Genetic link between *p53* and genes required for formation of the zonula adherens junction

Masamitsu Yamaguchi,[1] Fumiko Hirose,[2] Yoshihiro H. Inoue,[3] Katsuhito Ohno,[1, 4] Hideki Yoshida,[1, 5] Yuko Hayashi,[6] Peter Deak[7] and Akio Matsukage[8]

[1]Department of Applied Biology, Faculty of Textile Science, [3]*Drosophila* Genetic Resource Center, [5]Venture Laboratory, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585; [2]Division of Biochemistry, [4]Division of Molecular Medicine, [6]Central Laboratory and Radiation Biology, Aichi Cancer Center Research Institute, Furoucho, Chikusa-ku, Nagoya 464-8681; [7]Department of Genetics, University of Cambridge, Cambridge CB2 3EH, England; and [8]Chemical and Biological Sciences, Faculty of Science, Japan Women's University, 2-8-1 Mejirodai, Bunkyo-ku, Tokyo 112-8681

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Ectopic expression of human p53 in *Drosophila* eye imaginal disc cells induces apoptosis and results in a rough eye phenotype in the adult flies. We have screened *Drosophila* stocks to identify mutations that enhance or suppress the p53-induced rough eye phenotype. One of the dominant enhancers of the p53-induced rough eye phenotype corresponds to a loss-of-function mutation of the *crumbs* gene, which is essential for the biogenesis of the zonula adherens junction and the establishment of apical polarity in epithelial cells. Enhancement of p53-induced apoptosis in the eye imaginal discs by a half-reduction of the *crumbs* gene dose was confirmed by a TUNEL method. Furthermore, mutations of genes for Shotgun (*Drosophila* E-cadherin) and Armadillo (*Drosophila* β-catenin), the two main components of the adherens junction, also strongly enhanced the p53-induced rough eye phenotype. These results suggest that human p53 senses subtle abnormality at the adherens junction or in signals derived from the junction, and consequently induces apoptosis to remove abnormal cells from tissue. Thus p53 likely plays a role as a guardian of the tissue not only by sensing the damaged DNA, but also by sensing signals from the adherens junction. (Cancer Sci 2004; 95: 436–441)

The p53 tumor suppressor is a multifunctional protein which exerts a variety of different effects in response to genotoxic stress, including DNA damage.[1–3] Its importance for securing the stability and integrity of the genome of normal cells underlies its being named the "guardian of the genome".[4] DNA damage has been shown to stabilize the p53 protein, which in turn, can either cause growth arrest, permitting completion of DNA repair processes, or alternatively, can drive cells to undergo apoptosis.[5–9] In fact, p53 has been suggested to be involved in the mechanism that senses damaged DNA, and in the control of its repair.[10, 11] Various p53-target genes involved in cell cycle regulation (*p21*, *MDM-2, 14-3-3σ*), DNA repair (*Gadd45*), cell proliferation (*IGF-BP*), apoptosis (*Bax*, *GML, P2XM, Killer/DR5, PAG608*), angiogenesis inhibition (*TSP-1, BAI1*) and cellular stress response (*TP53TG1, CSR, PIG3*) have already been identified.[2, 3, 12] Although p53-deficient mice exhibit only a marginal defect during development, mice deficient in other *p53* family member genes, *p73* and *p63*, exhibit severe defects. Mice deficient in all p73 isoforms exhibit profound defects in hippocampal dysgenesis, hydrocephalus, chronic infections and inflammation and impaired pheromone sensory pathways.[13] The p63-deficient mice exhibit severe limb truncations and lack all squamous epithelia and their derivatives, including hair, whiskers and teeth, as well as the mammary, lacrimal and salivary glands.[14, 15] These data suggest that p53 and/or its family proteins likely play a critical role in organization of tissues and organs during development.

