Am J Cancer Res 2012;2(1):36-44
www.ajcr.us /ISSN:2156-6976/ajcr0000087

Original Article

The warts gene as a novel target of the *Drosophila* DRE/DREF transcription pathway

Shunsuke Fujiwara[1,2], Hiroyuki Ida[1,2], Yasuhide Yoshioka[1,3], Hideki Yoshida[1,2], Masamitsu Yamaguchi[1,2]

[1]Department of Applied Biology; [2]Insect Biomedical Research Center; [3]Venture laboratory, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

Received September 15, 2011; accepted October 21, 2011; Epub November 22, 2011; Published January 1, 2012

Astract: The Hippo tumor suppressor pathway in *Drosophila* represses expression of DIAP1 and Cyclin E via inactivation of the transcription co-activator Yorkie, resulting in cell cycle arrest and induction of apoptosis. The warts (wts) gene is well known as a core kinase in this pathway, but its transcriptional regulation has yet to be clarified. In Drosophila, DREF binds to a target sequence named DRE (5'-TATCGATA) and regulates transcription of cell proliferation-related genes containing the DRE sequence in their promoter regions. Here we found half reduction of the wts gene dose to enhance the DREF-induced rough eye phenotype, suggesting a DREF genetic interaction with the Hippo pathway in vivo. Three DREs indentified in the wts gene promoter region exhibited strong promoter activity with a luciferase transient expression assay in Drosophila S2 cells, this decreasing under DREF-RNAi conditions. In addition, knockdown of DREF in S2 cells reduced the level of endogenous wts mRNA. Chromatin immunoprecipitation assays with anti-DREF antibody revealed that DREF binds specifically to the wts gene promoter region containing DREs in vivo. These results indicate that the DRE/DREF pathway is required for transcriptional regulation of the wts gene, indicating a novel link between the DRE/DREF and the Hippo pathways.

Keywords: DRE, DREF, warts, Hippo pathway, transcription, tumor suppressor

Introduction

The Hippo pathway has been found to restrict cell proliferation by inducing apoptosis and cell cycle arrest [1-3]. The core components of this pathway are Hippo (Hpo), Warts (Wts), Salvador (Sav), Mob as tumor suppressor (Mats), and Yorkie (Yki). Hpo and Wts are serine/threonine kinases, while Sav functions as a scaffold to support their activity. Mats regulates Wts phosphorylation by Hpo and Yki as a transcriptional coactivator [3-7]. By phosphorylation of Yki, this signaling pathway inhibits Yki transcriptional effects on target genes such as the cell cycle regulator cyclin E and the inhibitor of apoptosis gene product DIAP1 [8]. In this way, the Hippo pathway controls cell number in growing tissues of *Drosophila*. Accordingly, mutations in Hpo, Wts, Sav, Mats or overexpression of Yki induce similar tumor-like phenotypes in Drosophila epithelial tissues. These components are conserved between the fly and vertebrates and mutations in these factors also result in tumorigenesis in mice. Additional components such as membrane-associated protein Merlin (Mer) and Expanded (Ex) function upstream of Hpo to promote its phosphorylation and activation of Wts [9]. Both Mer and Ex contain FERM domains linking with cytoskeletal and transmembrane proteins [10]. Thus, it is suggested that they might transmit signals from membranes. A recent study showed that the Hippo pathway also regulates proliferation of intestinal stem cells in *Drosophila* midgut [11], playing an essential role in maintaining homeostasis and regeneration in response to tissue damage. Furthermore a variety of other factors have been identified which interact with the Hippo pathway, indicating wide-ranging functions [12]. However, the transcriptional regulation of genes encoding these factors is largely unknown and poorly studied.

Promoters of many DNA replication- and proliferation-related genes such as *PCNA, DNA polymerase α (the 180kDa and 73kDa subunit)*,

dE2F, cyclin A, D-ras, D-raf, orc2, rfc1, elf4A, osa and moira contain a common 8 base pair (bp) palindromic sequence (5'-TATCGATA) named the DNA replication-related element (DRE) [13-23]. The requirement of DRE for promoter activity has been confirmed in both cultured cells and transgenic flies [24, 25] and a specific DRE-binding factor (DREF) has been identified [25, 26]. Molecular cloning of its cDNA has revealed that DREF is an 80kDa polypeptide of 709 amino acid residues transactivating DRE-containing genes [25]. It is also reported that DREF is a component of a transcription initiation complex containing TRF2 [27]. In addition, the chromatin remodeling factor dMi-2 and the homeodomain protein Distalless (Dll) interact with the DNA-binding domain of DREF and inhibit its DNA-binding activity, separately [28, 29]. Overexpression of the DREF in Drosophila eye imaginal discs induces ectopic S phase and apoptosis, while inhibiting photoreceptor cell differentiation, resulting in a rough eye phenotype in the adult [30]. Genetic screening taking advantage of this rough eye phenotype has identified many genes involved in cell proliferation and cell cycle [22]. Recently, transcriptional regulation of the Drosophila p53 gene by the DRE/DREF system was revealed by cytological and molecular biological studies [31]. For many years DRE/DREF system was thought to simply promote cell proliferation or growth via activation of gene transcription. However, since the p53 gene is well-known as a tumor suppressor gene, DRE/DREF may not only up-regulate but also down-regulate cell proliferation, suggesting roles in fine-tuning of tissue kinetics in Drosophila. In the present study we focused on the tumor suppressor wts gene, thereby obtaining evidence of a novel link between DRE/DREF and the Hippo pathway.

