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-Y (dNF-YA) in eye disc 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.

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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-YN 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′-CGATTGGY3′-3′ or 5′-YRRCCAATCAG-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 IIa*, *cyclin B1*, *CDK25C*, *E2F1*, *CDC2*, and *thyminde 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 been shown 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-Y (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 panner-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-YAIR231-399 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-Y4 double strand RNA (dsRNA) on dNF-Y4 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-Y4 dsRNA (Fig. 1D and E). Although dNF-Y4 is expressed ubiquitously in the eye imaginal 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-YAIR231-399 flies exhibit a pharate-adult lethal phenotype (Yoshioka et al., 2007). However, genetic crossing with other independently established UAS-dNF-YAIR231-399 transgenic strains revealed two out of five GMR-GAL4>UAS-dNF-YAIR231-399 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, penetration 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-YAIR63-228) (Table 1) (Yoshioka et al., 2008). This target sequence is different from that of the original UAS-dNF-YAIR231-399 transgenic fly strains (Table 1). Crossing one of these UAS-dNF-YAIR63-228 strains (strain 81) with the GMR-GAL4 driver also resulted in a rough eye phenotype, while the other two
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 and G). On crossing of UAS-HA-dNF-YA flies with the GMR-GAL4>UAS-dNF-YAIRstrains, 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-YAIR231–399 (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
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 convert 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 specifically recognize consensus sequences, 5'-CTGATTGG-YYRR-3' or 5'-YYRRCCAATCAG-3'.

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'. dNF-Y can also bind to the same consensus sequences in vitro (Yoshioka et al., 2007). A database 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 consensus (Fig. 4, region 2). 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-lucw ) (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 weak promoter activity in S2 cells (data not shown) and the sev enhancer located in the

Table 1. Transformants carrying UAS-dNF-YAIR transgene

| P-element plasmid | Strain | Chromosome linkage | Phenotype |
|-------------------|--------|--------------------|-----------|
| UAS-dNF-YAIR231-399 | 4      | III                | lethal    |
|                   | 5      | II                 | rough eye |
|                   | 7      | III                | no effect |
|                   | 39     | III                | lethal    |
|                   | 67     | II                 | rough eye |
| UAS-dNF-YAIR63-228 | 15     | III                | no effect |
|                   | 81     | III                | rough eye |
|                   | 82     | III                | no effect |

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

Table 1. Transformants carrying UAS-dNF-YAIR transgene

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|                   | 82     | III                | no effect |

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|                   | 7      | III                | no effect |
|                   | 39     | III                | lethal    |
|                   | 67     | II                 | rough eye |
| UAS-dNF-YAIR63-228 | 15     | III                | no effect |
|                   | 81     | III                | rough eye |
|                   | 82     | III                | no effect |

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

Table 1. Transformants carrying UAS-dNF-YAIR transgene

| P-element plasmid | Strain | Chromosome linkage | Phenotype |
|-------------------|--------|--------------------|-----------|
| UAS-dNF-YAIR231-399 | 4      | III                | lethal    |
|                   | 5      | II                 | rough eye |
|                   | 7      | III                | no effect |
|                   | 39     | III                | lethal    |
|                   | 67     | II                 | rough eye |
| UAS-dNF-YAIR63-228 | 15     | III                | no effect |
|                   | 81     | III                | rough eye |
|                   | 82     | III                | no effect |

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

Table 1. Transformants carrying UAS-dNF-YAIR transgene

| P-element plasmid | Strain | Chromosome linkage | Phenotype |
|-------------------|--------|--------------------|-----------|
| UAS-dNF-YAIR231-399 | 4      | III                | lethal    |
|                   | 5      | II                 | rough eye |
|                   | 7      | III                | no effect |
|                   | 39     | III                | lethal    |
|                   | 67     | II                 | rough eye |
| UAS-dNF-YAIR63-228 | 15     | III                | no effect |
|                   | 81     | III                | rough eye |
|                   | 82     | III                | no effect |

Eye morphology of adult flies obtained when each strain was crossed with the fly carrying the GMR-GAL4 transgene in X chromosome.
The 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). 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-YAIR231-399 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.
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
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 RosI, 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 RosI gene is involved in the MAPK cascade that is triggered by a variety of signals including...
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 ψ-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-lucZwt and psevPE-lacZwt and psevPE-lacZNF-Ymut1
the following oligonucleotides were synthesized.

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.

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-YAIR231-399/+.
(B, E) GMR-GAL4/w;hs-sev;UAS-dNF-YAIR/+.
(C, F) GMR-GAL4/w;UAS-dNF-YAIR231-399/+;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.
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'-AGCAGCCGCCCATGTGTACGGAAA
RPLP0-R: 5'-CATTTGGGTGCGCCGCAAARCGGTG
RP49-R.T-E: 5'-GCTTCTGGTTTCCGGCAAGCTTCAAG
RP49-R.T-E: 5'-GACCTCAAGCTGCACGTTGCGACCCGACG
Nhe1NF-YA-F: 50-CTAGCTAGCCATCAACAAGTACAATCCCAGAC
NF-YA-RXba1: 50-GCTTCTAGACTATTCCGATTTGACCCG
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 DpnI 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.
Fly stocks
Flies were cultured at 25 °C on standard food. The Oregon R or Canton S flies were used as the wild-type strain. The transgenic fly lines carrying *UAS-dNF-YAIR231-399*, which were used in the experiments, are maintained as described.
Chromatin immunoprecipitation was performed using a ChIP Assay kit as described earlier (Yoshizaki et al., 2007). The AE127 (svp-lacZ/TM6B, P62 (deadpan-lacZ)/CyO, B38 (Klignon-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 (Roberson et al., 1988). Three independent lines were established for psePE-lacZ2wt and psePF-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-YAIR231-399 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°C.

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 animals 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°C or 30 minutes at 25°C. 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°C and incubated with an anti-p-galactosidase mouse monoclonal antibody (DSHB) (1:500) or an anti-dNF-YA rabbit polyclonal (1:500) antibodies at 4°C 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°C. 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 transfection of 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 (Yoshizaki 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°C and incubated with the anti-dNF-YA antibody at 1:500 dilution, or an anti-z tubulin monoclonal antibody (Sigma) at 1:2,000 dilution at 4°C 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 1:10,000 dilution for 1 hour at 25°C. Detection was performed with ECL Western blotting detection reagents (GE healthcare), and images were analyzed with a LuminoVision Pro HSI 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 machine. cDNA fragments were amplified using primers specific for the Act5C gene as an endogenous reference (Tue et al., 2010). Experiments were performed in triplicate for each of three RNA batches isolated separately.

Acknowledgements

We thank Dr. Y. H. Inoue for technical advice, Dr. Y. Hiromi for supplying AE127 (svp-lacZ/TM6B, P62 (deadpan-lacZ)/CyO, B38 (Klignon-lacZ) lines, Dr. T. Adachi-Yamada for supplying fly strains hs-flp and Act5C>FRT y FRT>GAL4, UAS-GFP, Dr. M. Moore for comments on the English language in the manuscript and all members in our laboratory for helpful discussions. This study was partially supported by Grants-in-Aid from KIT, JST, JSPS and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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