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Research Article

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Overexpression of *Drosophila* RFX3 Induces Apoptosis and Interferes with Differentiation of Photoreceptor Cells

Toshimi Sugimoto[1], Takako Ueda[2], Hideki Yoshida[1,3], Akira Murakami[2] and Masamitsu Yamaguchi[1]*

[1]*Department of Applied Biology, Kyoto Institute of Technology, Japan*
[2]*Department of Bio-molecular Engineering, Kyoto Institute of Technology, Japan*
[3]*Insect Biomedical Research Center, Kyoto Institute of Technology, Japan*

Abstract

Regulatory Factor X (RFX) is a protein containing a characteristic DNA binding domain that is called RFX domain. Members of the RFX family are known to be present in human, mouse, *Drosophila, Caenorhabditis elegans, Schizosaccharomyces pombe* and *Saccharomyces cerevisiae.* Two RFX proteins, *Drosophila* RFX (dRFX) and dRFX2 have been identified in *Drosophila* so far. It is known that dRFX is involved in the differentiation of central and peripheral nerves, while dRFX2 is essential to the cell cycle progression and it may be involved in regulation of apoptosis. A novel protein, dRFX3 carrying RFX domain was identified by a *Drosophila* genome database search. dRFX3 is a likely homolog to the human RFX5, because of a significant sequence similarity in RFX domains between these two proteins. Two lines of transgenic fly carrying the HA-dRFX3 cDNA were established. Adult flies expressing dRFX3 specifically in the eye and the wing imaginal discs exhibited severe rough eye and atrophied wing phenotypes, respectively. 5-Bromo-2'-deoxyuridine incorporation assays and the immunological detection using anti-Cyclin B antibody indicated that the overexpression of dRFX3 in the eye imaginal discs exerted no effect on the cell cycle progressions. On the other hand, overexpression of dRFX3 in the eye imaginal discs interfered with the differentiation of R2/R5 photoreceptor cells and also induced apoptosis. These data suggest that the dRFX3 plays a negative role for the differentiation of R2/R5 photoreceptor cells and plays a positive role in the regulation of apoptosis.

Introduction

Regulatory Factor X (RFX) is a protein containing a characteristic DNA binding domain that is called RFX domain. Members of the RFX family are known to be widely present in eukaryotes [1,2], such as mammalian RFX1 to 5 in human and mouse, dRFX and dRFX2 in *Drosophila,* DAF-19 in *C. elegance,* Sak1 in *S. pombe,* and Crt in *S. cerevisiae.*

Drosophila RFX (dRFX) is homologous to human RFX1-3 (hRFX1-3) and to DAF-19 [3-5]. hRFX1 is a protein which binds to the X box (5'-GTNRCC N_{1-3} RGYAAC-3') of the Major Histocompatibility Complex (MHC) class II genes, and it is also involved in the regulation of human *PCNA* (proliferating cell nuclear antigen) gene promoter [6-9]. Furthermore, it is known that hRFX1 and hRFX2/hRFX3 form a heterodimer [10]. Both of them bind to the promoter region of IL-5α receptor gene, and thereby regulate its expression [11]. The X-ray crystal structure shows that the RFX domain of hRFX1 is a winged helix domain. DAF-19 is a protein that regulates *OSM-1, OSM-5* and *OSM-6*. *OSM-1* and *OSM-6* encode proteins of Intraflagellar Transport (IFT), and *OSM-5* encodes a protein engaged in cilium assembly [12-14]. dRFX is expressed in the Central Nervous System (CNS), in the Peripheral Nervous System (PNS), and in the testis during *Drosophila* development [3,4]. And the loss of dRFX is accompanied by a severe disorganization of the cilia of the sensory neuron dendrites. Thus it is known that dRFX is a transcription factor and involves in differentiation of CNS, PNS and testis [3,4,15]. A number of studies also indicate that RFX family proteins play major roles in regulation of genes involved in ciliogenesis [12,16]. However, functions of RFX family proteins other than ciliogenesis have been also reported [17].

RFX5, the most divergent mammalian member, regulates major histocompatibility class II gene expression and mutations in it results in the bare lymphocyte syndrome [17]. In addition, RFX4 has been implicated in dorsal patterning of brain development in mice and may participate in circadian rhythm regulation in humans [18-21]. dRFX2 is also reported to be involved in cell cycle progression and regulation of apoptosis. The *Drosophila PCNA* gene promoter contains URE (Upstream Regulatory Element), CFDD (Common Regulatory Factor for DNA Replication) and DREF (DNA Replication-Related Element Binding Factor) sites, DRE and E2F-binding sites [22-24]. A number of transcription factor such as UREF (Grainyhead: GRH), CFDD, DREF and E2F, all of which bind to their specific sites [22,25-27]. In addition to these regulatory elements, a novel regulatory element that is located between URE and DRE has been identified. dRFX2 was identified as a binding protein to this element and demonstrated that it is involved in the normal progression from G1 to S phase of cell cycle and in regulation of apoptosis [28].