Materials and methods

Fly strains

Fly strains were maintained at 25°C on standard food. The fly line $st^{1}in^{1}kni^{ri\text{-}1}p^{p}wts^{3\text{-}17}$/ TM3, Sb^{1} was obtained from the Bloomington, Indiana stock center. The UAS-DREF line was described earlier as well as the transgenic fly line carrying GMR-GAL4 on the X chromosome [21].

Scanning electron microscopy

Adult flies were anesthetized, mounted on stages and observed under a VE-7800 scanning electron microscope (Keyence Inc.) in the high 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 three times. No significant variation in eye phenotype among individuals was apparent.

Plasmid construction

To construct the plasmid p5'-wtswt-luc, PCR was performed using *Drosophila* genomic DNA as a template and the following primer combination: wts5'KpnI: 5'-ACCGGTACCGGCTACGGTGACCTTG ATCT; wts3'XhoI: 5'GATCTCGAGAGACAGAGATC-TAGGGAGGAG;

PCR products were digested with KpnI and XhoI and inserted between the KpnI and XhoI sites of the PGVB plasmid (Toyo Ink).

Base-substituted derivatives of p5'-wtswt-luc were constructed using a QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. The following oligonucleotide pairs carrying base substitutions in DRE were used as primers: wtsDRE1mutF: 5'-GGTCAAATCCTcgCGgcAC CTGATGTGGTTGCCAG; wtsDRE1mutR :5'-CTGGC AACCACATCAGGTgcCGcgAGGATTTGACC; wtsDRE2mutF :5'-GTTTTCAGTATAATcgCGgcGTTT ATATACCACGGTC; wtsDRE2mutR :5'-GACCGTGG TATATAAACgcCGcgATTATACTGAAAAC; wtsDRE3mutF :5'-CCATGCAGATACGATAGCTTcgC G g c A G C G C T G T T C T G G A A C G C A G C ; wtsDRE3mutR:5'-GCTGCGTTCAGAACAGCGCTgc CGcgAAGCTATCGTATCTGCATGG.

DNA transfection into S2 cells and luciferase assays

Approximately 2×10^{5} cells were plated 24h before plasmid transfection, then 500ng of reporter plasmid and 1ng of pAct5C-seapansy as an internal control were cotransfected into the cells using CellFectin II reagent (Invitrogen). After 48h from transfection, S2 cells were harvested and luciferase activities were measured with the Dual-Luciferase Reporter assay system (Promega). All assays were performed within the range of linear relation of activity to incubation time and values were normalized to Renilla luciferase activity. Transfections were performed several times with at least two inde-

pendent plasmid preparations.

For dsRNA interference (RNAi) experiments, 1×10^5 cells were plated in 24-well dishes in the presence of 3μg/well of DREF-dsRNA, YFP-dsRNA and mock for control (dsRNA free incubation) in fetal bovine serum (FBS) free M3 medium for 1h. After the incubation, three volumes of M3 containing 10% FBS were added to each well. Four days after RNAi treatment, cells were co-transfected with the luciferase reporter plasmid (500ng) and pAct5C-seapancy plasmid (1ng) as an internal control then harvested after 48h for measurement of luciferase activities according to the standard instructions with the kit (Promega).

Quantitative RT-PCR

1×10^6 S2 cells were plated in 6-well dishes in 2ml M3 medium containing 30μg/well of double-strand RNAs for DREF (DREFdsRNA) or LacZ (LacZdsRNA) for 1h. After the incubation, 3ml of M3 medium containing 10% FBS was added to each well. At 5 days after the RNAi treatment, total RNA was isolated from cells using Trizol Reagent (Invitrogen) and 1μg aliquots were reverse transcribed with oligo dT primer 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 1μl of reverse transcribed sample per reaction. β-tubulin mRNA was chosen as a negative control and Rp49 was used as an endogenous reference gene. To carry out quantitative real-time PCR, the following PCR primers were chemically synthesized: Wts2ndPrimer 5'-GTTAGTGTGCGGA GCATTTC; Wts3rd Primer 5'-GCCGTCATCATCTT TGGCACA; DREF-F 5'-GGCAATCTCCGTTGAAT GACG; DREF-R 5'-TTCACCTCCGAGAAGCCCTT; β-tubulin-F 5'-AGTTCACCGCTATGTTCA; β-tubulin-R 5'-CGCAAAACATTGATCGAG; RP49-F 5'-GCTTCTGGTTTCCGGCAAGCTTCAAG; RP49-R 5'-GACCTCCAGCTCGCGCACGTTGTGCACCAGGAAC.

