Transcription factor NF-Y is involved in differentiation of R7 photoreceptor cell in *Drosophila*

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Summary

The CCAAT motif-binding factor NF-Y consists of three different subunits, NF-YA, NF-YB and NF-YC. Knockdown of *Drosophila* NF-YA (dNF-YA) in eye discs with *GMR*-GAL4 and UAS-*dNF-YAIR* resulted in a rough eye phenotype and monitoring of differentiation of photoreceptor cells by *LacZ* expression in *seven up-LacZ* and *deadpan-lacZ* enhancer trap lines revealed associated loss of R7 photoreceptor signals. In line with differentiation of R7 being regulated by the *sevenless (sev)* gene and the MAPK cascade, the rough eye phenotype and loss of R7 signals in dNF-YA-knockdown flies were rescued by expression of the *sev* gene, or the *D-raf* gene, a downstream component of the MAPK cascade. The *sev* gene promoter contains two dNF-Y-binding consensus sequences which play positive roles in promoter activity. In chromatin immunoprecipitation assays with anti-dNF-YA antibody and S2 cells, the *sev* gene promoter region containing the NF-Y consensus was effectively amplified in immunoprecipitates from transgenic flies by polymerase chain reaction, indicating that dNF-Y is necessary for appropriate *sev* expression and involved in R7 photoreceptor cell development.

Key words: NF-Y, *sevenless*, R7 photoreceptor, chromatin immunoprecipitation, MAPK

Introduction

The CCAAT motif-binding factor NF-Y (nuclear factor Y-box, also called CBF) consists of three different subunits, NF-YA (CBF-B), NF-YB (CBF-A) and NF-YC (CBF-C) (Mantovani, 1999). Although NF-YA contains a DNA binding domain in its C-terminal region, the other two subunits, NF-YB and NF-YC are also required for DNA-binding, both containing a histone fold motif through which they interact to form a heterodimer (Mantovani, 1999). This then interacts with NF-YA to form a heterotrimeric NF-Y transcription factor. Absence of any of the NF-Y subunits results in loss of binding of the NF-Y complex to DNA (Yoshioka et al., 2007) and therefore of NF-Y-directed transcription (Mantovani, 1999). The CCAAT box is one of the most common elements in eukaryotic promoters, found in a forward or reverse orientation. Among the various DNA binding proteins that interact with this sequence, only NF-Y has been shown to absolutely require all five nucleotides (Mantovani, 1998). NF-Y also specifically recognizes the consensus sequences 5′-CTGATTGGYYRR-3′ or 5′-YYRRCCAATCAG-3′ (Y, pyrimidines and R, purines) present in the promoter region of many constitutive, inducible, and cell-cycle-dependent eukaryotic genes (Matsuoka and Chen, 1999).

It has been established that the CCAAT motif is present in promoters of many mammalian genes, including those expressed in specific cell types during the cell cycle, such as *topoisomerase IIα, cyclin B1, CDC25C, E2F1, CDC2,* and *thymidine kinase* genes (Hu et al., 2002). It is reported that NF-Y regulates transcription of *Hoxb4*, *γ-globin*, *Major histocompatibility (MHC) class II*, *TGF-β* receptor *II*, and *Sox* family genes (Fang et al., 2004; Gilthorpe et al., 2002; Grujicic et al., 2005; Huang et al., 2005; Niimi et al., 2004; Reith et al., 1994; Wiebe et al., 2000). Histone deacetylase 4 (HDAC4) is known to be recruited on NF-Y-dependent repressed promoters and a relationship between p53 and HDAC4 recruitment following DNA damage has also been noted (Basile et al., 2005). Recently, it was reported that recruitment of HDAC1 to the TBP-2 promoter is mediated by a protein complex, consisting of the RET finger protein (RFP; also called TRIM27) and the trimeric transcription factor NF-Y, which regulates the sensitivity of cancer cells to oxidative stress (Kato et al., 2009). NF-Y is itself activated by ER stress and assembled into a transcriptional complex to regulate stress response genes (Liu and Howell, 2010). While NF-Y activity is clearly present in all mammalian tissues, genes that are actually regulated by NF-Y in vivo have still to be determined in detail. The fact that knock out of mouse NF-YA results in early embryonic lethality indicates essential roles in early development (Bhattacharya et al., 2003).

To study NF-Y function in vivo, we have focused on the *Drosophila* NF-YA (dNF-YA) subunit containing a DNA-binding domain using established transgenic fly lines carrying UAS-*HA-dNF-YA* or the UAS-*dNF-YA* inverted repeat (IR) (Yoshioka et al., 2007). Utilizing the GAL4-UAS targeted expression system (Brand and Perrimon, 1993), we earlier demonstrated over-expression or knockdown of dNF-YA to be lethal at various developmental stages, suggesting that dNF-YA indeed participates in various gene regulatory pathways during

Drosophila development (Yoshioka et al., 2007). Expression of dNF-YA with *eyeless*-GAL4 mainly resulted in a headless phenotype in pharate-adults. Reduction of the *eyeless* gene dose enhanced the dNF-YA-induced phenotype, while reduction of the *Distal-less* gene dose suppressed the phenotype. In contrast, crossing the dNF-YA over-expressing flies with a *Notch* mutant resulted in no apparent effect. From these results we concluded that dNF-YA can disturb eye disc specification, but not eye disc growth (Yoshioka et al., 2007). On the other hand, specific knockdown of dNF-YA by pannier-GAL4 induced a thorax disclosed phenotype and we found that dNF-Y directly regulates *Drosophila JNK* gene *basket* (*bsk*) transcription (Yoshioka et al., 2008). It is reported that the other dNF-Y subunit, dNF-YC, is involved in photoreceptor neuron development. In the absence of NF-YC, R7 axons terminate in the same layer as R8 axons (Morey et al., 2008).

Here we examined the effect of knockdown of dNF-YA in the eye-antennal disc with a *GMR*-GAL4 driver, demonstrating a rough eye phenotype and loss of R7 precursor cells in eye imaginal discs. Differentiation of R7 photoreceptor is regulated by Sevenless (Sev) and the ERK pathway in *Drosophila* (Nagaraj and Banerjee, 2004) and in *sev* mutants the R7 photoreceptor is missing from each ommatidium (Tomlinson and Ready, 1986). Sev is a receptor tyrosine kinase whose activation induces intracellular changes in presumptive R7 cells to adopt an R7 rather than a cone cell fate (Basler and Hafen, 1988). However, expression of Sev is not restricted to the presumptive R7 cell (Tomlinson and Ready, 1987; Banerjee et al., 1987) but also features in R3/R4, R7, R1/R6 photoreceptors and cone cells (Tomlinson and Ready, 1987). Although expression patterns of *sev* in photoreceptors have been extensively studied, transcriptional regulatory elements of the *sev* gene promoter and transcription factors regulating its transcription have yet to be identified.

In the present study, we performed a genome data base search and found that the 5′ flanking region of the *sev* gene carries dNF-Y-binding consensus sequences, suggesting dNF-Y to be involved in *sev* gene transcription. These observations combined with other cytological, genetical and molecular biological studies indicate that dNF-Y regulates *sev* gene expression during *Drosophila* R7 photoreceptor development.

Results

Effects of knockdown of dNF-YA on *Drosophila* eye development

We earlier established seventeen independent UAS-*dNF-YAIR*$_{231\text{-}399}$ transgenic fly strains targeting between aa231 and aa399 (Yoshioka et al., 2007; Yoshioka et al., 2008). Using these strains, we revealed that dNF-YA participates in various gene regulatory pathways during *Drosophila* development (Yoshioka et al., 2007). Furthermore, analyses of ectopic expression of dNF-YA with *eyeless*-GAL4 revealed disturbed eye disc specification, but not eye disc growth (Yoshioka et al., 2007). However precise roles of dNF-YA during eye development have yet to be clarified. We therefore tried to knockdown dNF-YA with GMR-GAL4 to carry out more detailed studies. As noted previously, the *GMR*-GAL4 driver strain specifically expresses GAL4 in the domain of eye-antennal discs (Ishimaru et al., 2004). Specific effects of *dNF-YA* double strand RNA (dsRNA) on *dNF-YA* expression in the eye-antennal disc were confirmed by a flip-out experiment (Fig. 1) (Sun and Tower, 1999). Cells marked with GFP expressed *dNF-YA* dsRNA (Fig. 1D and E). Although *dNF-YA* is expressed ubiquitously in the eye imaginal