In addition, a novel protein, dRFX3 that contains RFX domain has been found by searching of a *Drosophila* genome database. In the present studies, we established transgenic fly carrying the HA-dRFX3 cDNA and utilized GAL4-UAS targeted expression system to examine effects of overexpression of HA-dRFX3 on the development [29]. Overexpression of dRFX3 in the eye imaginal discs had no effect on the cell cycle progressions of S phase and M phase, respectively. However it interfered with the differentiation of R2/R5 photoreceptor cells.

***Corresponding author:** Masamitsu Yamaguchi, Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan, Tel: +81 75 724 7781; E-mail: myamaguc@kit.ac.jp

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Moreover overexpression of dRFX3 induced apoptosis in eye imaginal disc cells. These data suggest that the dRFX3 plays a negative role for the differentiation of R2/R5 photoreceptor cells and plays a positive role in the regulation of apoptosis.

Materials and Methods

Fly stocks

Fly stocks were maintained at 25°C on standard food. The yellow white fly was used as a control strain. The GAL4 driver strains and mutant strain, *reaper* (*rpr*), which were from Bloomington Stock Center or Kyoto *Drosophila* Genetic Resource Center. The enhancer trap lines carrying the *lacZ* markers X63 (inserted in *rhomboid*), AE127 (inserted in *seven-up*), and P82 (inserted in *deadpan*) were obtained from Y. Hiromi. These lines express the β-galactosidase markers in photoreceptor cells of R8/R2/R5, R3/R4/R1/R6, and R3/R4/R7, respectively.

Establishment of transgenic flies

P element-mediated germ line transformation was carried out as described earlier. F1 transformants were selected on the basis of white eye color rescue. Two independent lines carrying UAS-HA-dRFX3 on the second chromosome were used in the present studies.

Scanning electron microscopy

Adult flies were anesthetized, mounted on the stages, and observed under a Scanning Electron Microscope (SEM) VE-7800 (Keyence Inc.) in the low vacuum mode. In every experiment, the eye phenotype of at least five adult flies of each line was simultaneously examined by scanning electron microscopy, and these experiments were repeated 3 times. In the experiments, no significant variation in eye phenotype among the five individuals was observed.

5-Bromo-2'-deoxyuridine (BrdU) labeling

Detection of cells in S phase was performed using a BrdU-labeling method as described previously with minor modifications [30-33]. Third instar larvae were dissected in *Drosophila* Ringer's solution and the eye imaginal discs were suspended in Grace's insect medium, then incubated in the presence of 75 μg/ml BrdU (Roche) at 25°C for 30 min. The samples were fixed in Carnoy's fixative (ethanol/acetic acid/chloroform, 6:1:3) at 25°C for 20 min, and further fixed in 80% ethanol/50 mM glycine buffer (pH 2.0) at -20°C for 16 h. Incorporated BrdU was visualized using an anti-BrdU antibody and an anti-mouse Alexa 594 antibody (Invitrogen) at 25°C for 2 h.

Immunohistology

Third instar larvae were dissected in *Drosophila* Ringer's solution and the eye imaginal discs were fixed in 4% paraformaldehyde/PBS (Phosphate-Buffered Saline) at 25°C for 30 min. After washing with PBS, the samples were blocked with 10% normal goat serum in PBST (PBS containing 0.15% Triton X-100) at 25°C for 20 min. Then the samples were incubated with culture supernatant of hybridoma cells producing mouse anti-HA monoclonal antibody (supplied by Dr. M. Inagaki) at a 1:100 dilution, mouse anti-β-galactosidase monoclonal antibody (Promega) at a 1:500 dilution or rabbit anti-Cyclin B polyclonal antibody (Santa Cruz Biotechnology, Inc.) at a 1:200 dilution at 4°C for 16 h. After washing with PBST, the samples were incubated with the second antibody, an alkaline phosphatase-conjugated goat anti-mouse Immunoglobulin G (IgG) (Promega) at a 1:500 dilution or anti-rabbit Alexa 594 antibody (Invitrogen) at a 1:400 dilution at 25°C for 2 h. After washing with PBST, the samples were mounted and analyzed or the color was developed in a solution containing NBT (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.34 mg/ml nitroblue tetrazolium salt), and BCIP (0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt). The samples were washed with PBS and mounted in 90% glycerol/PBS for microscopic observation. In the case of immuno-staining photoreceptor cells, the F1 third instar larvae from the cross of *GMR-GAL4; +*, or *GMR-GAL4; UAS-HA-dRFX3* with each enhancer trap line were dissected.

Detection of apoptotic cells

The third instar larvae were dissected and the eye imaginal discs were immunostained. The primary antibody, rabbit anti-cleaved caspase-3 polyclonal antibody (Cell Signaling Technology) was used at a 1:200 dilution at 4°C for 16 h. The second antibody, anti-rabbit Alexa 594 antibody (Invitrogen) was used at 25°C for 2 h. After washing with PBST, the samples were mounted and analyzed.

Quantification of the data

Intensities of immunofluorescent signals were quantified with Image J software. Relative area size of the adult compound eyes were also quantified with Image J software.