Chromatin immunoprecipitation

We performed chromatin immunoprecipitation using a Chip assay kit as recommended by the manufacturer (MILLIPORE). Approximately 1×10^7 S2 cells were fixed in 1% formaldehyde at 37°C for 10 min and then quenched in 125mM glycine for 5 min at 25°C. Cells were washed twice in PBS containing protease inhibitors (1mM PMSF, 1μg/ml aprotinin and 1μg/ml pepstain A), lysed in 200μl of SDS lysis buffer, sonicated to break DNA into fragments of less than 1kb and centrifuged at 15,300×g for 10 min at 4°C. The sonicated cell supernatants were diluted 10-fold in Chip Dilution Buffer and pre-cleared with 80μl salmon sperm DNA/Protein A agarose-50% slurry for 30 min at 4°C. After brief centrifugation, each supernatant was incubated with 1μg of the rabbit IgG or anti-DREF polyclonal antibodies [25] for 16h at 4°C. Salmon sperm DNA/Protein A agarose-50% slurry was added with incubation for 1h at 4°C. After washing, immunoprecipitated DNA was eluted with elution buffer containing 1% SDS and 0.1M $NaHCO_3$. Then the protein-DNA crosslinks were reversed by heating at 65°C for 4h. After deproteinization with proteinase K, DNA was recovered by phenol-chloroform extraction and ethanol precipitation. Quantitative real-time PCR was performed as described above. To carry out chromatin immunoprecipitation, the following PCR primers were chemically synthesized: ChIPwts5'Primer 5'-CCGATAACATTTACTTGCTCTC-3'; ChIPwts3'Primer 5'-GACCGATATCGATAGACA GAG-3'; PCNAP 5'-GATGAATGATTAACGTGGGCTG; PCNAantiP 5'-GAAATAAATATACTCTGTAAAAAGTG TGAAC; actin5CF5'-CTCCATCATGAAGTGTGATG TG; actin5CR 5'-CGTACTCCTGCTTGGACGTC.

Results

Half reduction of the wts gene dose enhances the DREF-induced rough eye phenotype

Recently, it is revealed that DRE/DREF regulates transcription of the *Drosophila p53* gene [31], suggesting that DRE/DREF is responsible for balanced cell proliferation by activating and deactivating it simultaneously. Since both DRE/DREF system and the Hippo pathway regulate cell proliferation, we were interested in examining the relationship between these two pathways. Overexpression of DREF induces ectopic DNA synthesis and apoptosis and inhibits photoreceptor cell differentiation in eye imaginal disc so that adult flies exhibit a severe rough eye phenotype [30]. Since eye phenotype does not impair viability or fertility, these flies serve as an excellent genetic tool to screen for mutations that modify the rough eye phenotype. As a first step, we carried out modifier test of DREF-induced rough eye phenotype by a mutant of one of the genes involved in the Hippo pathway.

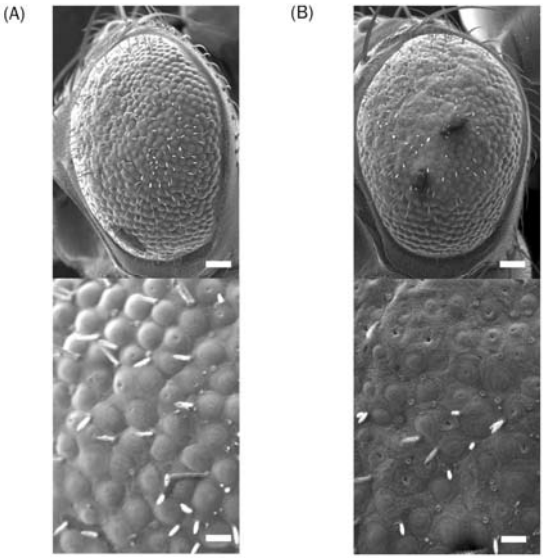

Figure 1. The wts gene genetically interacts with DREF. Scanning electron micrographs of adult eyes. (A) GMR-GAL4/Y; UAS-DREF/+, (B) GMR-GAL4/Y; UAS -DREF/+; +/wts$^{3\text{-}17}$. Lower panels indicate higher magnification images of the upper panels. Scale bars are for 12.5 μm and 50 μm, respectively.

As expected, overexpression of DREF induced a rough eye phenotype (**Figure 1A**), regarded as a reference. Compared with this, we found that half reduction of the wts gene dose promoted more pronounced fusion of ommatidia and loss of sensory hairs, resulting in strong enhancement of the DREF-induced rough eye phenotype (**Figure 1B**). The results indicate that the wts gene genetically interacts with DREF *in vivo*.

