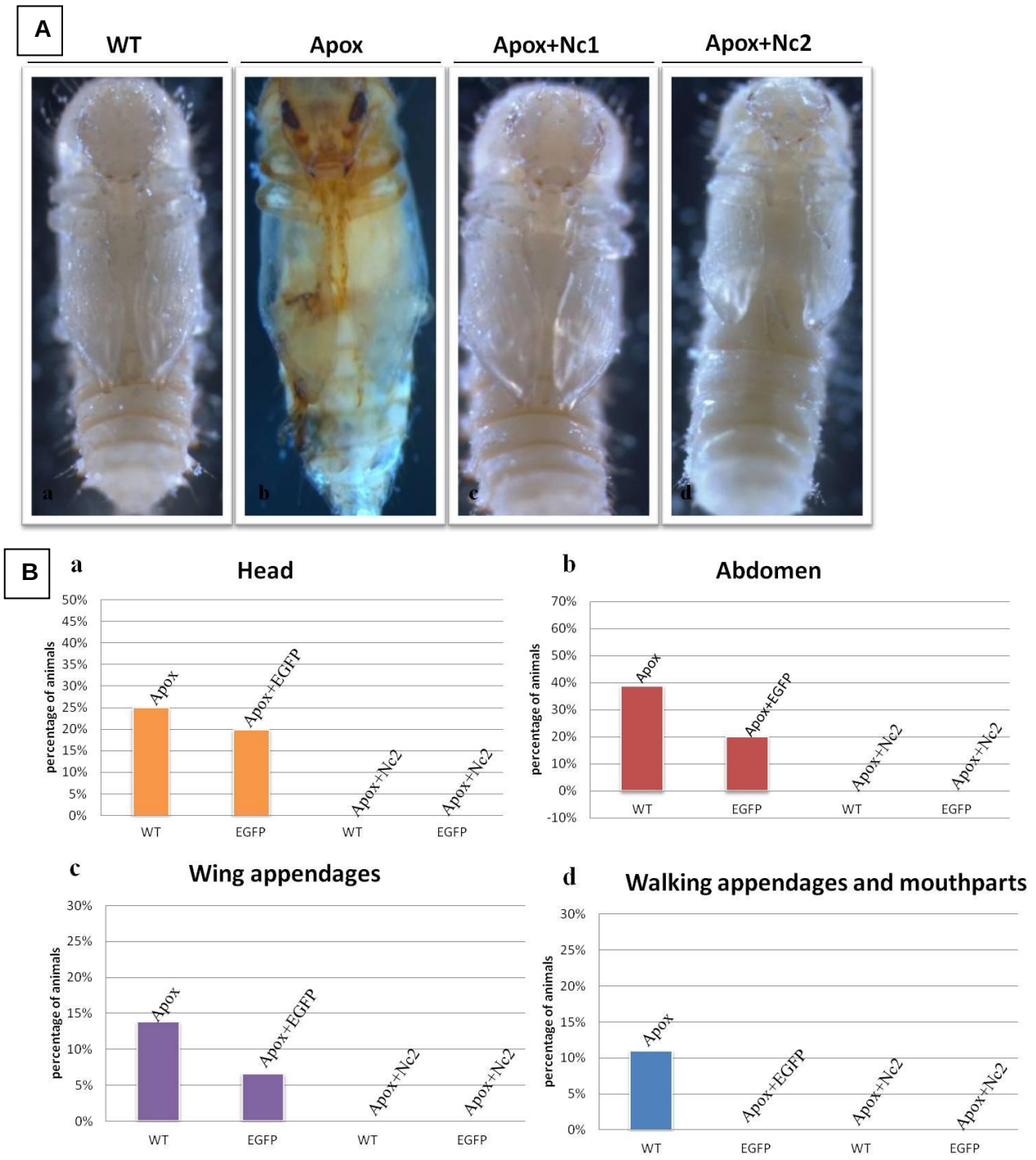

Fig. 13A: Effect of double knockdown of Apox and Nc1 or Nc2 dsRNA on Tribolium. Ventral view of 48 h old pupa. (a) WT, (b) Apox KD, (c) Apox+Nc1 KD and (d) Apox+Nc2 KD **B. Quantitative analysis of phenotype frequency in double KD and single KD experiments in WT and 3XP3-EGFP transgenic strain**.Y-axis shows percentage of pupae with blackening of tissue in (a) head, (b) abdomen, (c) wing appendages and (d) walking appendages and mouthparts. X-axis shows the *Tribolium* strain used.

in the developing eye (Fig. 10). Consistent with the rescue of the pupal Apox KD phenotype by Nc1 or Nc2 dsRNA co-injection, the pupal Apox KD eye phenotype was also completely abolished in Apox+Nc1 and Apox+Nc2 KD animals (Fig. 10c and d).