Results and Discussion

Overexpression of dRFX3 disrupts normal development in Drosophila

Based on a comparison of the RFX domain between human and *Drosophila*, dRFX3 shows the highest homology with hRFX5 (46.5% identity and 67% similarity) (Figure 1). hRFX1 and hRFX2/hRFX3 form a heterodimer, both of which bind to the promoter region of IL-5α receptor gene, and thereby regulate its expression [9,10]. However, hRFX5 has no dimerization motifs as well as dRFX3. Therefore dRFX3 may have similar functions to hRFX5. To investigate the effects of overexpression of dRFX3 in living flies, we utilized the GAL4-UAS targeted expression system. Two independent transgenic lines, strain 18 and strain 53 carrying UAS-dRFX3 with HA-tag (UAS-HA-dRFX3) were established, and crossed them with 40 lines of GAL4 drivers expressing GAL4 in specific tissues and developmental stages in *Drosophila*.

The *sd-GAL4>HA-dRFX3*, *MS1096-GAL4>HA-dRFX3*, *en-GAL4>HA-dRFX3*, *vg-GAL4>HA-dRFX3*, *48Y-GAL4>HA-dRFX3*, *arm-GAL4>HA-dRFX3* and *645-GAL4>HA-dRFX3* induced abnormal wing phenotypes such as atrophied, crumpled or unclosed wing phenotypes, respectively (Figure 2A) (Table 1). When the *pnr-GAL4* that expressed GAL4 at presumptive notum region in wing discs was crossed with *UAS-HA-dRFX3*, split thorax phenotype was induced (Figure 2B) (Table 1). Overexpression of dRFX3 with *GMR-GAL4* (*GMR-GAL4>HA-dRFX3*) and *eyeless-GAL4* (*eyeless-GAL4> HA-dRFX3*) drivers induced rough eye and small eye phenotypes, respectively (Figure 3) (Table 1). Size of the adult compound eye (*eyeless-GAL4> HA-dRFX3*) was reduced to 93% with strain 18 and 70% with strain 53 in compared to the control *eyeless-GAL4* flies. It is known that *389-GAL4* driver strain express GAL4 in embryonic CNS. When *389-GAL4* was crossed with *UAS-HA-dRFX3*, multiple phenotypes such as rough eye, small head, unclosed wing and crumpled wing phenotypes were induced (Table 1). Altogether the overexpression using strain 53 of transgenic fly carrying the HA-dRFX3 cDNA exhibited severer phenotypes than the strain 18. This is very likely due to the higher expression of HA-dRFX3 in strain 53 than in strain 18 (Figure 3,

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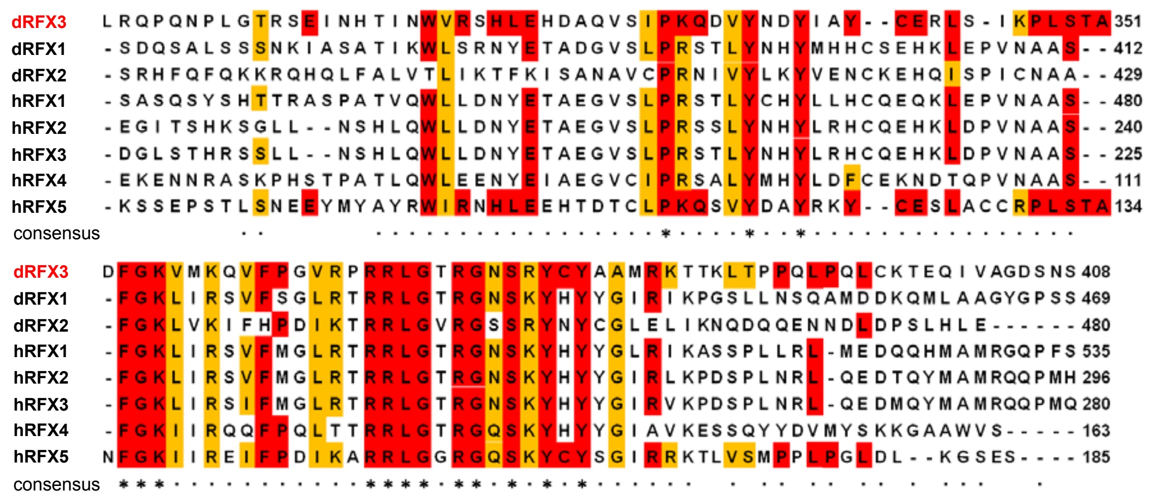

Figure 1: Multiple sequence alignment of RFX domains. Multiple sequence alignment of the conserved RFX domain between human and Drosophila RFX family proteins. Red and yellow boxes represent identical and similar amino acids between dREF3 and hRFX5.