The 5'-flanking region of the wts gene contains DRE and DRE-like sequences

To examine the possibility that transcription of the wts gene is directly regulated by DREF, we searched for DRE sequences within 1.4kb genome region from the transcription initiation site of the wts gene from the *Drosophila* genome database, FlyBase (http://flybase.org/). Previous studies demonstrated that DRE sequences within such regions can up-regulate transcription of many target genes, and the central 6 bp of DRE (5'-ATCGAT) are essential for DREF binding and activation of promoter [13, 24]. In the 5'-flanking region of the wts gene, within 1.4kb of the transcription initiation site, we found one DRE-like and two DRE sequences, named DRE1 (-49 to -56), DRE2 (-79 to -86),

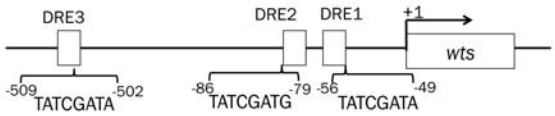

Figure 2. The wts gene carries DRE and DRE-like sequences in the 5'-flanking region. The transcription initiation site is indicated by the arrow and designated as +1. The positions and nucleotide sequences of DRE1, DRE2 and DRE3 are shown.

and DRE3 (-502 to -509) (**Figure 2**). DRE1 and DRE3 perfectly match the consensus DRE sequence and DRE2 matches 7 out of 8bp DRE.

Roles of DREs in wts promoter activity

To investigate the role of the DREs in wts promoter activity, we carried out luciferase transient expression assays with cultured *Drosophila* S2 cells. We constructed a wts promoter-luciferase fusion plasmid and derivatives carrying mutations in one or more of DRE1, DRE2, and DRE3. These plasmids were transfected into S2 cells and luciferase activity was measured. A mutation in DRE1 (wts-DRE1mut) almost completely abolished wts promoter activity, as was also the case with mutations in both DRE1 and DRE2 (wts-DRE1, 2mut), DRE1 and DRE3 (wts-DRE1,3mut), and in all three DREs (wts-DREallmut). Mutation in DRE2 (wts-DRE2mut) and DRE3 (wts-DRE3mut) alone reduced the promoter activity by 34% and 43%, respectively, relative to that of the wild-type promoter (**Figure 3A**). These results suggest that DRE1 is essential for wts gene promoter activity, while DRE2 and DRE3 might have supporting roles.

To further examine the requirement of DREF for wts promoter activity, we performed luciferase transient expression assays in DREF-knockdown cells. DREF-dsRNA or YFP-dsRNA were added to S2 cells. Western immunoblot analyses with anti-DREF antibody revealed that DREF protein was not detectable at 3 and 5 days after DREF-dsRNA treatment, while no significant change was evident in mock and YFP-dsRNA-treated cells (**Figure 3C**). When luciferase reporter plasmids were transfected into S2 cells at 4 days after RNAi treatment, wild type wts promoter activity was decreased to 56% in DREF-dsRNA-treated cells compared with no-dsRNA treatment cells (mock), while the promoter activity was only a slightly reduced in the YFP-dsRNA-treated cells (**Figure 3B**).