Previously, we have ectopically expressed human p53 in eye imaginal disc cells using the GAL4-UAS targeted expression system[16] in *Drosophila*.[17] The expression of human p53 abolished the S-phase zone behind the morphogenetic furrow in the eye imaginal discs, induced ectopic apoptosis in the region posterior to the furrow, and resulted in a rough eye phenotype in adult flies.[17] The results indicated conservation of signaling systems corresponding to human p53 pathways in *Drosophila*.[17] In fact, a *Drosophila p53* orthologue, *Dmp53*, was discovered by the *Drosophila* genome project.[18, 19] By utilizing human p53 transgenic fly lines, we have identified several genomic regions whose deletions modify the rough eye phenotype induced by expression of p53.[17]

In the present study, we have screened lethal P-element insertion mutants mapped to those genomic regions to identify mutations that enhance or suppress the p53-induced rough eye phenotype. One of the mutations that strongly enhanced the p53-induced rough eye phenotype corresponds to that of *crumbs* gene.[20] Ectopic induction of apoptosis by human p53 in the eye imaginal discs was further enhanced by a half-reduction of the *crumbs* gene dose. The Crumbs protein is essential for the biogenesis of the zonula adherens junction and the establishment of apical polarity in epithelial cells.[21–24] Furthermore, mutations of genes for Shotgun (*Drosophila* E-cadherin) and Armadillo (*Drosophila* β-catenin), the two main constituents of the adherens junction[25, 26] also strongly enhanced the p53-induced rough eye phenotype. These results suggest that human p53 senses abnormality in the adherens junction and induces apoptosis to exclude abnormal cells from the tissue. This might represent an important property of p53 as a guardian of the tissue.

Materials and Methods

Fly strains. Unless otherwise specified, flies were reared at 25°C on standard food. Canton S was used as the wild-type strain. P-element insertion mutations of essential genes on the third chromosome of *Drosophila melanogaster* were described previously.[27] A transgenic fly line carrying pGMR-GAL4 on the X chromosome (line 16) and those carrying pUAS-human p53 wild type were as described,[17] although the p53 transgenic line 3 was mainly used in this study. The other stocks, including mutant strains for *crumbs* (*crb*^{1} and *crb*^{2}), *shotgun* (*shg*^{2}) and *armadillo* (*arm*^{1} and *arm*^{4}), were obtained from the Bloomington, Indiana stock center.

Scanning electron microscopy. Adult flies were anesthetized, mounted on the stage and observed under a Hitachi S-100 scanning electron microscope in the low vacuum mode.

Apoptosis assay. Third instar larvae were dissected in *Drosophila* Ringer's solution and imaginal discs were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, endogenous peroxidase activity was

E-mail: myamaguc@ipc.kit.ac.jp

blocked by treatment with methanol containing 0.3% H_2O_2 at room temperature for 30 min. The samples were then washed with PBS and permeabilized by incubation in a solution containing 0.1% sodium citrate and 0.1% Triton X-100 on ice for 2 min. After extensive washing, the TUNEL reaction was carried out using an *in situ* Cell Death Detection Kit, POD (Boehringer) according to the manufacturer's recommendations.

Reversion analysis. To test whether the P-*lacW* integrated at 95F is responsible for enhancement of the p53-induced rough eye phenotype, reversion analyses were carried out by mating *w*; P{*lacW*}*l(3)*0104/09/*TM3, Sb* P(*Δ2,3*) dysgenic males with *w*; *sr e Pr ca*/*TM6*. After w^- revertant lines were established, flies from each revertant line were individually crossed with transgenic flies expressing p53 to examine phenotypic reversion in the eye as well. The compound eyes of their progeny were observed under a scanning electron microscope.

Plasmid rescue. Genomic DNA was isolated from 30 adult flies as described[28] and digested with *Eco*RI or *Bam*HI. Plasmid rescue from genomic DNA was performed as described.[29] Oligonucleotides complementary to sequences close to the 3′-(5′-CTTGCCGACGGGACCACCTTATGTTATT) and 5′-ends (5′-GCTATCGACGGGACCACCTT) of the P-element were used to determine nucleotide sequences of DNA flanking the insertion site using an ABI PRISM 377 sequencer.