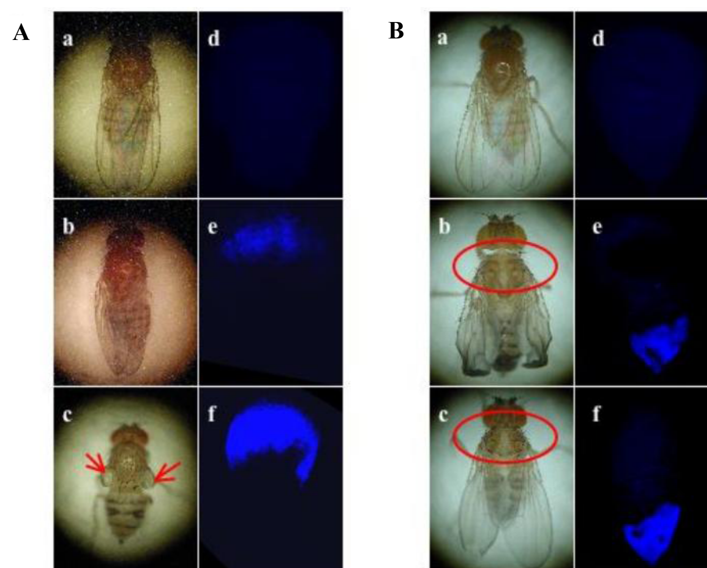

Figure 2: Adult flies expressing dRFX3 in wing imaginal disc exhibited atrophied wing and split thorax phenotypes. (a)~(c) Micrographs of adult flies. (d)~(f) Immunostainning of wing imaginal discs with anti-HA antibody. (A) (a, d) sd-GAL4/+; +/+, (b, e) sd-GAL4/+; UAS-HA-dRFX3 (strain 18) /+, (c, f) sd-GAL4/+; UAS-HA-d RFX3 (strain 53) /+, (B) (a, d) +/+; pnr-GAL4/+, (b, e) +/+; pnr-GAL4/UAS-HA-dRFX3 (strain 18) /+, (c, f) +/+; pnr-GAL4/UAS-HA-dRFX3 (strain 53) /+. These flies were developed at 28°C. HA signals are detected in ventral regions (A-e and A-f) or dorsal regions (B-e and B-f) of wing discs. Arrows indicate the atrophied wing. Red-circled regions indicate the split thorax of the adult fly.

Panels A-g and B-g). The other 15 GAL4 drivers exerted no detectable abnormality (Table 1). Since cell cycle regulation and differentiation have been well characterized during eye development in *Drosophila*, we focused on analyses of the rough eye phenotype induced by dRFX3 in the following studies.

Overexpression of dRFX3 in eye imaginal discs exerts no effect on the cell cycle progression.

In third instar larvae, the Morphogenetic Furrow (MF) appears at the end of posterior in the eye imaginal discs, and then it moves to the anterior direction. Cells in the anterior to the MF proliferate asynchronously. Once those cells are on MF, they are arrested synchronously at G0/G1 phase. Cells in the posterior to MF undergo one more cell cycle such as S, G2, M, which in turn differentiate into photoreceptors of the adult ommatidium [30].

In *GMR-GAL4*, it is known that GAL4 is expressed in the region within and posterior to MF by GMR carrying transcription factor Glass-binding sites. The flies with *GMR-GAL4>HA-dRFX3* showed specific expression of HA-dRFX3 in the region posterior to MF that was detected by immunostaining with anti-HA antibody (Figures 3A and 3B (d-f)).

The rough eye phenotype may arise from the ectopic induction or arrest of cell cycle in the eye imaginal discs. To examine effect of dRFX3 overexpression on DNA synthesis in the eye imaginal disc cells, at first, we carried out the BrdU incorporation assay that can detect

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GAL4 line	Chromosome linkage	Expression pattern	Lethal phase	Phenotype
GMR(#16)	X	eye		rough eye
GMR	Ⅱ	eye		rough eye
eyeless	Ⅱ	eye		rough eye
eyeless/CyO	Ⅱ	eye		rough eye
sev/CyO	Ⅱ	eye	larval,pupal	
sd	X	wing		atrophied wing(#53)
MS1096	X	wing		crumpled wing(#18),atrophied wing(#53)
Hs/CyO	Ⅱ	wing, nerve		no effect
Hs	Ⅱ	wing, nerve		no effect
en	Ⅱ	wing, imaginal discs	embryo(#53)	crumpled wing(#18)
en/CyO	Ⅱ	wing, imaginal discs	embryo(#53)	unclosed wing(#18)
ve	Ⅱ	wing		no effect
vg	Ⅱ	wing		crumpled or atrophied wing
ap/CyO	Ⅱ	wing, nerve	embryo	
wg	Ⅱ	wing		no effect
pnr/TM3	Ⅱ	wing		split back
elav	X	embryonic nerve system		no effect
389	Ⅱ	embryonic CNS		rough eye,small head,unclosed wing,crumpled wing(#53)
91Y	Ⅱ	CNS		no effect
twist	X	mesoderm		low viability(#53)
48Y	Ⅱ	endoderm		unclosed wing(#53)
dpp/CyO	Ⅱ	endo/mesoderm		no effect
dll	Ⅱ	early embryo		no effect
dll/CyO	Ⅱ	early embryo		no effect
arm	Ⅱ	segments, ubiquitous		unclosed wing(#53)
Cg	Ⅱ	blood cells	larval	
Hml	Ⅱ	larval hemocyte		no effect
c323a	X	SG, gut, embryo, follicle cells	larval	
T155	Ⅱ	follicle cells	embryo(#53)	low viability(#18)
55B	Ⅱ	follicle cells		no effect
198Y	Ⅱ	follicle cells, oocyte		no effect
645	Ⅱ	oocyte		crumpled wing(#53)
69B	X	larval discs		no effect
T80/CyO	Ⅱ	imaginal discs	larval	
Act25C/CyO・GFP	Ⅱ	imaginal discs	larval	
Act5C/TM6B	Ⅱ	all tissues		crooked body(#53)
da	Ⅱ	ubiquitous		no effect
kr/TM3	Ⅱ	embryo		no effect
hh/TM6B	Ⅱ	embryo		low viability
tub/TM3	Ⅱ	all tissues	larval	