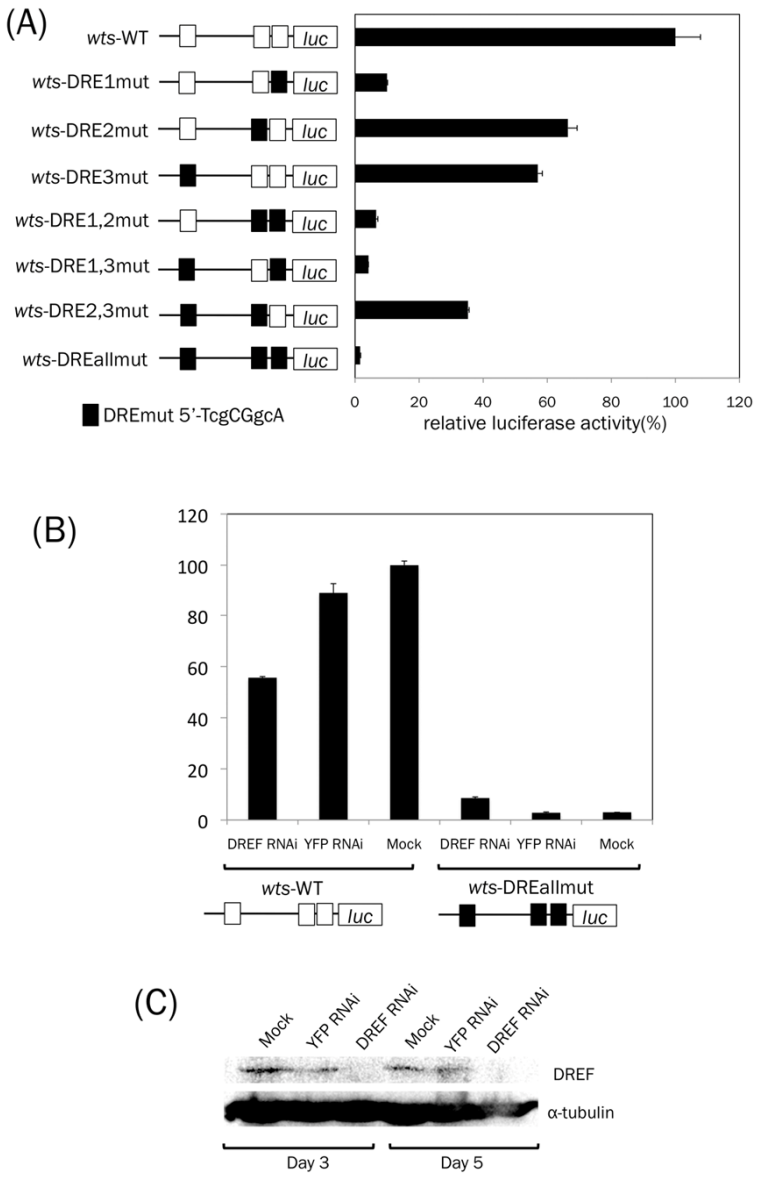

Figure 3. DREs play essential roles in wts gene promoter activity in cultured cells. (A) Schematic features of the wts promoter-luciferase fusion plasmid wts-WT-luc, and its base-substituted derivatives (wts-DRE1mut, wts-DRE2mut, wts-DRE3mut, wts-DRE1,2mut, wts-DRE1,3mut, wts-DRE2,3mut and wts-DREallmut) are illustrated. DRE is represented by an open box and mutated DRE by a closed box. Plasmids were transfected into S2 cells and luciferase activities measured at 48 h thereafter. Luciferase activity was normalized to Renilla luciferase activity and expressed relative to that of wts-WT-luc. The mean activities with standard deviations from three independent transfections are shown. (B) Three days after treatment with DREF dsRNA (DREF RNAi) or YFP dsRNA (YFP RNAi), S2 cells were transfected with 0.5 μg each of wts-WT-luc or wts-DREallmut-Luc. Promoter activities were measured at 48 h after transfection. The luciferase activity was normalized to Renilla luciferase activity and expressed relative to that of wts-WT-luc in non-RNA treated cells (Mock). (C) Western immunoblot analysis showing decreased DREF protein level in S2 cells after DREF dsRNA treatment. The upper panel shows data for the DREF band with anti-DREF polyclonal antibody. The lower panel shows findings for α-tubulin as a loading control.

To further confirm dependence of promoter activation by DREF, we determined luciferase expression with the wts promoter carrying mutations in all three DREs in DREF-knockdown cells. The data indicated no reduction of promoter activity in DREF-knockdown cells (**Figure 3B**). These results again confirmed a positive role of DREF in wts promoter activity.

Knockdown of the DREF gene reduces endogenous wts gene expression in cultured S2 cells

To examine whether DREF affects expression of the endogenous wts gene, we carried out DREF RNA interference experiments in Drosophila S2 cells. Total RNAs from dsRNA-treated S2 cells were isolated at 5 days after addition of dsRNA for assessment by quantitative RT-PCR. The β-tubulin gene was used as a negative control. The DREF mRNA level was extensively reduced to 2% by DREF-dsRNA treatment as compared with no-dsRNA treated cells (mock) (**Figure 4**). The wts mRNA level in DREF dsRNA treated cells was reduced to 31% relative to no dsRNA treated cells, while no significant change was observed with LacZ-dsRNA-treated cells (**Figure**

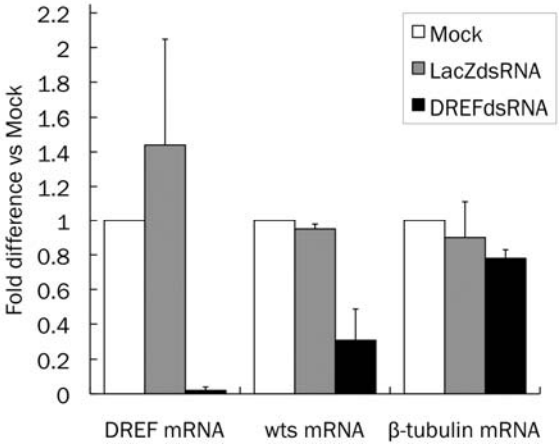

Figure 4. DREF dsRNA treatment reduces endogenous wts mRNA levels in S2 cells. DREF mRNA and wts mRNA in DREF dsRNA-treated cells were measured by quantitative RT-PCR and compared with values for non-dsRNA treated cells (Mock). The mRNA for β-tubulin was used as a negative control.

4). These results indicate that DREF is truly required for wts gene expression.

DREF binds to the genomic region containing DRE and DRE-like sequences of the wts gene in vivo

To test whether DREF binds to the DRE-containing 5'-flanking region of the wts gene in S2 cells, chromatin immunoprecipitation assays with anti-DREF polyclonal antibodies were carried out (**Figure 5**). Since it is well known that the PCNA gene is regulated by DRE/DREF system it was here used as a positive control, and the Actin5C genomic region not carrying any DREs was used as a negative control.