Further, the quantitative analysis of eye size revealed no difference of eye size between Apox+Nc1 KD animals (95) and control injected animals, suggesting a weakening effect of Nc1 KD on the Apox KD eye size-reducing effect (Fig. 10e). Animals hatching from double injection of Apox+Nc2 dsRNA exhibited a very similar increase in average eye size like Nc2 single KD animals: 101+/-2.6 compared to control injected animals (Fig. 10e). This result revealed that while the Nc2 KD ameliorated the effects of Apox KD, the reverse was not the case, which indicated that Apox was not antagonizing PCD by directly affecting the expression or activity of Nc2 during normal development.

3.11. Effect of combinatorial knockdown of Apox and initiator caspases on adult photoreceptor survival

Further informative differences between the adult eye phenotypes emerged by confocal image analysis in the transgenic strain 3XP3-EGFP *Tribolium* animals injected with Nc2+EGFP, Apox+EGFP and Apox+Nc2. (Fig. 11). As in the case of adult animals phenotypic for the Apox KD (Fig. 11), Apox+EGFP double KD animals exhibited a reduced field of facets without detectable photoreceptors (not shown). In Nc2+Apo double KD adults, however, a large eye area with intact photoreceptor clusters per ommatidium was detectable (Fig. 11c and g), consistent with a rescue of photoreceptor apoptosis by the reduction of caspase activity.

Interestingly, the distance between the photoreceptor clusters of neighboring ommatidia was more pronounced than in untreated animals (compare Fig. 11 e and g). The same was true

for the adult eye of Nc2+EGFP KD animals (Fig. 14h). The consistently wider interommatidial space in both KD experiments that targeted the Nc2 homolog suggested that this additional phenotype was most likely due to the differentiation of surplus accessory cells a consequence of the suppression of PCD.

4. Discussion

4.1. Apox suppresses programmed cell death during Tribolium development

In this study I show that the postembryonic knockdown of the zinc finger transcription factor gene Apox leads to blackening in external tissues of the pupal body. This effect could be due to cell death via caspase-dependent or caspase-independent mechanisms. The effect of Apox KD on pupal development was rescued by simultaneous downregulation of the initiator caspases Nc1 or Nc2, which facilitate the execution of the PCD pathway. This finding provided the key evidence that Apox antagonizes PCD to suppress cell death in specific pupal tissues.

The suppression of PCD by Apox during postembryonic and embryonic development is spatially regulated. During the development of the *Tribolium* pupa, Apox prevents cell death in tissues including head epidermis, wing and walking appendages, mouthparts and terminal abdomen. During larval development, Apox appears to suppress cell death in head, thoracic and abdominal segments. I speculate that these Apox KD-sensitive tissues undergo extensive cell proliferation and differentiation during metamorphosis in *Tribolium*.

Consistent with this, previous studies in other holometabolous insect species have shown that cell proliferation and PCD play important roles during morphogenesis. Previous investigation of wing development has shown that, during pre-pupal development, wing disc of

holometabolous insects rapidly differentiate and proliferate (Fristrom and Fristrom 1993; Fujiwara and Hojyo 1997). Studies in the pupal wing development of Lepidoptera have shown that PCD occurs in the pupal wing periphery shaping the outline of the adult wing in moths and butterflies (Dohrmann and Nijhout 1988; Kodama et al. 1995).

In *Drosophila*, hormonal signals trigger the destruction of obsolete larval tissue at late larval stage (Jiang, Baehrecke et al. 1997). The abdominal epidermis forms from small groups of cells called histoblasts, which are present in the larval midgut. During abdominal morphogenesis, dynamic tissue rearrangements take place, which lead to apoptosis of 10-20% of histoblasts (Bischoff and Cseresnyes 2009). 12 h after the onset of metamorphosis, another wave of hormonal activation causes destruction of larval salivary glands (Jiang, Baehrecke et al. 1997). Along with apoptosis, extensive cell proliferation, cell elongation and differentiation take place in the wing imaginal cells, leg imaginal cells and the larval epidermis during insect metamorphosis (Fristrom 1972).