Table 1: Summary of effects of HA-dRFX3 expression with each GAL4 driver lines.

the cells in S phase (Figure 4A). In eye discs expressing GAL4 alone, BrdU incorporation was observed in the whole anterior region to the MF and in a line just posterior to the MF, which cannot discriminate from the pattern of BrdU incorporation in wild-type fly discs (Figure 4A (a)). In the eye imaginal discs overexpressing dRFX3, the pattern of BrdU incorporation was indistinguishable from the one expressing GAL4 alone (Figure 4A). The quantification of BrdU-positive cells also indicates no significant difference between control and dRFX3-overexpressing flies.

Start of M phase is regulated by the phosphorylation of MPF (M-Phase Promoting Factor) that is comprised of Cyclin B and Cdc2. And the Cyclin B is abolished at the end of M phase. We therefore carried out the immunological detection of Cyclin B with anti-Cyclin B antibody to examine the effect of dRFX3 overexpression on the progression of M phase (Figure 4B). In eye discs expressing GAL4 alone, Cyclin B signal was detected in the whole anterior region to the MF and in a line posterior to the MF, which cannot discriminate from the pattern of wild-type fly discs (Figure 4B (a)). In the eye imaginal discs overexpressing dRFX3, the pattern of Cyclin B signal was indistinguishable from the one expressing GAL4 alone (Figure 4B). These results suggest that the overexpression of dRFX3 in the eye imaginal discs exerts no effect on the cell cycle progression of S phase and M phase.

Overexpression of dRFX3 in eye imaginal discs interferes with the differentiation of R2/R5 photoreceptor cells

In *Drosophila* development, photoreceptor cells are generated in specific order with the MF crosses the eye imaginal discs from posterior to anterior: R8 photoreceptor is generated first after passing of the MF, then R2/R5, R3/R4, R1/R6, and R7 that is the last cell, all of which are generated sequentially [31,32]. Thereafter, each photoreceptor cell forms rhabdomere that receives light and then transduces it to chemical signal.