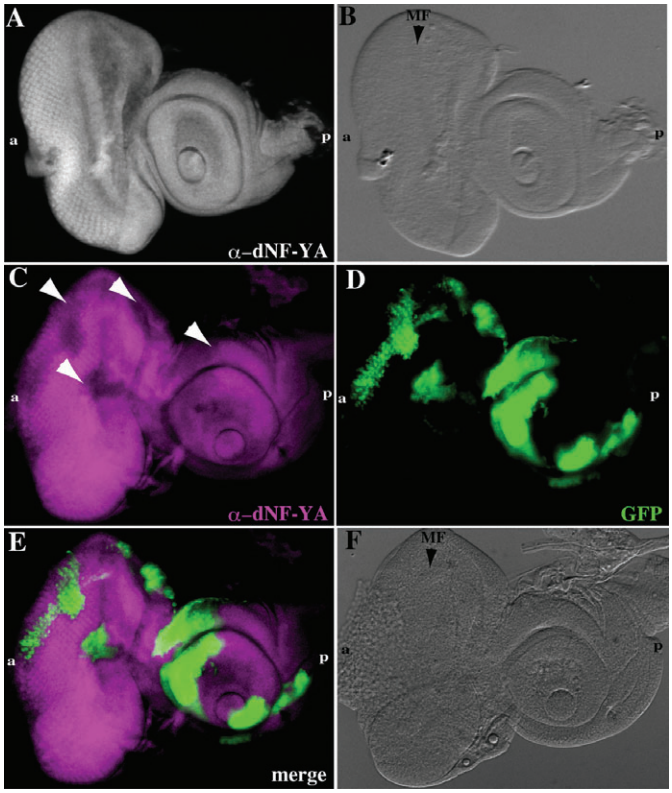

Fig. 1. Expression of *dNF-YA*dsRNA reduces dNF-YA levels in eye-antennal imaginal discs. (A, B) Expression pattern in eye imaginal discs. **(A)** Immunostaining of eye discs with anti-dNF-YA IgG. **(B)** Nomarski image of the wing disc shown in panel A. **(C–F)** Flip out experiments. In the dNF-YA RNAi clone area, levels of dNF-YA signals are reduced (white arrowheads). **(C)** Immunostaining of an eye-antennal disc with anti-dNF-YA IgG. **(D)** Cells expressing *dNF-YA*dsRNA are marked with GFP (Green). **(E)** Merged image of anti-dNF-YA and GFP signals. **(F)** Nomarski image of the eye disc. a, anterior; p, posterior; MF, morphogenetic furrow.

disc (Fig. 1A), in the RNAi clone area, the level of dNF-YA signals was specifically reduced (Fig. 1C and E). These results pointed to specific knockdown of dNF-YA in the eye-antennal disc by expression of *dNF-YA* dsRNA, as observed previously in other tissues (Yoshioka et al., 2008).

Previously we reported that the *GMR*-GAL4>UAS-*dNF-YAIR*$_{231\text{-}399}$ flies exhibit a pharate-adult lethal phenotype (Yoshioka et al., 2007). However, genetic crossing with other independently established UAS-*dNF-YAIR*$_{231\text{-}399}$ transgenic strains revealed two out of five *GMR*-GAL4>UAS-*dNF-YAIR*$_{231\text{-}399}$ fly lines to exhibit a rough eye phenotype (Fig. 2B, C and Table 1). The strength of rough eye phenotype roughly correlates with the extent of dNF-YA knockdown examined by immunostaining of the eye disc with anti-dNF-YA antibody (Fig. 2I–K). Although the strength of rough eye phenotype was different among independent transgenic lines, penetrance of the phenotype was almost 100 %. To exclude the possibility of off-target effects, three independent UAS-*dNF-YAIR* strains were previously established, targeting between aa63 and aa228 (UAS-*dNF-YAIR*$_{63\text{-}228}$) (Table 1) (Yoshioka et al., 2008). This target sequence is different from that of the original UAS-*dNF-YAIR*$_{231\text{-}399}$ transgenic fly strains (Table 1). Crossing one of these UAS-*dNF-YAIR*$_{63\text{-}228}$ strains (strain 81) with the *GMR*-GAL4 driver also resulted in a rough eye phenotype, while the other two

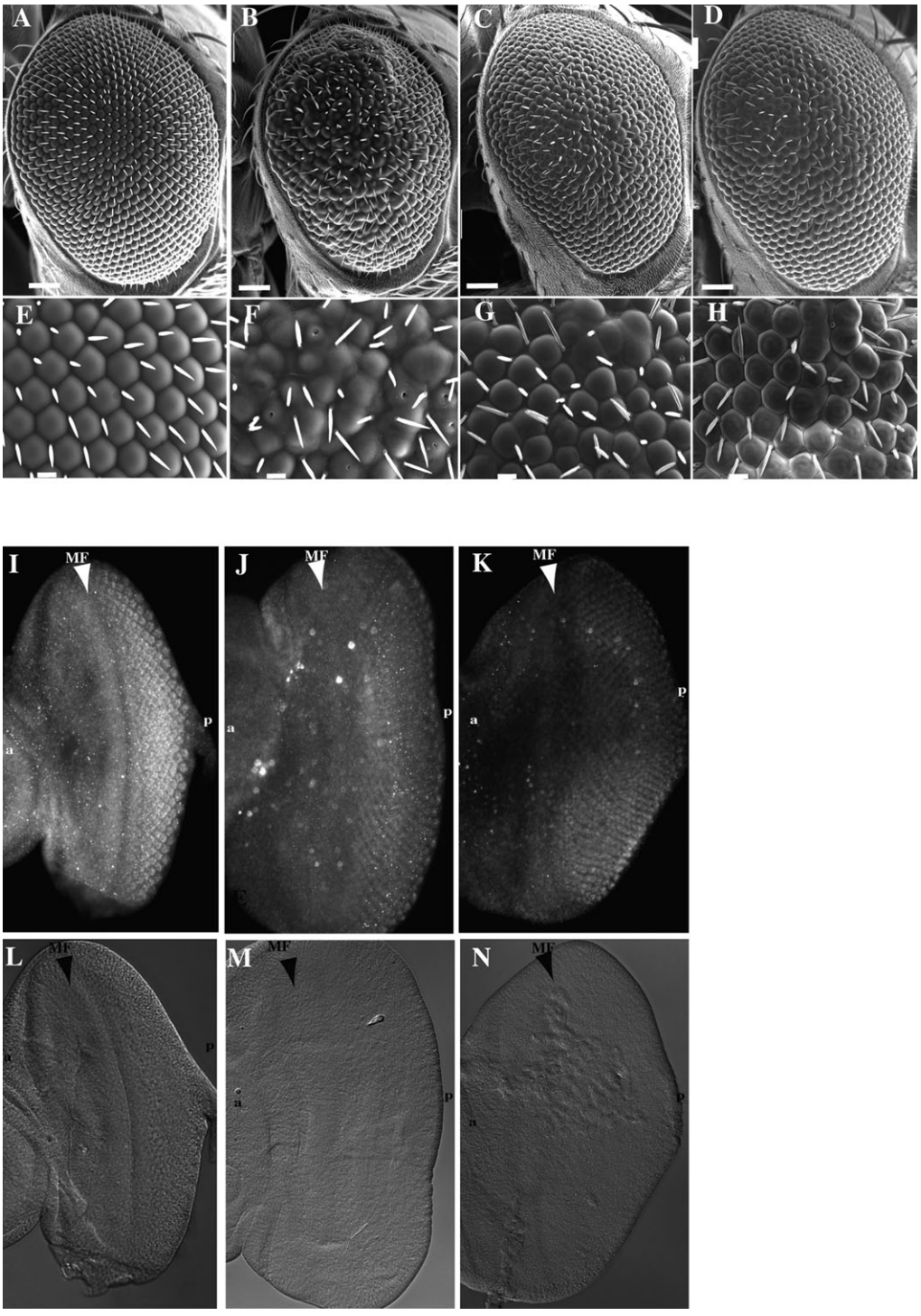

Fig. 2. Knockdown of dNF-YA induced a rough eye phenotype. **(A–H)** Scanning electron micrographs of an adult compound eye. **(I–K)** Immunostaining of the eye imaginal discs with anti-dNF-YA antibody. **(L–N)** Nomarski images of the eye discs shown in panels I to K. **(A, E, I, L)** *GMR*-GAL4/*w*. **(B, F, J, M)** *GMR*-GAL4/*w*; UAS-*dNF-YAIR*$_{231-399}$/+ (strain 67). **(C, G, K, N)** *GMR*-GAL4/*w*; UAS-*dNF-YAIR*$_{63-228}$/+ (strain 81). **(D, H)** *GMR*-GAL4/*w*; UAS-*dNF-YAIR*$_{231-399}$/+; UAS-*HA-dNF-YA*/+. Scale bars are for 50 µm in (A) to (D) and 10 µm in (E) to (H). The arrowhead indicates morphogenetic furrow (MF). a, anterior; p, posterior.

exerted no apparent effect on eye morphology (Fig. 2 and Table 1). Differences in phenotype of *GMR*-GAL4>UAS-*dNF-YAIR* fly lines likely reflect differences in knockdown levels of dNF-YA. Additionally, we examined whether increasing the dNF-YA level suppresses the rough eye phenotype. The *GMR*-GAL4>UAS-*dNF-YAIR* flies exhibited a rough eye phenotype (Fig. 2B, C, F and G). On crossing of UAS-*HA-dNF-YA* flies with the *GMR*-GAL4>UAS-*dNF-YAIR*$_{231-399}$ strain, the progeny flies exhibited an apparently normal eye phenotype (Fig. 2D and H). These results, taken together, indicate that the rough eye phenotypes observed in *GMR*-GAL4>UAS-*dNF-YAIR* flies are due to reduction in the dNF-YA protein level. Strain 67 carrying UAS-*dNF-YAIR*$_{231-399}$ (Table 1) was mainly used for the following detailed studies.