Immunohistochemistry. Third instar larvae were dissected in *Drosophila* Ringer's solution and imaginal discs were fixed in PBS containing 4% paraformaldehyde for 20 min at 4°C. Samples were washed with PBS containing 0.3% Triton X-100 (PBS-T), then blocked with PBS-T containing 10% normal goat serum for 30 min at room temperature and incubated with a mouse anti-human p53 monoclonal antibody DO-7 (DAKO) at a 1:100 dilution at 4°C for 16 h. After extensive washing with PBS-T, the imaginal discs were incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG (Promega) at a 1:2000 dilution for 2 h at room temperature. After extensive washing with PBS-T, color was developed as described previously.[17]

Results

Transgenic fly lines expressing human p53 in the eye imaginal discs exhibit a rough eye phenotype, but normal viability and fertility.[17] This p53 expression system, therefore, can be used as a genetic tool to identify mutations that modify the rough eye phenotype. First, by genetic crosses between the transgenic flies expressing p53 and a collection of *Drosophila* deficiency stocks, we found 10 genomic regions on the third chromosome that modified the p53-induced rough eye phenotype when they were heterozygous for deletions, 65F3; 66B7-8, 67F2-3; 68C7-10, 71E5-6; 71F, 76A3; 76B2, 83C1-2; 83F, 85B7; 85C-D, 87B11-13; 87E8-11, 93B; 93C2, 94A; 94F and 95F7; 96A17-18.[17] Second, in order to identify each gene in these genomic regions that are responsible for modification of the p53-induced rough eye phenotype, recessive lethal mutants with P-element insertions mapped in these deficiencies were collected[27] and used to cross to transgenic flies expressing p53.

Out of 40 independent mutant lines examined, four lines suppressed, while three lines enhanced the rough eye phenotype when they were heterozygous for the P-element insertion. The other P-element insertional lines apparently exerted no detectable effect on the rough eye phenotype induced by p53 expression. The strongest enhancement of the rough eye phenotype was observed with the line 0104/09[27] (Fig. 1). The enhancement could be reverted under dysgenic conditions (Fig. 1C), suggesting that the mutation induced by the P-element insertion is responsible for the enhancement. *In situ* hybridization to polytene chromosomes confirmed a single P-element at the 95F region (data not shown).

To identify the gene responsible for the enhancement, genomic DNA surrounding the P-element insertion was cloned by plasmid rescue and the nucleotide sequence of the insertion site was determined. A comparison of the sequence with the Berkeley *Drosophila* genome project database (http://