Interestingly, the steroid hormone 20-hydroxyecdysone (ecdysone) is responsible for regulating differentiation, tissue remodeling and programmed cell death in order to completely transform the larvae into a mobile adult (Baehrecke 2000). Ecdysteroid and juvenile hormone are the effector hormones which control transition from larva to pupa and subsequently the adult in holometabolous insects (Bollenbacher, Smith et al. 1981; Jindra, Malone et al. 1996; Riddiford 1996). It is therefore reasonable to assume that some of the ecdysteroid signal dependent PCD events in *Drosophila* are evolutionarily related to patterning events in the *Tribolium* pupa.

4.2 Possible molecular mechanisms of Apox function in programmed cell death regulation

The sequence analysis of Apox revealed a putative SET domain along with five zinc finger domains (Fig. 4), identifying Apox as a member of the Prdm family of proteins (Keller and Maniatis 1991). This suggests that Apox acts through chromatin modification during transcriptional regulation. Apox might recruit histone methyltransferase (HMT) through the zinc finger domains. HMTs methylate arginine or lysine residues of histone H3, inducing repression of chromatin. This leads to a model in which apoptosis in specific tissues of the pupal body is prevented by Apox through transcriptional repression.

Further informative is the fact that the Nc2 KD mediated increase in eye size was not affected in the double KD experiments that simultaneously targeted Apox. This finding implies that Apox does not directly affect caspase transcription or activity during normal development. In combination, the data suggest that Apox represses pro-apoptotic target genes which modulate caspase activity.

4.3 The role of Apox and programmed cell death during Tribolium adult eye development

Two kinds of patterning defects were observed in the developing pupal eye of *Tribolium* due to Apox KD. In one case, ommatidia formed an irregular pattern compared to WT. In the other case, the differentiating photoreceptor clusters dislocated into the gena and the antenna. This is consistent with the expression of Apox in the early developing retina and later in the head cuticle. These findings together suggest that in the Apox KD pupal eye of *Tribolium*, mispatterning is due to increase in cell death in the developing retina and the surrounding head epidermis. All these strongly phenotypic animals show pupal lethality due to overall increase in cell death.

In the developing adult eye, Apox KD leads to photoreceptor cell death during late differentiation after the eye facets have been formed. The clear retina in the Apox KD adults further indicates the occurrence of PCD in the pigment cells. These findings are consistent with the expression Apox in the early developing retina. This suggests that Apox promotes normal eye development by suppressing pigment and photoreceptor cell death in *Tribolium.*

This study reveals important functions of PCD during adult eye development in *Tribolium*. The KD data show that anti-apoptotic genes like Apox and pro-apoptotic genes like Nc1 and Nc2 regulate the final eye size and photoreceptor cell number in *Tribolium*. These functions are also required for the correct formation of a regular pattern of ommatidia in the compound eye.

Previous studies have shown that inter-ommatidial cells are eliminated in the developing *Drosophila* retina one day after pupation (Miller and Cagan 1998). Subsequently, ommatidia are removed from the edge of the eye by activation of pro-apoptotic genes hid, *grim and reaper* through Wg signaling (Lin, Rogulja et al. 2004). The function of programmed cell death and proper patterning of the eye is well understood in *Drosophila*. I propose that PCD occurs during the following processes during normal eye development in *Tribolium*. The first one is removal of surplus interommatidial cells across the eye. After the photoreceptor cells have been specified and differentiated, one round of apoptosis may take place to remove extra cone cells and pigment cells. At mid pupation (36-48 h APF), another round of programmed cell death may take place to remove incomplete ommatidia from the periphery of the eye. The latter is essential for proper patterning of the eye margin. It appears that these functions of apoptosis in Tribolium eye development are conserved in *Drosophila*. Further studies on the retinal structure and cell death pathways in *Tribolium* will shed light on functions of PCD in the adult eye development in this

important insect model. Interestingly, in zebrafish and mouse the disruption of Prdm1 family members leads to photoreceptor cell death (Wilm and Solnica-Krezel 2005; Briknarova, Zhou et al. 2008). It will be important to study in more detail how these functionalities are evolutionarily related.