Knockdown of dNF-YA specifically interferes with R7 photoreceptor cell differentiation

Knockdown of dNF-YA by *GMR*-GAL4 exerted no apparent effect on cell cycle progression examined by BrdU incorporation assay (data not shown). Therefore we examined differentiation of

Table 1. Transformants carrying *UAS-dNF-YAIR* transgene

P-element plasmid	Strain	Chromosome linkage	Phenotype
UAS-*dNF-YAIR*$_{231-399}$	4	III	lethal
	5	II	rough eye
	7	III	no effect
	39	III	lethal
	67	II	rough eye
UAS-*dNF-YAIR*$_{63-228}$	15	III	no effect
	81	III	rough eye
	82	III	no effect

Eye morphrogy of adult flies obtained when each strain was crossed with the fly carrying the GMR-GAL4 transgene in X chromosome

photoreceptor cells in the dNF-YA knockdown fly. In wild-type discs, developmentally uncommitted cells are sequentially recruited into clusters that comprise ommatidial precursors. Cluster formation is first observed within the MF, where cells are in G1. Cells either leave the cell cycle and differentiate or undergo a final synchronous round of cell division. Overt ommatidial organization starts in the MF when cells are grouped into equally spaced concentric aggregates, which converted into preclusters. Photoreceptor cells (R) are generated in a stereotyped order. Firstly R8 cells are formed with movement posterior from the furrow, after which cells are added pairwise, R2 and R5, R3 and R4, and R1 and R6. R7 cell is the last photoreceptor to be added to each cluster. Several enhancer trap lines expressing a nuclear-localized form of *E. coli* β-galactosidase depend on the specific enhancer-promoter located nearby the P-element. They were here used to determine the identities of each photoreceptor. We employed two enhancer trap lines, AE127

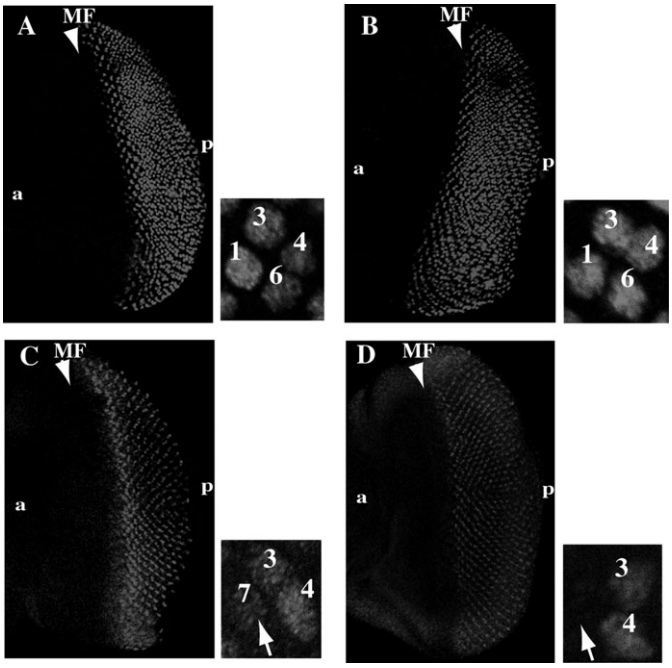

Fig. 3. Expression of *dNF-YA*dsRNA inhibits R7 development in eye imaginal discs. R3, R4, R1, R6 photoreceptor cells are marked by the AE127 (*svp-lacZ*) enhancer trap line and the R3, R4, R7 photoreceptor cells are marked by the P82 (*deadpan-lacZ*) enhancer trap line. **(A)** *GMR*-GAL4/*w*; *svp-lacZ*/+. **(B)** *GMR*-GAL4/*w*; UAS-*NF-YAIR*/+; *svp-lacZ*/+. **(C)** *GMR*-GAL4/*w*; *deadpan-lacZ*/+. **(D)** *GMR*-GAL4/*w*; UAS-*NF-YAIR*/*deadpan-lacZ*. The insets illustrate ommatidia. The arrow indicates the position of an R7 cell. a, anterior; p, posterior; MF, morphogenetic furrow.

(inserted in *seven-up*) and P82 (inserted in *deadpan*), specifically expressing the β-galactosidase marker in photoreceptor cells of R3/R4/R1/R6 and R3/R4/R7, respectively. Eye imaginal discs from F1 larva from mating of enhancer trap lines and *GMR*-GAL4>UAS-*dNF-YAIR*$_{231-399}$ transgenic flies were immunohistochemically stained with the anti-β-galactosidase antibody. In ommatidia of *GMR*-GAL4>UAS-*dNF-YAIR* flies, nuclei of R3/R4/R1/R6 demonstrated a similar staining pattern as nuclei of control ommatidia (Fig. 3A and B). With P82 the ommatidia of *GMR*-GAL4>UAS-*dNF-YAIR* flies were found to contain R3 and R4 signals, but no R7 signals were detected (Fig. 3C and D). Loss of R7 signals in dNF-YA-knockdown flies was also confirmed with an R7 specific enhancer trap line B38 (inserted in the *klingon* gene) as described below (Fig. 9). The results suggest that expression of *dNF-YAIR* specifically inhibited the differentiation of R7 photoreceptor cells.

The 5′ flanking region of the *sevenless* gene contains a NF-Y consensus sequence and dNF-YA binds to genomic regions containing this motif in cultured cells

NF-Y is a major CCAAT-binding transcription factor that specifically recognizes consensus sequences, 5′-CTGATTGG-YYRR-3′ or 5′-YYRRCCAATCAG-3′ (Y, pyrimidines and R, purines), present in promoter regions (Matsuoka and Chen, 1999). dNF-Y can also bind to the same consensus sequences in vitro (Yoshioka et al., 2007). A data base search revealed that the 5′ flanking region of the *sev* gene contains two CCAAT motifs at −10 and −310 with respect to the transcription initiation site (Fig. 4). These two sites match 11 out of 12 and 8 out of 12 NF-Y-binding consensus sequences, respectively. To obtain further insight into dNF-Y-binding to these CCAAT motifs in the *sev* gene, we performed chromatin immunoprecipitation assays with anti-dNF-YA IgG. Immunoprecipitated samples were subjected to quantitative real-time PCR using primers to amplify the *sev* gene promoter region containing the NF-Y-binding consensus (Fig. 4, region 1). In this ChIP assay two CCAAT motifs at −10 and −310 could not be distinguished, since average size of genomic DNA fragments subjected to the immunoprecipitation were 500∼1,000 bp. The 2.5 kb upstream region from the transcription initiation site of the *sev* gene was chosen as a negative control, because it does not contain a NF-Y-binding consensus (Fig. 4, region 2).

Amplification of the *sev* gene promoter region (Fig. 4, region 1) in the immunoprecipitates with anti-dNF-YA IgG was 12.8-fold higher than that with the control rabbit IgG (Fig. 4). In contrast, no amplification was observed for the 2.5 kb upstream region from the transcription initiation site of *sev* (Fig. 4, region 2). These results indicate that dNF-YA binds to the *sev* gene promoter region containing two CCAAT boxes and suggest that dNF-Y regulates *sev* gene expression.

dNF-YA is required for *sevenless* gene promoter activity

To examine role of NF-Y-binding consensus sequences in *sev* gene promoter activity, we constructed the plasmid carrying the *sev* gene promoter (−1,000 to +60) and *sev* enhancer (Basler et al., 1989) fused with the luciferase reporter gene (psevPE-lucwt) (Fig. 5A) and a derivative carrying mutations in the NF-Y consensus 1 (psevPE-lucNF-Ymut1), 2 (psevPE-lucNF-Ymut2) and 1, 2 (psevPE-lucNF-Ymut1,2). The *sev* gene promoter without the *sev* enhancer showed very weak promoter activity in S2 cells (data not shown) and the *sev* enhancer located in the