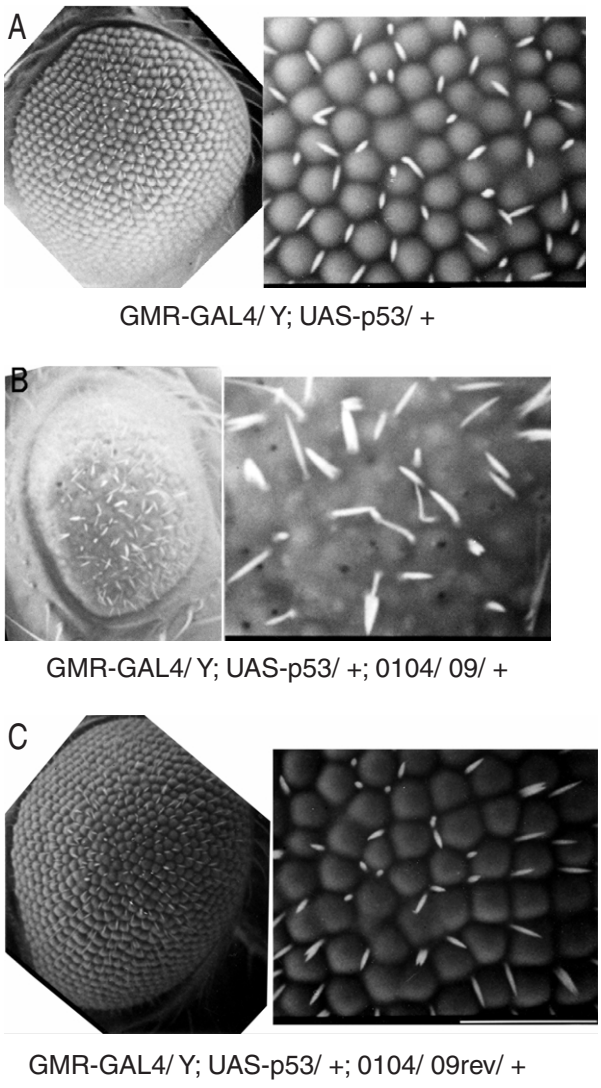

Fig. 1. Scanning electron micrographs of adult compound eyes. (A) *GMR-GAL4/Y; UAS-p53*/+, (B) *GMR-GAL4/Y; UAS-p53*/+; *0104/09*/+, (C) *GMR-GAL4/Y; UAS-p53*/+; *0104/09 rev*/+. The rough eye phenotype is enhanced in panel B. Scale bars for 50 μm (right panel) are indicated.

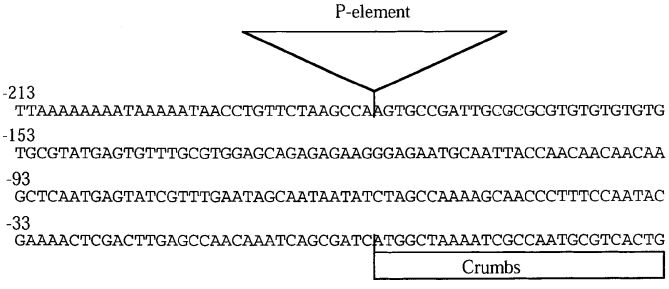

Fig. 2. Nucleotide sequence of the genomic region surrounding the P-element insertion in the *0104/09*. The open box represents the coding region of the *crumbs* gene and the nucleotide position is defined as the distance from the ATG codon of the *crumbs* gene.

www.fruitfly.org/blast) revealed that the P-element was inserted 180 bp upstream of the first ATG of the *crumbs* gene (Fig. 2). No other transcription unit was identified in the region flanking the P-element insertion. This result suggests that a reduction of the *crumbs* gene expression by the P-element insertion is responsible for the enhancement of the p53-induced rough eye phenotype. This conclusion is further supported by the fact that two other alleles of *crumbs* mutation (crb^1 and crb^2) also strongly enhanced the rough eye phenotype induced by the p53 (Fig. 3). Compound eyes of the flies heterozygous for the *crumbs* mutations without p53 overexpression appeared normal (Fig. 3, panels C and E). From these results, we conclude that a half-reduction of the *crumbs* gene dose can enhance the p53-induced rough eye phenotype.

Previously we reported that the induction of apoptosis by expression of p53 is mainly responsible for the rough eye phenotype.[17] We therefore investigated whether a half-reduction of the *crumbs* gene dose can further enhance apoptosis in eye imaginal disc cells. Apoptotic cells in the third instar larval eye discs were detected by the TUNEL method. In eye discs of flies heterozygous for the *crumbs* mutation, there were very few apoptotic cells (Fig. 4A). The eye discs of flies carrying one copy each of GMR-GAL4 and UAS-*p53* showed an increase of cell death in the very posterior region of the eye disc (Fig. 4B). In eye discs of flies carrying one copy each of GMR-GAL4 and UAS-*p53* and heterozygous for the *crumbs* mutation, a much more extensive increase of cell death was detected (Fig. 4C). These results indicate that a half-reduction of the *crumbs* gene dose can enhance the p53-induced apoptosis in eye imaginal disc cells.