Amplification of the *PCNA* gene promoter region containing DRE in the immunoprecipitates with the anti-DREF IgG was 97-fold higher than that with control IgG (**Figure 5**). In contrast, no significant amplification of the Actin5C gene region was observed. Amplification of the wts gene region containing DRE1, 2 from the immunoprecipitates with the anti-DREF IgG was about 319-fold higher than that with the control IgG (**Figure 5**). We also could detect binding of DREF to the DRE3 region of the wts promoter, but this binding was weaker than was the case for the DRE1, 2 containing region. These results indicate that DREF mainly binds to the genomic region con-

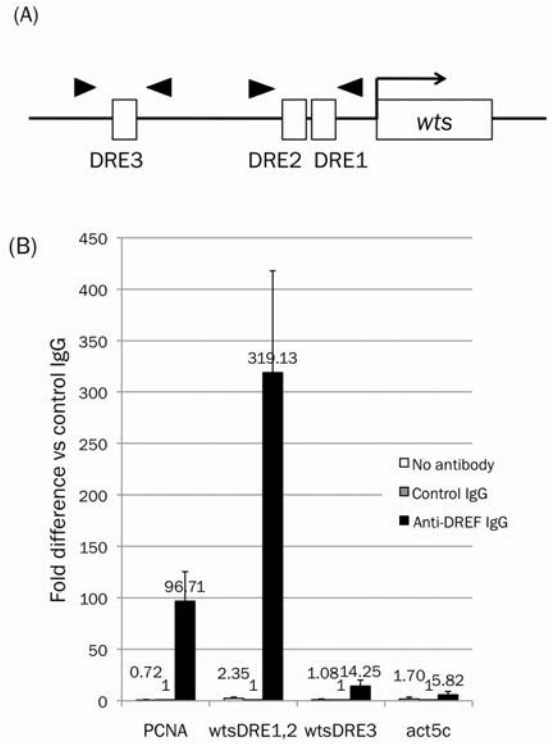

Figure 5. Binding of DREF to the DRE-containing genomic region of the wts gene. (A) Schematic of the 5'-flanking region of the wts gene. Arrowheads indicate positions of the primers used for real-time PCR reactions. (B) Crosslinked chromatin of S2 cells was immunoprecipitated with either anti-DREF IgG or the control rabbit IgG. Genomic regions containing wtsDRE1, 2 or wtsDRE3 were amplified by PCR, and compared with amplicons from immunoprecipitates with the control rabbit IgG.

taining DRE1, 2 and less effectively to DRE3 under cellular conditions.

Discussion

In this study, we obtained evidence that the wts gene is a DREF-target, featuring two DREs and one DRE-like sequence in its 5'-flanking region. Luciferase transient expression assays showed wts promoter activity to depend on both DREs and DREF levels. In addition, the level of wts mRNA was reduced in DREF knockdown S2 cells treated with DREF dsRNA. Furthermore, chromatin immunoprecipitation assays showed DREF to bind to the genomic region of the wts gene containing DREs. These data provide compelling evidence that DREF is a positive regulator of the wts gene, suggesting that wts gene

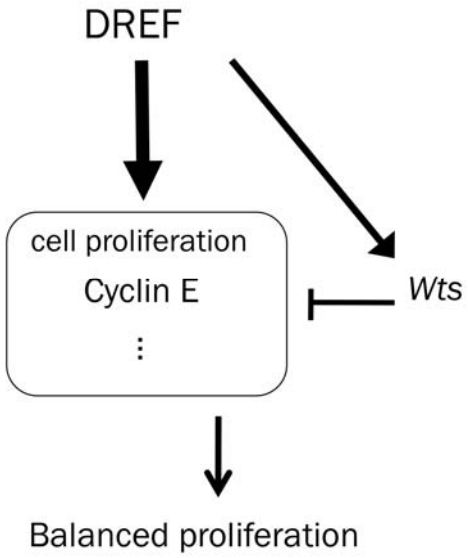

Figure 6. Model of balanced cell proliferation via the DRE/DREF system. DRE/DREF may simultaneously function as both strong enhancer and mild suppressor of cell proliferation.

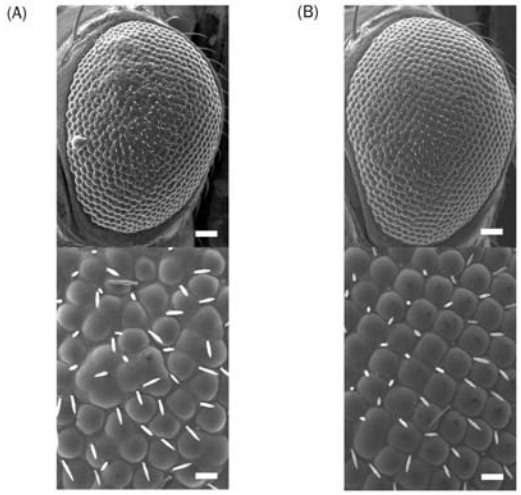

Figure 7. The *wts* gene genetically interacts with DREF. Scanning electron micrographs of adult eyes. (A) UAS-DREFIR/+; GMR-GAL4/+, (B) UAS-DREFIR/+; GMR-GAL4/+; + /wts$^{3\text{-}17}$. Scale bars are for 50 μm in upper panels, and 12.5μm in lower panels.

expression is directly activated by the DRE/DREF pathway.