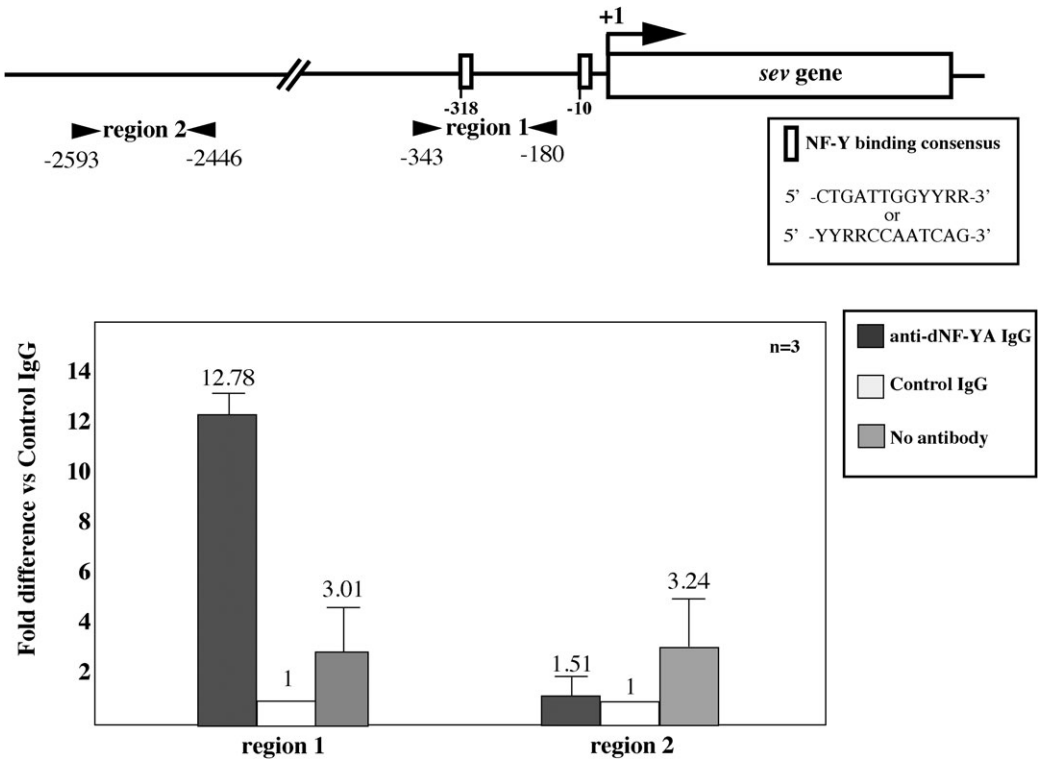

Fig. 4. Examination of NF-YA-binding in the 5′-flanking region of the *sev* gene by chromatin immunoprecipitation. (A) Schematic illustration of NF-Y consensus in the 5′-flanking region of the *sev* gene. The transcription initiation site is indicated by the arrow and designated as +1. Arrowheads show the positions of primers used for the chromatin immunoprecipitation assays for two genomic regions (regions 1 and region 2). The boxes indicate NF-Y-binding consensus sites located at −10 and −318. **(B)** Chromatin immunoprecipitation for two genomic regions of the *sev* gene. The data shown are derived from quantitative real-time PCR analysis of two genomic regions, 1 and 2, of the *sev* gene shown in panel A. Chromatin from S2 cells was immunoprecipitated with either anti-dNF-YA IgG or control rabbit IgG. The fold difference values are for anti-dNF-YA immunoprecipitated samples (shown as anti-dNF-YA IgG column) compared to the corresponding control rabbit IgG immunoprecipitated samples (control IgG column) defined as 1. A sample without antibody treatment was also included as a negative control (no antibody column). Mean values with standard deviations from three independent immunoprecipitations are shown.

second intron of the *sev* gene is known to be required for eye disc-specific expression (Basler and Hafen 1988). When our plasmids were transfected into S2 cells, and 48 h later, luciferase activities were determined (Fig. 5B), the base-substituted mutations in the NF-Y consensus 1 were found to reduce *sev* gene promoter activity by 86% (Fig. 5B). In contrast, mutations in the NF-Y consensus 2 exerted no significant effects on *sev* gene promoter activity. Mutations in both NF-Y consensus 1 and 2 reduced *sev* gene promoter activity to a similar level as that with mutations in NF-Y consensus 1 (Fig. 5B). These results indicate that the proximal NF-Y consensus 1 plays a major role in *sev* gene promoter activity in cultured cells.

Furthermore, we performed *lacZ* reporter assays in living transgenic flies carrying the *sev* promoter, enhancer and *lacZ* fusion gene (Fig. 5C). The transgenic fly line carrying the wild type *sev* promoter and enhancer showed high expression of lacZ in photoreceptor cells appearing in the region posterior to the morphogenetic furrow (MF), as reported previously (Fig. 5D) (Basler et al., 1989). The transgenic fly line carrying a mutation in site 1 exhibited reduced *lacZ* expression, especially in the region proximal to MF (Fig. 5F). These results again suggest that NF-Y-binding consensus 1 plays a critical role in appropriate *sev* promoter activity in vivo.

To further explore the requirement of dNF-YA for *sev* gene promoter activity, dNF-YA RNA interference experiments in S2 cells were carried out (Fig. 6). Measuring levels of dNF-YA proteins by the Western immunoblot analysis confirmed efficient knockdown of the dNF-YA gene after treatment with dsRNA, as observed previously (Fig. 6B) (Yoshioka et al., 2008). Transient luciferase expression assays were conducted with the wild type *sev* gene promoter-luciferase reporter gene, after treating S2 cells with *dNF-YA*dsRNA (dsdNF-YA) or *LacZ*dsRNA (dsLacZ). Treatment of cells with *dNF-YA*dsRNA reduced the wild type *sev* gene promoter activity by 27.4% as compared to control *LacZ*dsRNA treatment (Fig. 6A). Although the extent of reduction was not great, it was statistically significant. In contrast, mutant type *sev* promoter activity was not changed by *dNF-YA*dsRNA as compared to *LacZ*dsRNA treatment. These results indicate that dNF-YA is required for *sev* gene promoter activity in cultured cells.

To further examine roles of dNF-YA in endogenous *sev* gene expression in living flies, the level of *sev* mRNA was quantified by real time PCR (Fig. 7). In the experiments, the *Rp49* gene carrying no NF-Y-binding consensus was used as a negative control (Fig. 7, Rp49 columns). The dNF-YA mRNA level in *Act5C*-GAL4/UAS-*dNF-YAIR*$_{231\text{-}399}$ larvae was 23 % of that of the wild type Canton S (Fig. 7, *dNF-YA* columns), confirming efficient knockdown of dNF-YA in the transgenic larvae. The *sev* mRNA level in the dNF-YA knockdown larvae was 30 % of that of the wild type Canton S (Fig. 7, *sev* columns). However, no such reduction of *sev* mRNA levels was observed in transgenic flies carrying *Act5C*-GAL4 alone. These results further support that dNF-YA is required for endogenous *sev* gene expression in vivo.