The rough eye phenotype may arise from the interference of

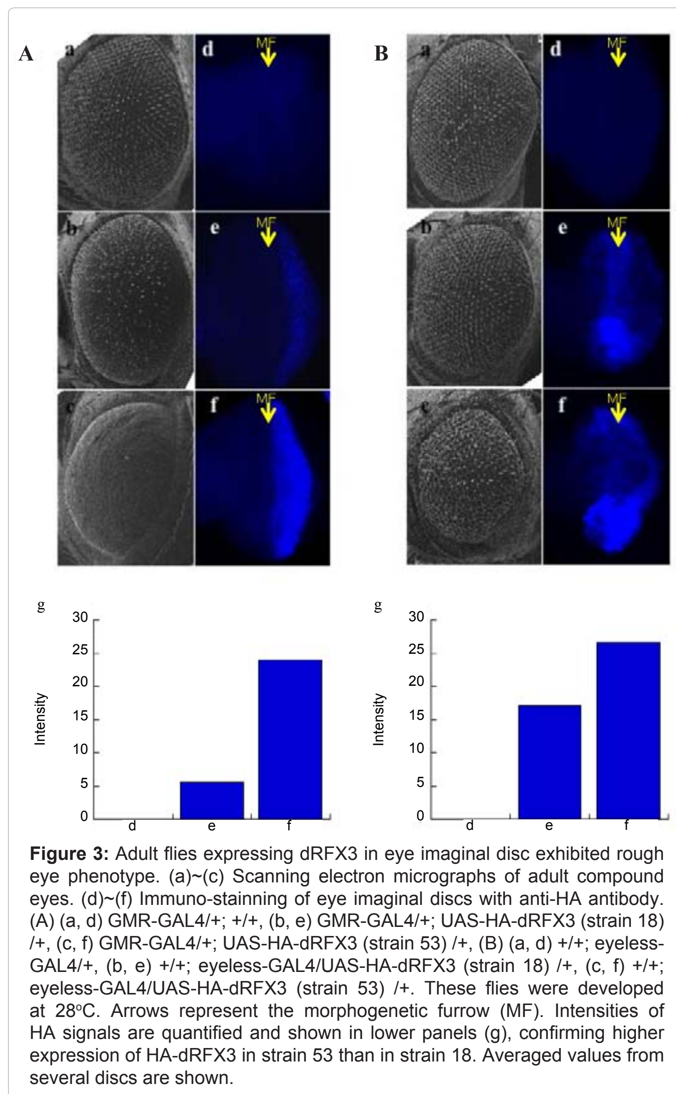

Figure 3: Adult flies expressing dRFX3 in eye imaginal disc exhibited rough eye phenotype. (a)~(c) Scanning electron micrographs of adult compound eyes. (d)~(f) Immuno-stainning of eye imaginal discs with anti-HA antibody. (A) (a, d) GMR-GAL4/+; +/+, (b, e) GMR-GAL4/+; UAS-HA-dRFX3 (strain 18) /+, (c, f) GMR-GAL4/+; UAS-HA-dRFX3 (strain 53) /+, (B) (a, d) +/+; eyeless-GAL4/+, (b, e) +/+; eyeless-GAL4/UAS-HA-dRFX3 (strain 18) /+, (c, f) +/+; eyeless-GAL4/UAS-HA-dRFX3 (strain 53) /+. These flies were developed at 28°C. Arrows represent the morphogenetic furrow (MF). Intensities of HA signals are quantified and shown in lower panels (g), confirming higher expression of HA-dRFX3 in strain 53 than in strain 18. Averaged values from several discs are shown.

photoreceptor cell differentiation in the eye imaginal discs. We therefore carried out the immunological detection of photoreceptor cells using enhancer trap lines expressing a nucleus-localized form of β-galactosidase markers in photoreceptor cells. In this analysis, we utilized three enhancer trap lines, X63, AE127 and p82, specifically expressing β-galactosidase in R8/R2/R5, R3/R4/R1/R6 and R3/R4/R7, respectively. The eye imaginal discs of F1 third instar larvae from the cross of *GMR-GAL4; +,* or *GMR-GAL4; UAS-HA-dRFX3* with each enhancer trap line were dissected, and then immunostained with anti-β-galactosidase antibody (Figures 5A-I). In eye imaginal discs overexpressed dRFX3, only R8 photoreceptor cell was stained by cross with X63 enhancer trap line (Figures 5B and 5C). On the other hand, when the enhancer trap lines AE127 and p82 were used, none of the photoreceptor cells was stained (Figures 5E, F, H and I). These results indicate that the overexpression of dRFX3 in the eye imaginal discs interfere with the differentiation of R2/R5 and consequently the following R3/R4, R1/R6 and R7 differentiation, but exerted no effect on that of R8. Therefore overexpression of dRFX3 may have disturbed expression of genes responsible for differentiation of R2/R5 photoreceptor cells, although further analyses are necessary to identify the responsible genes. Genetic screen to identify mutations that can suppress the rough eye phenotype induced by dRFX3-overexpression may allow us to identify such genes. In any event, the transgenic flies established in this study would be useful to perform such genetic screen.

Overexpression of dRFX3 in eye imaginal discs induces caspase-dependent apoptosis

Overexpression of dRFX3 in the eye imaginal discs interfered with the differentiation of photoreceptor cells. Since there is a possibility that interference of differentiation process lead to apoptosis, we investigated whether apoptosis is induced in the eye imaginal discs, by immunological detection of the activated caspase-3. In this assay, *GMR-GAL4/+; GMR-rpr* that is expressing GAL4 and Reaper, a *Drosophila* cell death factor, was used for a positive control. And *GMR-GAL4/+* flies expressing GAL4 alone was used for a negative control. In the eye imaginal discs of *GMR-GAL4/+*, cell death was barely detectable (Figure 6b). In contrast, innumerable apoptotic signals were detected in the region within and posterior to the MF in the eye imaginal discs of *GMR-GAL4/+; GMR-rpr* (Figure 6a). When dRFX3 was overexpressed in the eye imaginal discs, a large number of apoptotic signals were detected in the region posterior to the MF (Figures 6c and 6d). Furthermore, when the flies overexpressing dRFX3 were crossed with the flies expressing the Caspase inhibitors such as P35 and DIAP1, the rough eye phenotypes were effectively suppressed in their progenies (Figures 7A and 7B (a,b,d,e,g,h)) and the apoptosis signals detected in dRFX3-overexpression (Figures 7A(c) and 7B(c)) also disappeared from their eye imaginal discs (Figures 7A (f,i) and 7B(f,i)). These results indicate that overexpression of dRFX3 in eye imaginal discs induces the Caspase-dependent apoptosis.