In general, half-dose reduction of DREF target genes results in suppression of the DREF-induced rough eye phenotype [21, 23]. However, with the wts gene, it was not reduced but rather enhanced. Although proof is lacking at present, we can propose a model to explain these observations (**Figure 6**). In general, the DRE/DREF system mainly up-regulates genes which promote cell proliferation like CycE [32]. The Wts is known to down-regulate CycE expression. Therefore, DRE/DREF may simultaneously function as both strong enhancer and mild suppressor of cell proliferation. Overexpression of DREF results in the rough eye phenotype, and half dose reduction of the wts gene might weaken inhibitory effects on cell proliferation inducing a severer rough eye. Consistent with this interpretation, we confirmed that half dose reduction of the *wts* gene suppressed the rough eye phenotype induced by DREF-knockdown (**Figure 7**).

In addition to the wts gene, we found DRE-like sequences on the 5'-flanking regions of other Hippo pathway genes such as the *sav*, hpo and *ex* genes, suggest that these three are also DREF targets. It is therefore possible that a tight link exists between the DRE/DREF and Hippo pathways. Since the Hippo pathway restricts cell proliferation via cell cycle arrest and inducing apoptosis [1, 2], it is possible that DREF simultaneously activates both positive and negative regulators of cell proliferation as suggested previously [23, 33] and evidenced by its positive regulation of the p53 gene. The p53 transcriptional activator has three major functional areas: cell cycle arrest, cellular senescence and apoptosis [34]. The so-called 'the guardian of the genome', regulates cell proliferation negatively and the fact of DREF influence suggest fine-tuning of appropriate cell proliferation.

In addition, the relationship between cell proliferation and cell death are complex. It has been known that increased proliferation via activation of oncogenes like *Myc* or *Ras* is often induced simultaneously with increased apoptosis [35]. Apoptosis functions as a built-in failsafe to repress inappropriate cell proliferation [3]. Thus, DREF may have important role to balance cell proliferation with apoptosis and maintain tissue kinetics.

Acknowledgements

We thank Dr Moore for comments on the English language in the manuscript. This study was partially supported by a scholarship and grants

from the KIT, and Grants-in-Aid for Scientific Research (A), (C) and Priority Research.

Address correspondence to: Dr. Masamitsu Yamaguchi, Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan E-mail: myamaguc@kit.ac.jp

References

[1] Edgar BA. From cell structure to transcription: Hippo forges a new path. Cell 2006; 124: 267-273.

[2] Saucedo LJ, Edgar BA. Filling out the Hippo pathway. Nat Rev Mol Cell Biol 2007; 8: 613-621.

[3] Wu S, Huang J, Dong J and Pan D. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell 2003; 114: 445-456.

[4] Xu T, Wang W, Zhang S, Stewart RA and Yu W. The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev 1995; 9: 534-546.

[5] Pantalacci S, Tapon N and Leopold P. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat Cell Biol 2003; 5: 853-855.

[6] Udan RS, Kango-Singh M, Nolo R, Tao C and Halder G. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat Cell Biol 2003; 5: 914-920.

[7] Wei X, Shimizu T and Lai ZC. Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. EMBO J 2007; 26: 1772-1781.

[8] Huang J, Wu S, Barrera J, Matthews K and Pan D. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell 2005; 122: 421-434.

[9] Hamaratoglu F, Willecke M, Kango-Singh M, Nolo R, Hyun E, Tao C, Jafar-Nejad H and Halder G. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat Cell Biol 2006; 8: 2265-2277.

[10] McClatchey AI, Giovannini M. Membrane organization and tumorigenesis-the NF2 tumor suppressor, Merlin. Genes Dev 2005; 19: 2265-2277.

[11] Ren F, Wang B, Yue T, Yun EY, Ip YT and Jiang J. Hippo signaling regulates Drosophila intestine stem cell proliferation through multiple pathways. Proc Natl Acad Sci USA 2010; 107: 21064-21069.

[12] Halder G, Johnson RL. Hippo signaling: growth control and beyond. Development 2011; 138: 9-22.

[13] Hirose F, Yamaguchi M, Handa H, Inomata Y and Matsukage A. Novel 8-base pair sequence (Drosophila DNA replication-related element) and specific binding factor involved in the expression of Drosophila genes for DNA polymerase a and proliferating cell nuclear antigen. J Biol Chem 1993; 268: 2092-2099.

[14] Lightfoot K, Maltby L, Duarte R, Veale R and Segev O. Conserved cis-elements bind a protein complex that regulates Drosophila ras2/rop bidirectional expression. Br J Cancer 1994; 69: 264-273.

[15] Matsukage A, Hirose F, Hayashi Y, Hamada K and Yamaguchi M. The DRE sequence TATCGATA, a putative promoter-activating element for Drosophila melanogaster cell-proliferation-related genes. Gene 1995; 166: 233-236.