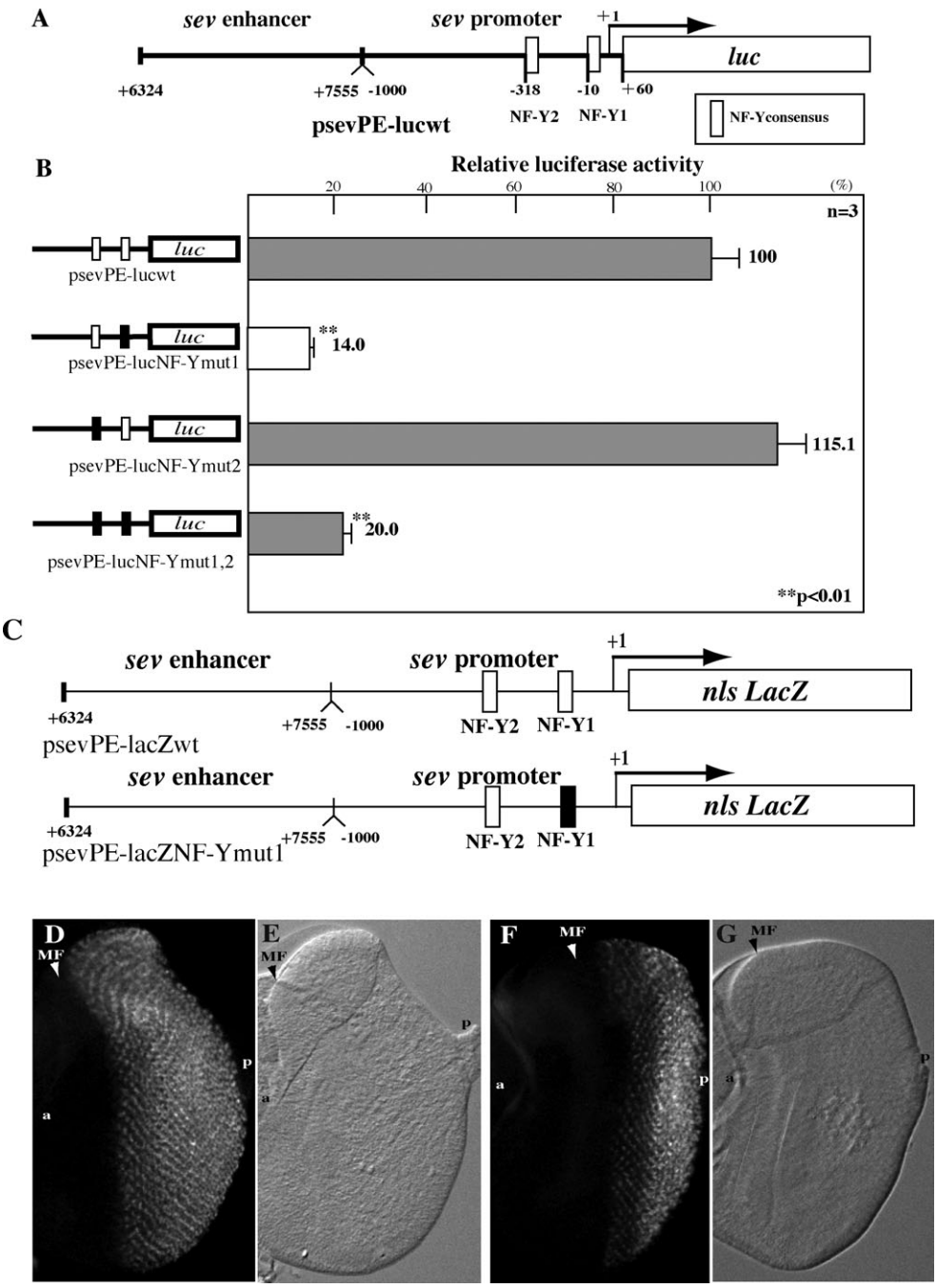

Fig. 5. Roles of NF-Y consensus sequences in promoter activity of the *sev* gene in S2 cells and in eye imaginal discs. **(A)** Schematic features of the *sev* promoter-enhancer-*luciferase* fusion plasmid, psevPE-lucwt, are illustrated. NF-Y consensus 1 and 2 are indicated by open boxes. **(B)** The psevPE-lucwt and its base-substituted derivatives, psevPE-lucNF-Ymut1, psevPE-lucNF-Ymut2 and psevPE-lucNF-Ymut1,2 are shown, with mutated CCAAT indicated by closed boxes. Plasmids were transfected into S2 cells and promoter activities measured 48 h thereafter. Luciferase activity was normalized to Renilla luciferase activity and expressed relative to that of psevPE-lucwt. The mean values with standard deviations from four independent transfections are shown. The P-value by Welch's *t*-test is also given. **(C)** Schematic features of the *sev* promoter-enhancer-*lacZ* fusion plasmids, psevPE-lacZwt and its base-substituted derivative, psevPE-lacZNF-Ymut1, are shown. **(D)** Expression of lacZ in the eye disc from the transgenic fly carrying the wild type *sev* promoter-enhancer-*lacZ* fusion gene. **(E)** Nomarski image of the eye disc in panel D. **(F)** Expression of lacZ in the eye disc from the transgenic fly carrying the mutant type *sev* promoter-enhancer-*lacZ* fusion gene. **(G)** Nomarski image of the eye disc shown in panel F. a, anterior; p, posterior; MF, morphogenetic furrow.

Expression of sev or D-raf suppresses the rough eye phenotype induced by knockdown of dNF-YA

To further confirm that the rough eye phenotype induced by knockdown of dNF-YA depends on *sev* gene transcription and activation of downstream MAPK signaling, we performed expression experiments with *sev* or its downstream gene *D-raf* in dNF-YA knockdown flies. Co-expression of Sev or D-raf rescued the rough eye phenotype induced by knockdown of dNF-YA (Fig. 8), suggesting that the rough eye phenotype is truly induced by reduction of sev levels and its downstream signaling.

Next, we examined R7 photoreceptor signals in these flies by crossing with an R7 specific enhancer trap line B38 (inserted in the *klingon* gene) (Fig. 9). The quantified data for R7 signals per eye discs are also shown (Fig. 9G). R7 signals were detected in eye discs of *GMR*-GAL4; B38/+ flies (Fig. 9A and G), but not in those from dNF-YA knockdown flies (Fig. 9B and G) and overexpression of D-raf recovered the R7 signals (Fig. 9C and G). These results also support the idea that dNF-YA regulates R7 photoreceptor cell differentiation by regulating *sev* gene transcription.

Discussion

Many *in vitro* studies have provided evidence that mammalian NF-Y regulates transcription of a number of genes related to biological processes like cell cycle regulation, development and immunity (Fang et al., 2004; Gilthorpe et al., 2002; Grujicic et al., 2005; Huang, et al., 2005; Niimi et al., 2004; Reith et al., 1994; Wiebe et al., 2000). While, genes that are actually regulated by NF-Y in vivo remain largely to be determined, the fact that

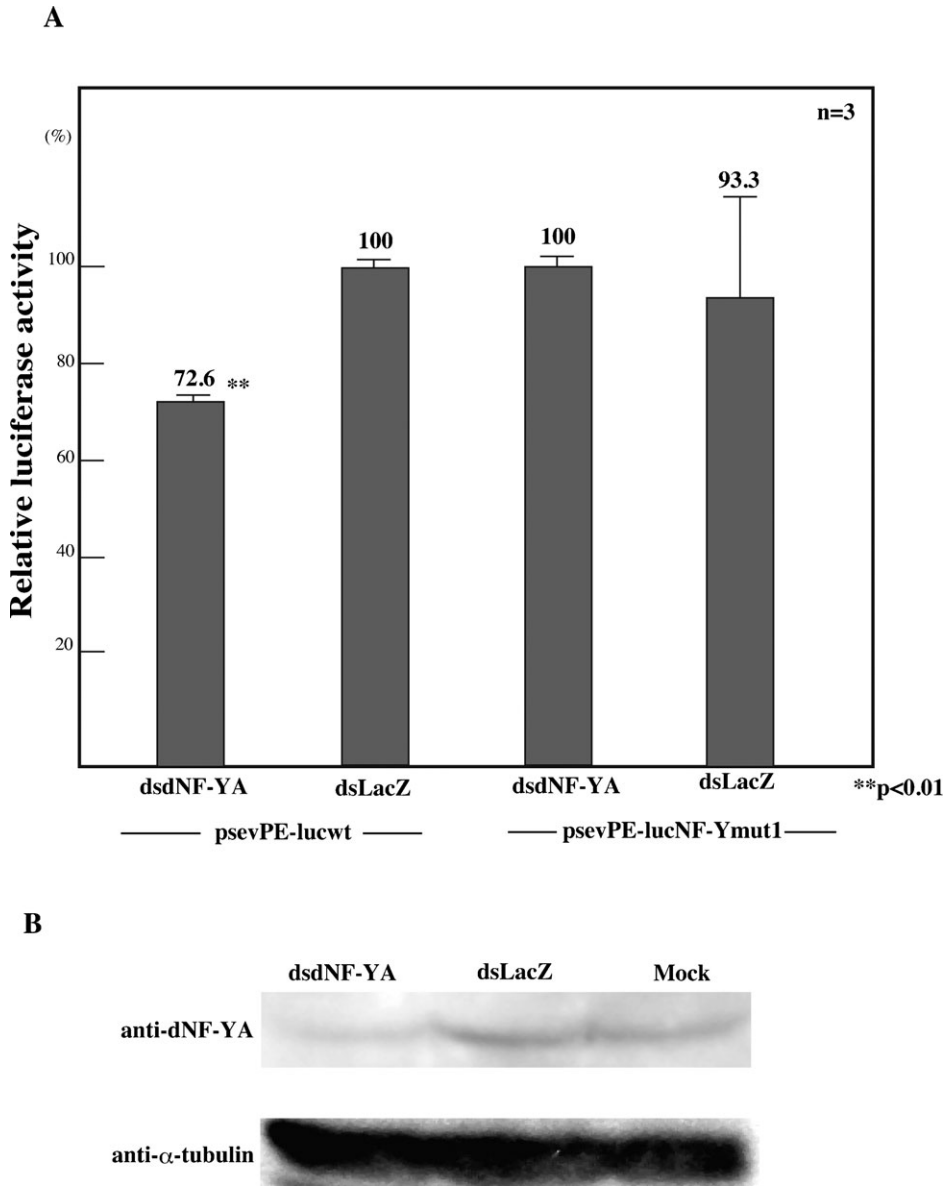

Fig. 6. Effects of dNF-YAdsRNA treatment on *sev* gene promoter activity in S2 cells. (A) Four days after treatment with dNF-YAdsRNA (dsdNF-YA) or LacZdsRNA (dsLacZ), S2 cells were transfected with 0.5 μg each of psevPE-lucwt or psevPE-lucNF-Ymut1. Promoter activity was measured at 48 h thereafter. The luciferase activity was normalized to Renilla luciferase activity. The luciferase activity relative to that of LacZdsRNA treated cells is shown. Mean activities with standard deviations from three independent transfections are shown, with the P-value by Welch's *t*-test. **(B)** Western immunoblot analysis of cells treated with dNF-YAdsRNA (dsdNF-YA), LacZdsRNA (dsLacZ) or no dsRNA (Mock). Proteins were probed with anti-dNF-YA antibody and anti-α-tubulin antibody.