APPENDIX A: BUFFERS AND SOLUTIONS

Table 1: Stock solution

Reagent	pH	Mw	Volume	Grams add	Notes
1M Tris-HCl	pH 8	121.14g/mol	500ml	For 500ml = 121.14/2= 60.5g in 400ml ddH2O	Adjust the pH with 1M HCl and then make up the volume to 500ml.
0.5M EDTA	pH 7.0-8.0	372.44g/mol	500ml	For 500ml= (372.44 X 0.5) /2=93.11g	Adjust the pH with 10N NaOH and it will dissolve only at pH 8. Then make up the volume to 500ml.
5M NaCl	N/A	58.44g/mol	500ml	For 500ml= (58.44X 5mol/L)/ 2 = 146.1g	Heat in the microwave for the salt to dissolve.
50X TAE	N/A	N/A	1L	Tris base 242g Glacial acetic acid 57.1ml 0.5M EDTA 100ml	After dissolving the Tris base make up the volume to 1L.
4M LiCl	N/A	42.39g/mol	50ml	For 50ml = (42.39g/mol X 5mol/L)/ 20 =10.60g	Dissolve 10.6g in 25ml ddH2O. Make up the volume and store in 100ml bottle. Autoclave and store at RT.
10%	N/A	N/A	10ml	Add 1ml	Keep at RT.

TritonX				stock solution to 9ml ddH2O	Do not autoclave.
10% Tween 20	N/A	N/A	10ml	Add 1ml stock solution to 9ml ddH2O	Keep at RT. Do not autoclave.
20% Tween	N/A	N/A	10ml	Add 2ml stock solution to 8ml ddH2O	Keep at RT. Do not autoclave.
1M MgCl2	N/A	203.30g/mol	100ml	For 100ml = (203.30g/mol X 1mol/L) / 10 = 20.33g	Do not autoclave.
10% SDS	N/A	N/A	500ml	For 500ml = 50g	Store at RT. Do not autoclave.

Table 2- TE (1X, 500ml)

Reagent	Stock	Volume added
10mM Tris-Cl	1M	5ml
1mM EDTA	0.5M	1ml
ddH2O		494ml

APPENDIX B*: IN SITU* HYBRIDIZATION BUFFERS

Table 1: SSC stock solution 20X, 1L, pH=7

Reagent	Amount added
NaCl	175g(3M final)
Trisodium citrate dehydrate	88g(3M final)
ddH2O	Adjust to 1 liter , Autoclave

Table 2: HybA-RNA buffer, 40ml

Buffer name	Reagent	Amount added
HybA-RNA I (for pupal heads)	Dextran sulphate	2g
	SSC 20X	8ml
	Denhardt's stock 20X	800ul
	Baker's yeast RNA stock 10mg/ml	1ml
	Formamide	20ml
	Tween 20 %	400ul
	Salmon sperm DNA 10mg/ml	2ml
	DEPC treated H2O	7.8ml
HybA-RNA II (for embryos)	Same as HybA-RNA I except do not add Dextran sulphate	

Note: Dissolve dextran sulphate in SSC and H_2O for 30min at 37^{o}C and then add all the other ingredients.

Table 3: HybB-RNA Buffer 40ml

Reagent	Amount added
SSC stock 20X	6ml
Formamide	15ml
Tween 20%	300ul
DEPC H2O	18.7ml

Table 4: 70% glycerol 50ml

Reagent	Amount added
Glycerol 100%	35ml
PBS 1X	15ml

Note: Mix thoroughly by flipping.

Table 5: PBS 1X, 50ml

Reagent	Amount added
PBS 10X stock	5ml
ddH2O	45ml

Table 6: PBT 1X, 50ml

Reagent	Amount added
PBS 1X	50ml
Triton X 0.1%	45ml

Note: PBS, PBT and H2O are DEPC treated using 1:1000 dilution. Add DEPC and shake on the shaker overnight and then autoclave.