[16] Ohno K, Hirose F, Sakaguchi K, Nishida Y and Matsukage A. Transcriptional regulation of the Drosophila cycA gene by the DNA replication-related element (DRE) and DRE binding factor (DREF). Nucleic Acids Res 1996; 24: 3942-3946.

[17] Ryu JR, Choi TY, Kwon EJ, Lee WH, Nishida Y, Hayashi Y, Matsukage A, Yamaguchi M and Yoo MA. Transcriptional regulation of the Drosophila-raf protooncogene by the DNA replication-related element (DRE)/DRE-binding factor (DREF) system. Nucleic Acids Res 1997; 24: 794-799.

[18] Sawado T, Hirose F, Takahashi Y, Sasaki T, Shinomiya T, Sakaguchi K, Matsukage A and Yamaguchi M. The DNA replication-related element (DRE)/DRE-binding factor system is a transcriptional regulator of the Drosophila E2F gene. J Biol Chem 1998; 273: 26042-26051.

[19] Okudaira K, Ohno K, Yoshida H, Asano M and Hirose F. Transcriptional regulation of the Drosophipla orc2 gene by the DREF pathway. Biochim Biophys Acta 2005; 1732: 23-30.

[20] Tsuchiya A, Inoue YH, Ida H, Kawase Y, Okudaira K, Ohno K, Yoshida H and Yamaguchi M. Transcriptional reguration of the Drosophila rfc1 gene by the DRE-DREF pathway. FEBS J 2007; 274: 1818-1832.

[21] Ida H, Yoshida H, Nakamura K and Yamaguchi M. Identification of the Drosophila eIF4A gene as a target of the DREF transcription factor. Exp Cell Res 2007; 313: 4208-4220.

[22] Matsukage A, Hirose F, Yoo MA and Yamaguchi M. The DRE/DREF transcriptional regulatory system: a master key for cell proliferation. Biochim Biophys Acta 2008; 1779: 81-89.

[23] Nakamura K, Ida H and Yamaguchi M. Transcriptional regulation of the Drosophila moira and osa genes by the DREF pathway. Nucleic Acids Res 2008; 36: 3905-3915.

[24] Yamaguchi M, Hayashi Y, Nishimoto Y, Hirose F and Matsukage A. A nucleotide sequence essential for the function of DRE, a common

promoter element for Drosophila DNA replication-related genes. J Biol Chem 1995; 270: 15808-15814.
[25] Hirose F, Yamaguchi M, Kuroda K, Omori A, Hachiya T, Ikeda M, Nishimoto Y and Matsukage A. Isolation and characterization of cDNA for DREF, a promoter-activating factor for Drosophila DNA replication-related genes. J Biol Chem 1996; 271: 3930-3937.
[26] Hirose F, Yamaguchi M and Matsukage A. Targeted expression of the DNA binding domain of DRE-binding factor, a Drosophila transcription factor, attenuates DNA replication of the salivary gland and eye imaginal disc. Mol Cell Biol 1999; 19: 6020-6028.
[27] Hochheimer A, Zhou S, Zheng S, Holmes MC and Tjian R. TRF2 associates with DREF and directs promoter-selective gene expression in Drosophila. Nature 2002; 420: 439-445.
[28] Hirose F, Ohshima N, Kwon EJ, Yoshida H and Yamaguchi M. Drosophila Mi-2 negatively regulates dDREF by inhibiting its DNA-binding activity. Mol Cell Biol 2002; 22: 5182-5193.
[29] Hayashi Y, Kato M, Seto H and Yamaguchi M. Drosophila Distal-less negatively regulates dDREF by inhibiting its DNA binding activity. Biochim. Biophys Acta 2006; 1759: 359-366.
[30] Hirose F, Ohshima N, Shiraki M, Inoue HY, Taguchi O, Nishi YAM and Yamaguchi M. Ectopic expression of DREF induced DNA synthesis, apoptosis, and unusual morphogenesis in the Drosophila eye imaginal disc: possible interaction with Polycomb and trithorax group proteins. Mol Cell Biol 2001; 21: 7231-7242.
[31] Tue NT, Thao DT and Yamaguchi M. Role of DREF in transcriptional regulation of the Drosophila p53 gene. Oncogene 2010; 29: 2060-2069.
[32] Hyun J, Jasper H and Bohmann D. DREF is required for efficient growth and cell cycle progression in Drosophila imaginal discs. Mol Cell Biol 2005; 25: 5590-5598.
[33] Suyari O, Ida H, Yoshioka Y, Kato Y, Hashimoto R and Yamaguchi M. Identification of the Drosophila Mes4 gene as a novel target of the transcription factor DREF. Exp Cell Res 2009; 315: 1403-1414.
[34] Levine AJ, Hu W and Feng Z. The p53 pathway: what questions remain to be explored? Cell Death Differ 2006; 13: 1027-1036.
[35] Green DR, Evan GI. A matter of life and death. Cancer Cell 2002; 1: 19-30.