knockout of mouse NF-YA results in early embryonic lethality indicates essential roles in early development (Bhattacharya et al., 2003). In our *Drosophila* system, we earlier found that dNF-Y participates in various gene regulatory pathways during development (Yoshioka et al., 2007). In addition, analyses dNF-YA overexpressing flies revealed that overexpressed dNF-YA can disturb eye disc specification, but not eye disc growth (Yoshioka et al., 2007). In the present study, we clarified a novel function of dNF-Y in regulation of the *sev* signal transduction pathway that has not been found in mammalian systems.

dNF-Y subunits are related to R7 photoreceptor development

In addition to the role of dNF-Y in positive regulation of the *sev* gene in R7 photoreceptor cells clarified in this study, participation in other processes in R7 cells has been demonstrated (Morey et al., 2008) . Targeting of *Drosophila* R7 and R8 photoreceptor axons to different synaptic layers in the brain has been used as a model to study the genetic program regulating target specificity. Loss of function mutation in the *dNF-YC* gene was identified by a genetic screen for R7 targeting mutants (Morey et al., 2008). In the *dNF-YC* mutant the R8-specific transcription factor Senseless (Sens) is ectopically expressed in a late stage of R7 differentiation that results in targeting defects in R7 axons. Therefore in R7 cells it is likely that dNF-Y positively regulates the *sev* gene and negatively regulates the *Sens* gene. It should be noted that NF-Y has been reported to act as both an activator and a repressor in other organisms (Morey et al., 2008). Differential effects of dNF-Y on transcription may depend on differences in the gene and/or chromatin context, although further analyses are necessary to address this point.

Does human *sevenless* homolog contain NF-Y-binding consensus sequences?

In humans, a *sevenless* homolog, oncogene *Ros1*, has been reported (Tessarollo et al., 1992), whose 5′ flanking region contains two CCAAT boxes at −227 and −339 with respect to the transcription initiation site. Therefore, human NF-Y might regulate its expression as in the *Drosophila* NF-Y case. The *Ros1* gene is involved in the MAPK cascade that is triggered by a variety of signals including

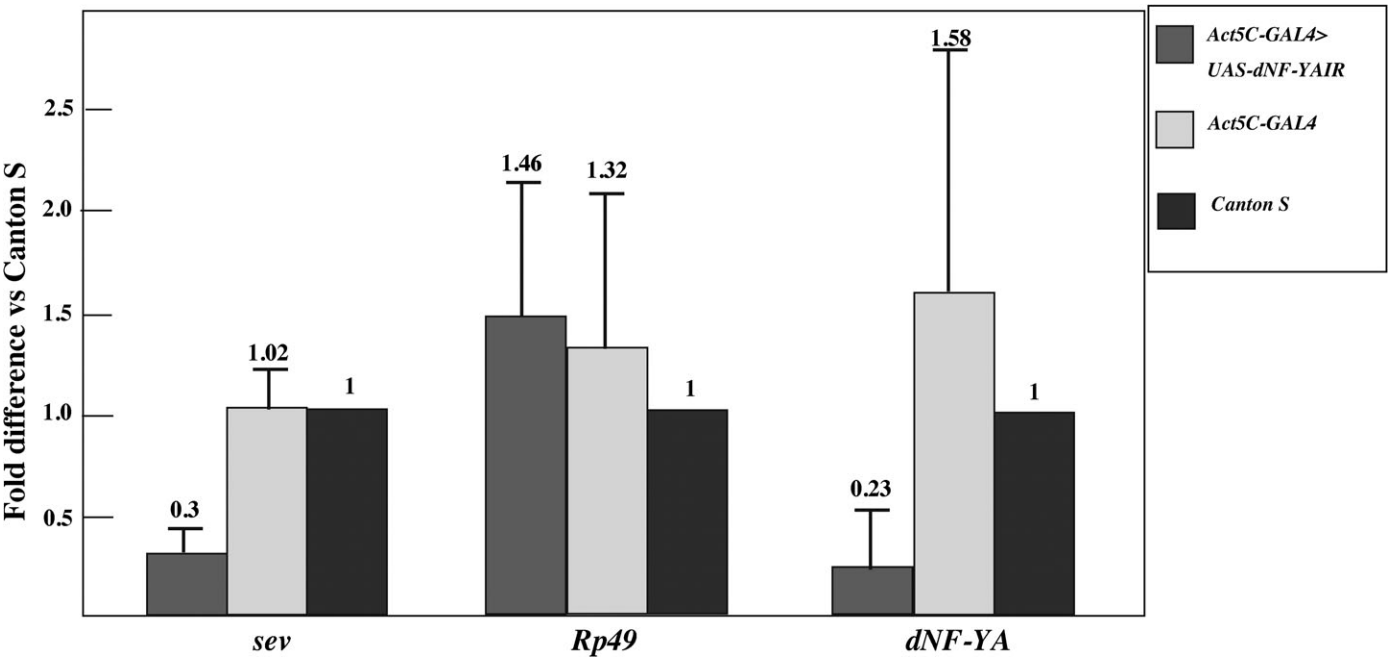

Fig. 7. Knockdown of dNF-YA reduces *sev* mRNA levels in third instar larvae. *dNF-YA* mRNA and *sev* mRNA levels were measured by quantitative RT-PCR. mRNA for *Rp49* was used as a negative control. Fold differences against the amplification with RNA samples from Canton S are shown with standard deviations from three independent preparations of RNA.

examples important for the immune systems or cell proliferation (Fang et al., 2004; Gilthorpe et al., 2002; Grujicic et al., 2005; Huang et al., 2005; Niimi et al., 2004; Reith et al., 1994; Wiebe et al., 2000). It should also be noted that mammalian NF-Y also regulates expression of various genes related to immune responses such as *γ-globin* and *Major histocompatibility (MHC) class II*.

Materials and Methods

Oligonucleotides

To construct the plasmids psevPE-lucwt, psevPE-lucNF-Ymut1, psevPE-lucNF-Ymut2 and psevPE-lucNF-Ymut1,2, psevPE-lacZwt and psevPE-lacZNF-Ymut1 the following oligonucleotides were synthesized.

sevUS1000MluI: 5′-CGGACGCGTTCCTCGGTAACAAAGCCCACAATGC
sevUS-60BglII: 5′-TCAGATCTTCTGGATGTGCGGATTCCCGAAGCGT
sevenhancerFKpnI: 5′-GGGGTACCCTCGAGGTCTCTCTCCCTGCTCCACA
sevenhancerRMluI: 5′-CGGACGCGTCTGAGATCCAGCTCCTCGCCGTGG
sevUS-60BamHI: 5′-CGGGATCCTTCATTCTGGATGTGCGGATTCCCGAAGCGT
sevNF-Ymut1F: 5′-CGCGCGATCGCAGCAGAACTGGCGTTCCGGCGAGCGGCGGCTTTT
sevNF-Ymut1R: 5′-AAAAGCCGCCGCTCGCCGGAACGCCAGTTCTGCTGCGATCGCGC
sevNF-Ymut2F: 5′-GACGCAGCAACTATGACGTCGCGTTCAGGGCAACCCCTAAACTGG
sevNF-Ymut2R: 5′-CCAGTTTAGGGGTTGCCCTGAACGCGACGTCATAGTTGCTGCTC