Taken together, our data suggests that the dRFX3 plays a negative role for the differentiation of R2/R5 photoreceptor cells and plays a positive role in the regulation of apoptosis. A number of studies indicate that RFX family proteins play major roles in regulation of genes involved in ciliogenesis [12,16]. However, this study shows

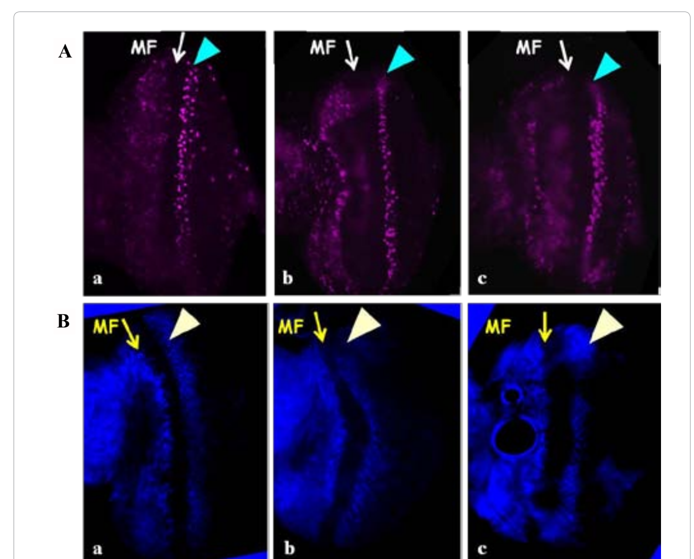

Figure 4: Overexpression of HA-dRFX3 in eye imaginal disc exerted no effect on the cell cycle progression. (A) After incorporation of BrdU, the eye imaginal discs were immuno-stained with anti-BrdU antibody. (a) GMR-GAL4/+; +/+, (b) GMR-GAL4/+; UAS-HA-dRFX3 (strain 18) /+, (c) GMR-GAL4/+; UAS-HA-dRFX3 (strain 53) /+. (B) Immunostaining of eye discs with anti-Cyclin B antibody. (a) GMR-GAL4/+; +/+, (b) GMR-GAL4/+; UAS-HA-dRFX3 (strain 18)/+, (c) GMR-GAL4/+; UAS-HA-dRFX3 (strain 53) /+. Arrows indicate morphogenetic furrow (MF). Blue arrowheads indicate BrdU-positive cells behind MF and white arrowheads indicate Cyclin B-positive cells behind MF.