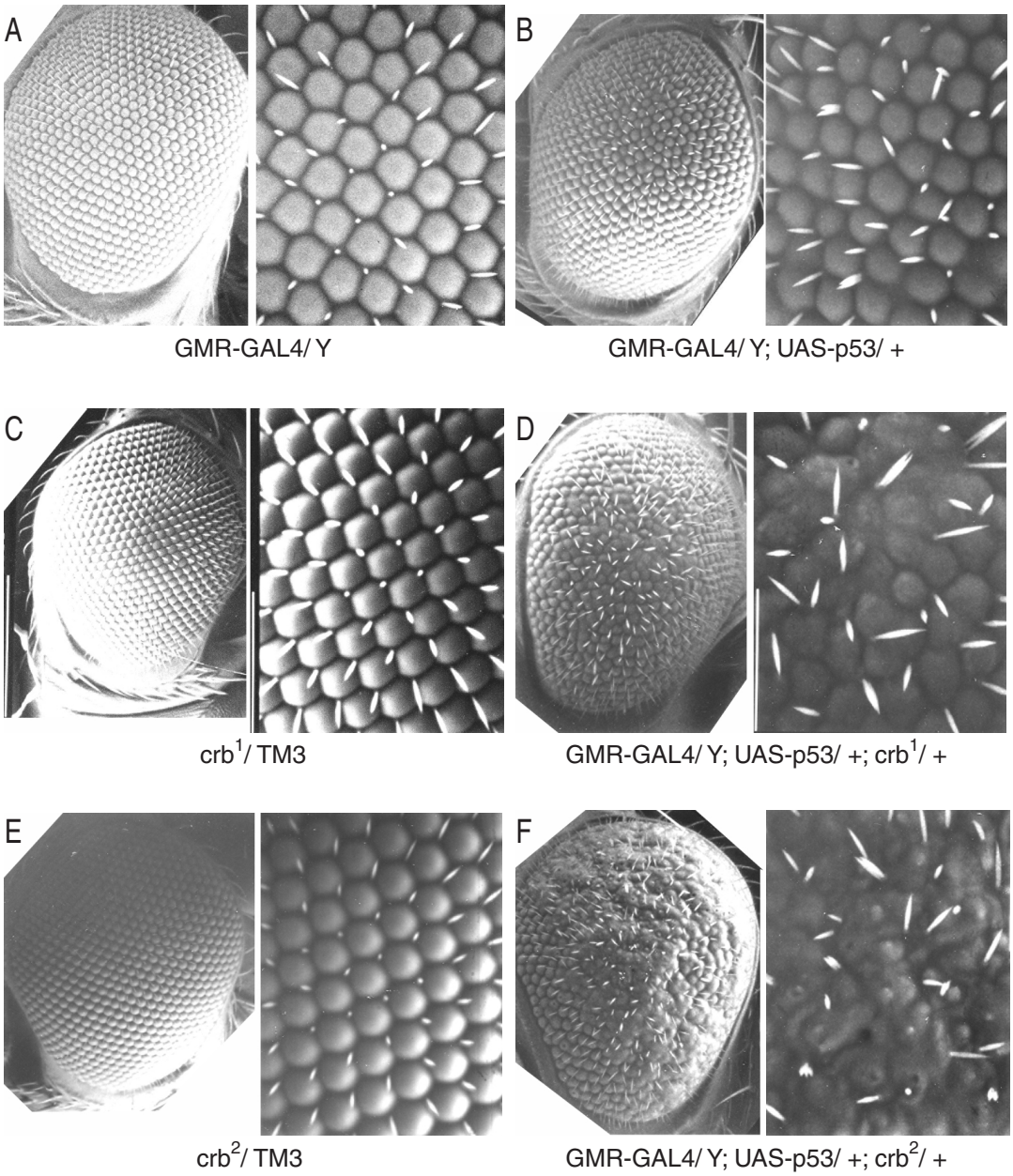

Fig. 3. Scanning electron micrographs of adult compound eyes. (A) *GMR-GAL4/Y*; +; +, (B) *GMR-GAL4/Y*; *UAS-p53*/+; +, (C) crb^1/*TM3*, (D) *GMR-GAL4/Y*; *UAS-p53*/+; crb^1/+, (E) crb^2/*TM3*, (F) *GMR-GAL4/Y*; *UAS-p53*/+; crb^2/+. The rough eye phenotype is enhanced in panels D and F. Scale bars for 50 μm (right panel) are indicated in panels C, D and F.