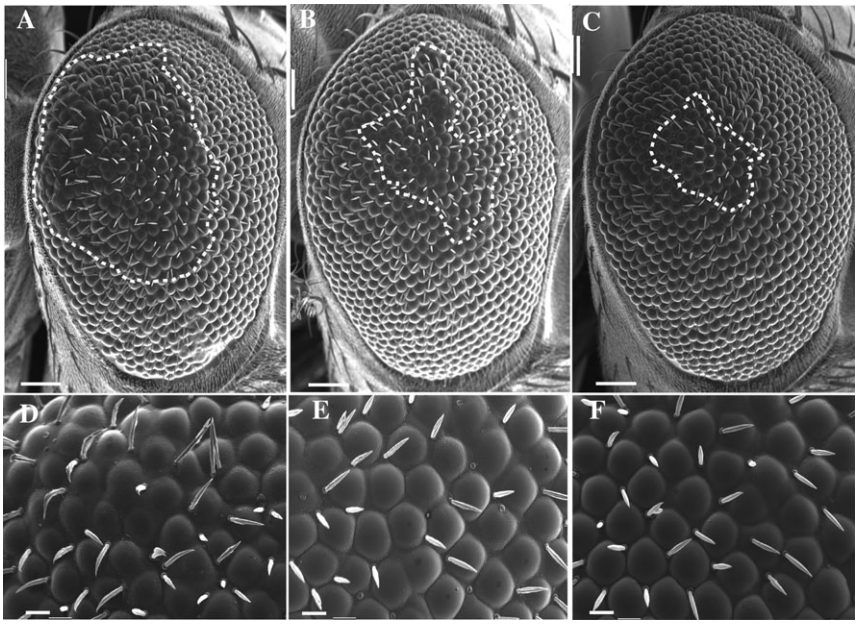

Fig. 8. Scanning electron micrographs of adult compound eyes. Expression of *sev* or *D-raf* suppressed the rough eye phenotype induced by knockdown of dNF-YA. **(A, D)** *GMR* -GAL4/*w*;UAS-*dNF-YAIR*$_{231-399}$/+. **(B, E)** *GMR*-GAL4/*hs-sev*;UAS-*dNF-YAIR*/+;. **(C, F)** *GMR*-GAL4/*w*; UAS-*dNF-YAIR*$_{231-399}$/+;*hs-D-raf* /+. Scale bars are for 50 μm in (A) to (C) and for 10 μm in (D) to (F). The rough areas of the compound eyes are marked with dot lines.

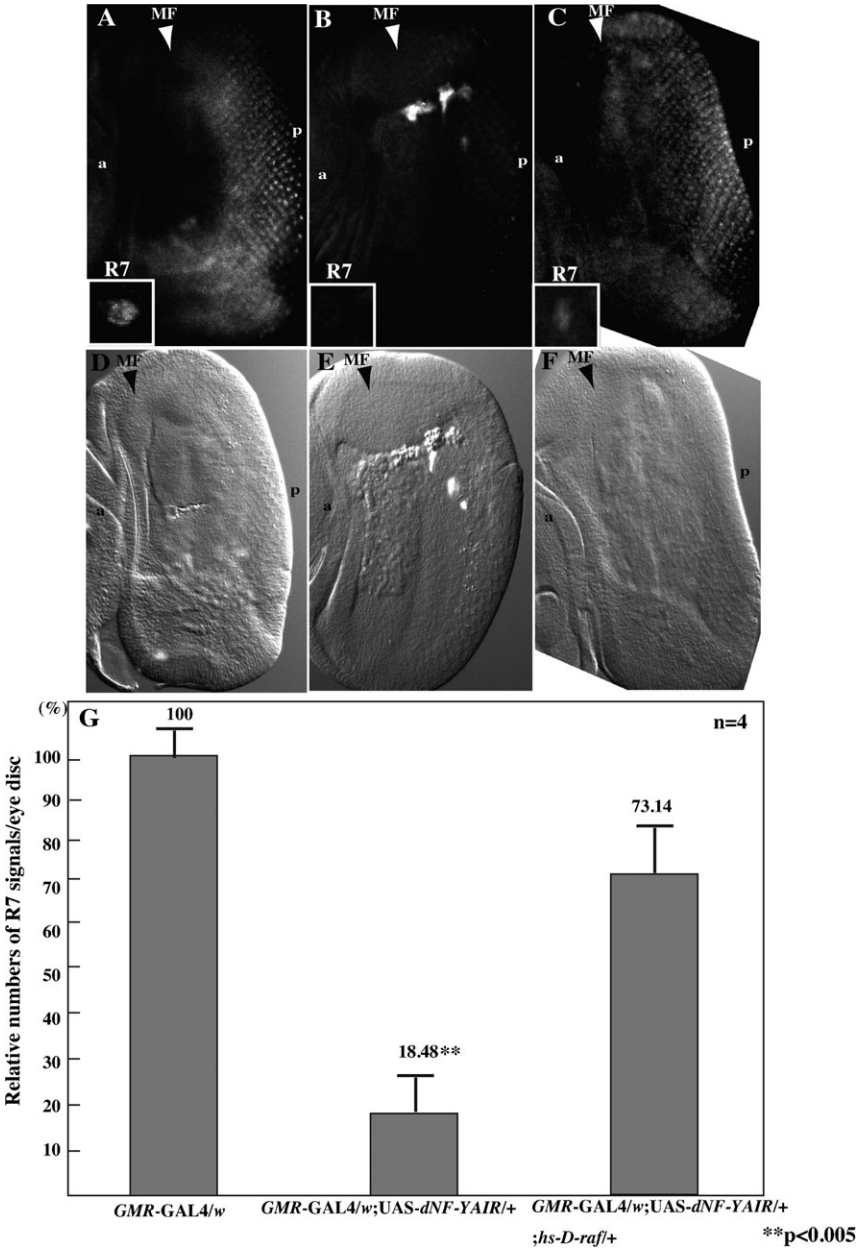

Fig. 9. Expression of *sev* or *D-raf* suppresses reduction of R7 signals induced by knockdown of dNF-YA. (A, B and C) R7 photoreceptor cells are marked by B38 (*klingon-lacZ*) enhancer trap line. **(A)** *GMR*-GAL4/*w*;B38/+. **(B)** *GMR*-GAL4/*w*; UAS-*dNF-YAIR*$_{231\text{-}399}$/+; B38/+. **(C)** *GMR*-GAL4/*w*;UAS-*dNF-YAIR*$_{231\text{-}399}$/+;B38/*hs-Draf*. **(D, E and F)** Nomarski images of the eye discs shown in panels A, B and C, respectively. The insets illustrate R7 cells. a, anterior; p, posterior; MF, morphogenetic furrow. **(G)** Summary of the numbers of R7 signals in the eye imaginal disc of *GMR*-GAL4/*w*;B38/+, *GMR*-GAL4/*w*; UAS-*dNF-YAIR*$_{231\text{-}399}$/+; B38/+ and *GMR*-GAL4/*w*;UAS-*dNF-YAIR*$_{231\text{-}399}$/+;B38/*hs-Draf* flies. Mean activities with standard deviations from four discs are shown, with the P-value by Welch's *t*-test.

To carry out chromatin immunoprecipitation, the following PCR primers were chemically synthesized. These primer sets were designed to amplify 150 bp amplicons. sevus180F: 5′-AACCGAACTGAACCGATCTTAA
sevus343R: 5′-TGAGTTGAGCTTTGACCCATGGAAAGA
sevus2446F: 5′-TATTATGCTGATTAGGCAGGTCAGACG
sevus2593R: 5′-GAACGCAACTTACGATGCTCTGCTTAT

To carry out quantitative real time PCR, the following oligonucleotides were synthesized.
sevRT-F: 5′-AGCAGCCGCCCATGTGTACGGAGAA
sevRT-R: 5′-CATTTGGGTGCGCCGCAAARCGGTG

To carry out quantitative real time PCR, the following oligonucleotides were also used (Tue, et al., 2010; Yoshioka et al., 2008).
RPLP0-F: 5′- AGCTGCTACCCCACATCAAG
RPLP0-R: 5′- TGTTCCCTTGGAAATTTTGG
RP49-RT.F: 5′- GCTTCTGGTTTCCGGCAAGCTTCAAG
RP49-RT.R: 5′- GACCTCCAGCTCGCACGTTGTGCACCAGGAAC
Nhe1NF-YA-F: 50-CTAGCTAGCCATCAACAAGTACAATCCCAGAC
NF-YA-RXba1: 50-GCTCTAGACTATTCCGATTTGATCGCCGT

Plasmid construction

To construct the plasmid psevPE-lucwt, PCR was performed using *Drosophila* genomic DNA as a template and sevUS1000MluI and sevUS-60XhoI primers in combination. PCR products were digested with *Mlu*I and *Xho*I and inserted between the *Mlu*I and *Xho*I sites of the PGVB plasmid (Toyo Ink). Then, PCR was performed using *Drosophila* genomic DNA as a template and sevenhancerFKpnI and sevenhancerRMluI primers in combination. PCR products were digested with *Kpn*I and *Bgl*II and inserted between the *Kpn*I and *Bgl*II sites.