Table 7: Fixation solution for Tcas thick Tissue (pupal heads)

Reagent	Amount added
Formaldehyde stock	2ml
0.5M EDTA pH=8	0.8ml
PBT,1X, DEPC treated	5.2ml
0.1% Triton X	80ul
DEPC H2O	1.92

Table 8: Fixation solution for Tcas embryos

Reagent	Amount added
PBS 1X DEPC treated	2ml
Formaldehyde stock	300ul
Heptane	6ml

APPENDIX C: REAGENTS AND KITS

Table 1: Reagents

Reagent	Product number	Vendor
Ampicillin solution	A5354	Sigma Aldrich
JM 109 competent cells *E. coli*	L2001	Promega
Ribonuclease	R6513	Sigma
PCR nucleotide mix,10X	C1141	Promega
Taq DNA polymerase	FB600045	Fisher Scientific
Go Taq Hotstart	9P1M512	Promega
1Kb DNA ladder	9P1G571	Promega
RNAase Inhibitor	N2111	Promega
10X PBS	BP3994	Fisher Scientific
Triton X-100	H5142	Promega
Tween-20	H5152	Promega
Ethanol 200 proof	2710	Decon Labs Inc
Methanol	A412-4	Fisher Scientific
Heptane	BP1115-500	Fisher BioReagents
Formaldehyde	F79-1	Fisher Scientific
Formamide	BP227500	Fisher BioReagents
DIG RNA labeling mix	1127073910	Roche Diagnostics
Anti-digoxigenin AP Fab fragment	11093274910	Roche Diagnostics
T7 RNA polymerase	AM2716	Ambion

Baker Yeast RNA	AM7118	Ambion
Salmon Sperm DNA	A2159,0005	AppliChem
DIG wash and Block Buffer set	11585762001	Roche Diagnostics
NBT	S380C	Promega
BCIP	S381C	Promega
Glycerol	BP229-1	Fisher Bioreagents
50X Denhardt's solution	750018	Invitrogen

Table 2: Kits

Kit	Product number	Vendor
MegaScript T7 High yield Transcription kit	AM1333	Ambion
MiniElute PCR Purification kit(50)	28004	Qiagen
RETROscript	AM1710	Ambion

APPENDIX D: PRIMERS

Primer name	Sequence
Tcas_nonamezf_A1	ATTGATAAGCTGGATGTCTACG
Tcas_nonamezf_B1	TGCGTCATTCGGTGGTAG
Tcas_nonamezf_A2:	TATTCGGCACCGTTTCGG
Tcas_nonamezf_T7_B2	TAATACGACTCACTATAGGGAAGGCTTTGTTGCATACTCCAC
Tcas_Nc2_A1	TTAAAACAGTGGTTAAGAAATTCTCG
Tcas_Nc2_B1	TATTGCATAATTAGTTTCGATCACG
Tcas_Nc2_A1T7	TGTAATACGACTCACTATAGGGCAAACAGTGGT
Tcas_Nc2_B1T7	TGTAATACGACTCACTATAGGGCTTGCATAATTAG
Tcas_Nc1_A1	CAGTTTTACGACACTGAAAGGTACA
Tcas_Nc1_B1	CTTTACTGAAGGGTGGCAGATG
Tcas_Nc1_A1T7	TGTAATACGACTCACTATAGGGGTTTTACGAC
Tcas_Nc1_B1T7	TGTAATACGACTCACTATAGGGTTACTGAAGG

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ABSTRACT

THE NOVEL PRDM GENE APOPTIX ANTAGONIZES PRORAMMED CELL DEATH IN *TRIBOLIUM*

by

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August 2012

Advisor: Dr.Markus Friedrich

Major: Biological Sciences

Degree: Master of Science

Previous studies in holometabolous insects have shown that programmed cell death and cell proliferation play important roles in insect metamorphosis. To elucidate the function of the newly identified *Tribolium* Prdm gene Apoptix (Apox), I performed a detailed analysis of Apox knockdown effects in the embryo, the pupa and the adult eye. My results revealed that Apox is required for the survival of retinal tissue after onset of differentiation and also generally in tissues which experience high amounts of proliferation and differentiation during pupal development. Further, combinatorial knockdown of Apox and initiator caspases produced evidence that Apox specifically protects from programmed cell death in *Tribolium* development. My results characterize a novel regulator of programmed cell death, which is highly conserved in arthropods but was lost during dipteran evolution.

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