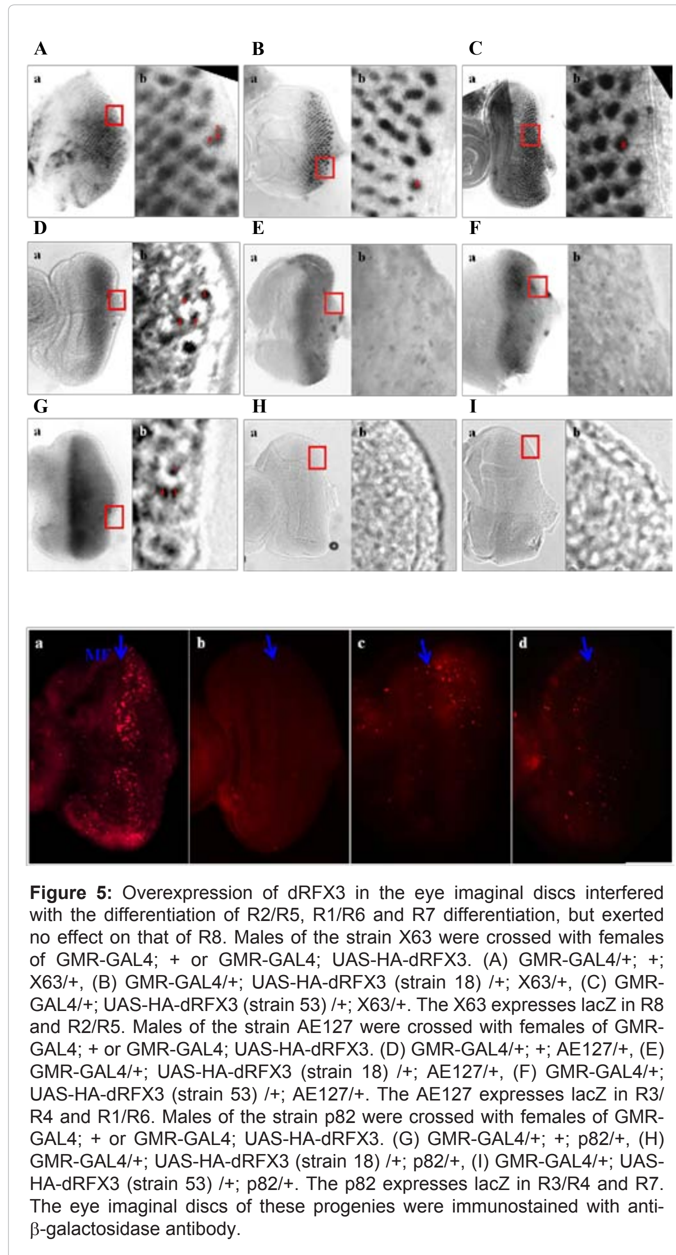

Figure 5: Overexpression of dRFX3 in the eye imaginal discs interfered with the differentiation of R2/R5, R1/R6 and R7 differentiation, but exerted no effect on that of R8. Males of the strain X63 were crossed with females of GMR-GAL4; + or GMR-GAL4; UAS-HA-dRFX3. (A) GMR-GAL4/+; +; X63/+, (B) GMR-GAL4/+; UAS-HA-dRFX3 (strain 18) /+; X63/+, (C) GMR-GAL4/+; UAS-HA-dRFX3 (strain 53) /+; X63/+. The X63 expresses lacZ in R8 and R2/R5. Males of the strain AE127 were crossed with females of GMR-GAL4; + or GMR-GAL4; UAS-HA-dRFX3. (D) GMR-GAL4/+; +; AE127/+, (E) GMR-GAL4/+; UAS-HA-dRFX3 (strain 18) /+; AE127/+, (F) GMR-GAL4/+; UAS-HA-dRFX3 (strain 53) /+; AE127/+. The AE127 expresses lacZ in R3/R4 and R1/R6. Males of the strain p82 were crossed with females of GMR-GAL4; + or GMR-GAL4; UAS-HA-dRFX3. (G) GMR-GAL4/+; +; p82/+, (H) GMR-GAL4/+; UAS-HA-dRFX3 (strain 18) /+; p82/+, (I) GMR-GAL4/+; UAS-HA-dRFX3 (strain 53) /+; p82/+. The p82 expresses lacZ in R3/R4 and R7. The eye imaginal discs of these progenies were immunostained with anti-β-galactosidase antibody.

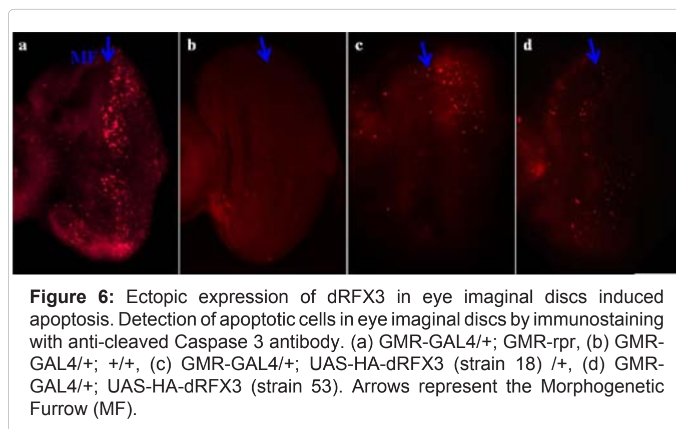

Figure 6: Ectopic expression of dRFX3 in eye imaginal discs induced apoptosis. Detection of apoptotic cells in eye imaginal discs by immunostaining with anti-cleaved Caspase 3 antibody. (a) GMR-GAL4/+; GMR-rpr, (b) GMR-GAL4/+; +/+, (c) GMR-GAL4/+; UAS-HA-dRFX3 (strain 18) /+, (d) GMR-GAL4/+; UAS-HA-dRFX3 (strain 53). Arrows represent the Morphogenetic Furrow (MF).

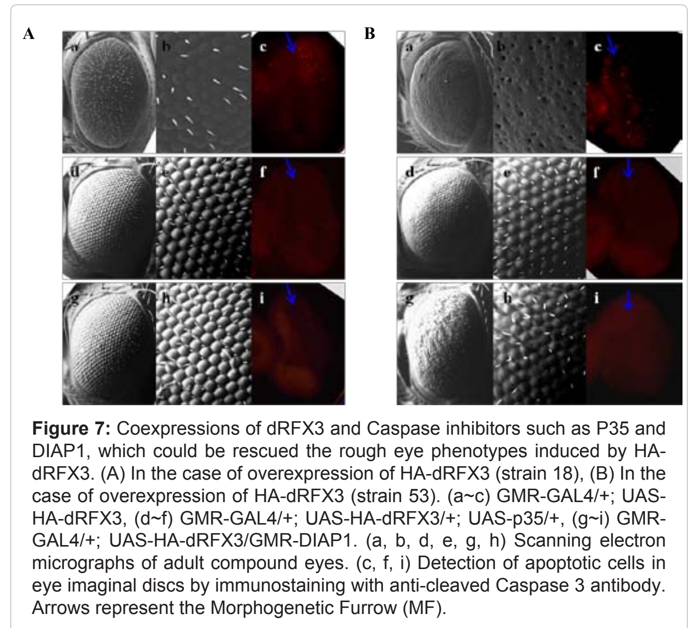

Figure 7: Coexpressions of dRFX3 and Caspase inhibitors such as P35 and DIAP1, which could be rescued the rough eye phenotypes induced by HA-dRFX3. (A) In the case of overexpression of HA-dRFX3 (strain 18), (B) In the case of overexpression of HA-dRFX3 (strain 53). (a~c) GMR-GAL4/+; UAS-HA-dRFX3, (d~f) GMR-GAL4/+; UAS-HA-dRFX3/+; UAS-p35/+, (g~i) GMR-GAL4/+; UAS-HA-dRFX3/GMR-DIAP1. (a, b, d, e, g, h) Scanning electron micrographs of adult compound eyes. (c, f, i) Detection of apoptotic cells in eye imaginal discs by immunostaining with anti-cleaved Caspase 3 antibody. Arrows represent the Morphogenetic Furrow (MF).

that the dRFX3, a homolog of RFX5 has other important function in *Drosophila*. Involvement of dRFX3 in apoptosis also implicates possible links with mutagenesis and cancer in which apoptosis plays critical roles.

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