For site-directed mutagenesis, PCR was carried out using a Quick Change Site-Directed Mutagenesis Kit (Stratagene). Oligonucleotide pairs carrying base-substitutions in the region of interest were used as primers and the psevPE-Lucwt DNA as a template for the PCR. Fully amplified PCR products were digested with *Dpn*I to remove the methylated template DNA and then transformed into *E. coli* XL-1 blue. The mutated nucleotide sequences were confirmed by nucleotide sequencing and the resultant plasmids were named psevPE-LucNF-Ymut1, psevPE-lucNF-Ymut2 and psevPE-lucNF-Ymut1,2. To construct the plasmid psevPE-lacZwt and psevPE-lacZNF-Ymut1, PCR was performed using psevPE-lucwt and psevPE-lucNF-Ymut1 plasmid DNA as a template and primers sevenhancerRKpnI and sevUS-60BamHI in combination. PCR products were digested with *Kpn*I and *Bam*HI and inserted between the *Kpn*I and *Bam*HI sites of the pOBP-lacZ plasmid (Galindo and Smith, 2001).

Fly stocks

Flies were cultured at 25 ℃ on standard food. The Oregon R or Canton S flies were used as the wild-type strain. The transgenic fly lines carrying UAS-*dNF-YAIR*$_{231\text{-}}$

$_{399}$, UAS-*dNF-YAIR*$_{63-228}$ and UAS-*HA-dNF-YA* were as described earlier (Yoshioka et al., 2007; Yoshioka et al., 2008). The transgenic fly lines carrying GMR-GAL4 were described earlier (Yoshioka et al., 2007). The AE127(svp-lacZ)/*TM6B*, *P82(deadpan-lacZ)/CyO*, *B38(Klingon-lacZ)* lines were kindly provided by Dr Y. Hiromi and the hs-*flp* and *Act5C>FRT y FRT>*GAL4, UAS-*GFP* lines by Dr T. Adachi-Yamada.

Establishment of transgenic flies

P-element-mediated germ line transformation was carried out as described earlier (Spradling, 1986) and F1 transformants were selected on the basis of white-eye color rescue (Robertson et al., 1988). Three independent lines were established for psevPE-lacZwt and psevPE-lacZNF-Ymut1, respectively. These independent transgenic lines showed essentially the same expression pattern of *lacZ* in eye imaginal discs.

Flip out experiments

RNAi clones in wing discs were generated with a flip-out system (Sun et al., 1999). Female flies with *hs-flp*; *Act5C>FRT y FRT>* GAL4, UAS-*GFP* were crossed with male flies with UAS-*dNF-YAIR*$_{231-399}$ and clones were marked by the presence of GFP expressed under control of the *Act5C* promoter. Flip-out was induced 24-48 hours after egg laying with 60 minutes heat shock at 37℃.

Scanning electron microscopy

Adult flies were anesthetized, mounted on stages and observed with a VE-7800 (Keyence Inc.) scanning electron microscope 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.

Immunohistochemistry

Third instar larvae were dissected in *Drosophila* Ringer's solution and imaginal discs were collected and fixed in 4% paraformaldehyde in PBS for 10 minutes at 4℃ or 30 minutes at 25℃. After washing with PBS containing 0.3% Triton X-100 (PBS-T), the samples were blocked with PBS-T containing 10% normal goat serum for 20 minutes at 25℃ and incubated with an anti-β-galactosidase mouse monoclonal (DSHB) (1:500) or anti-dNF-YA rabbit polyclonal (1:500) antibodies at 4℃ for 16 hours. After extensive washing with PBS-T, the imaginal discs were incubated with an anti-mouse IgG conjugated with Alexa 594 (Invitrogen) (1:400) for 16 hours at 4℃. After further washing with PBS-T and PBS, samples were mounted in Fluoroguard Antifade Reagent (Bio-Rad) and inspected with an Olympus BX-50 microscope equipped with a cooled CCD camera (Hamamatsu Photo).

Preparation of double stranded RNA for RNA interference experiments

The 355 nucleotides of cDNA spanning the DNA-binding domain (aa282 to aa399) of dNF-YA were cloned into pBluescript II SK(-) and the plasmid was used for synthesizing double stranded RNA (dsRNA), using RiboMax T7 (Promega) and MEGAscript T3 (Ambion) kits according to the manufacturer's instructions. RNA interference (RNAi) analysis was carried out as described earlier (Seto et al., 2006: Ida et al., 2007).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using a ChIP Assay kit as recommended by the manufacturer (Upstate) with minor modifications (Thao et al, 2006). Approximately 2×10^7 S2 cells were fixed in 1% formaldehyde at 37℃ for 10 minutes, quenched in 125 mM glycine for 5 minutes at 25℃, collected and washed twice in PBS containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatin A) and then lysed in 2 ml of SDS lysis buffer (Upstate). Lysates were sonicated to break DNA into fragments of less than 1 kb and centrifuged at $15,300 \times g$ for 10 minutes at 4℃. The sonicated cell supernatants were diluted 10 fold in ChIP Dilution Buffer (Upstate) and pre-cleared with 80 μl of salmon sperm DNA/protein G agarose–50% slurry for 30 minutes at 4℃. After brief centrifugation, supernatants were incubated with 4 μg of normal rabbit IgG (Sigma) or anti-dNF-YA IgG for 16 hours at 4℃. Salmon sperm DNA/protein G agarose–50% slurry was added, followed by incubation for 1 hour at 4℃. After washing, immunoprecipitated DNA was eluted with the elution buffer containing 1% SDS and 0.1M $NaHCO_3$. Then protein–DNA crosslinks were reversed by heating at 65℃ for 4 hours. After deproteinization with proteinase K, DNA was recovered. Then, the immunoprecipitated DNA fragments were detected by quantitative real time PCR using SYBR Green I (Takara) and the Applied Biosystems 7,500 Real Time PCR system. The $^{\Delta\Delta}$CT value of each sample was calculated by subtracting the CT value for the input sample from the CT value obtained for the immunoprecipitated sample. Fold differences of each sample relative to control non-immune IgG were then calculated by raising 2 to the $^{\Delta\Delta}C_T$ power. The $^{\Delta\Delta}C_T$ was calculated by subtracting the $^{\Delta}C_T$ value for the sample immunoprecipitated with control IgG (Morrison et al., 1998).

Luciferase transient expression assays

For luciferase transient expression assays, 1×10^5 S2 cells were plated in 24-well dishes. Transfection of various DNA mixtures was performed using Cell-Fectin reagent (Invitrogen) and cells were harvested 48 hours thereafter. Luciferase activity was measured as described earlier (Hayashi et al., 1999: Seto et al., 2006: Ida et al., 2007) and normalized to Renilla luciferase activity using pAct5C-seapansy (Sawado et al., 1998) as an internal control. All plasmids for transfection were prepared using a QIAGEN Plasmid Kit.

For dsRNA interference experiments, thirty μg of *dNF-YA*dsRNA or *LacZ*dsRNA were added to 1×10^6 S2 cells plated in each of 6-well dishes. Seventy-two hours after the RNAi treatment, the cells were transfected with various DNA mixtures and harvested 48 hours later for processing for the luciferase assay as described above.

All transient expression data reported in this paper are means from three independent experiments, each performed in triplicate. Average relative luciferase activity was graphed and statistically analyzed with the Welch's *t*-test.

Western immunoblot analysis

Whole cell extracts from S2 cells prepared as described earlier (Yoshioka et al., 2008) were applied to SDS-10% polyacrylamide gels and transferred to PVDF membranes. Blotted membranes were blocked with Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 8.3 and 150 mM NaCl) containing 10% skim milk for 1 hour at 25℃ and incubated with the anti-dNF-YA antibody at 1:500 dilution, or an anti-α tubulin monoclonal antibody (Sigma) at 1:2,000 dilution at 4℃ for 16 hours. After washing with TBS, the blots were incubated with a horseradish peroxidase-labeled anti-rabbit IgG and a horseradish peroxidase-labeled anti-mouse IgG (GE healthcare) at a 1:5,000 dilution for 1 hour at 25℃. Detection was performed with ECL Western blotting detection reagents (GE healthcare), and images were analyzed with a Lumivision Pro HSII image analyzer (Aisin Seiki).

Quantitative RT-PCR

Total RNA was isolated from third instar larvae (wandering stage) using Trizol®Reagent (Invitrogen) and one μg aliquots were reverse transcribed with oligo(dT) primers using a Takara high fidelity RNA PCR kit (Takara). Then, real-time PCR was performed with a SYBR Green I kit (Takara) and the Applied Biosystems 7500 Real Time PCR system using one μl of reverse transcribed sample per reaction. DNA fragments were amplified using sets of primers sevRT-F and sevRT-R, or dNF-YA691NheI and NF-YA1200XbaI (Yoshioka et al., 2007). Levels of mRNAs in transgenic flies carrying *Act5C*-GAL4/UAS-*dNF-YAIR*$_{231-399}$ or *Act5C*-GAL4/+ and those in Canton S were investigated by the C_T comparative method. The *Rp49* gene was chosen as a negative control (Tue et al., 2010) and the *RPLP0* gene as an endogenous reference (Tue et al., 2010). Experiments were performed in triplicate for each of three RNA batches isolated separately.

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