Crumbs protein is essential for the biogenesis of the zonula adherens junction in epithelial cells.[21–24] It is well known that Shotgun (*Drosophila* E-cadherin) and Armadillo (*Drosophila* β-catenin) are the two main constituents of the adherens junction. Although direct physical interaction between Crumbs and Shotgun or Armadillo has not been reported, the defect in *crumbs* mutants results in a misdistribution of Armadillo and Shotgun, resulting in a disruption of tissue integrity.[23] We therefore investigated whether a half-reduction of the *shotgun* or the *armadillo* gene dose can enhance the p53-induced rough eye phenotype. Under the scanning electron microscope, eyes of heterozygous *shotgun* or *armadillo* mutant flies appeared normal (Fig. 5, panels C and F). Flies carrying one copy of GMR-GAL4 and one copy of UAS-*p53* had abnormal eyes which were rough in appearance (Fig. 5, panels A and D). When these p53-expressing flies were made heterozygous for the *shotgun* (*shg*^{2}) or the *armadillo* (*arm*^{4}) mutation, the abnormality became much more extensive, many ommatidia appearing to be fused (Fig. 5, panels B and E). Essentially the same results were obtained with other allele *arm*^{1} (data not shown). These results indicate that a half-reduction of the *shotgun* or the *armadillo* gene dose can strongly enhance the p53-induced rough eye phenotype.

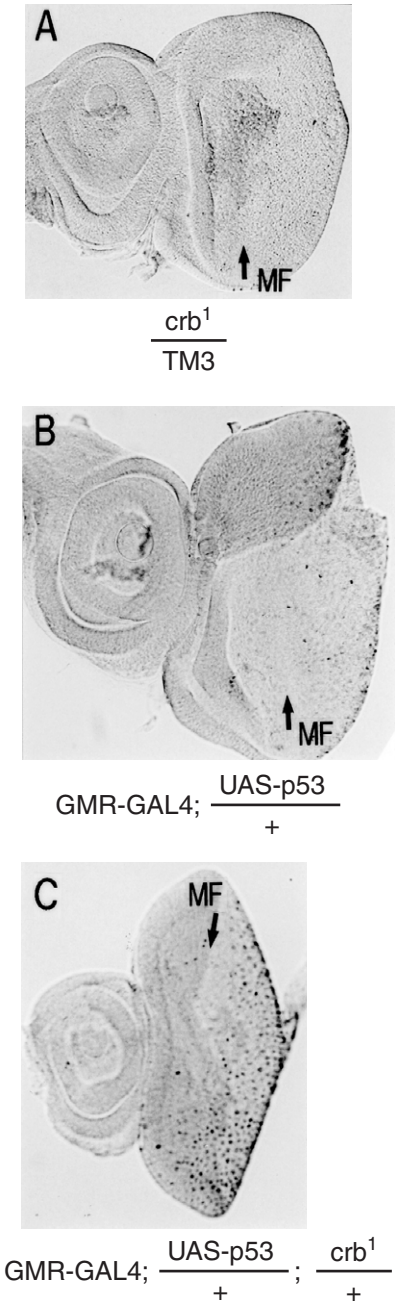

Fig. 4. Detection of apoptotic cells in eye imaginal discs. The TUNEL method was carried out with terminal deoxynucleotidyl transferase. (A) *crb*^{1}*/TM3,* (B) *GMR-GAL4*; *UAS-p53*/+; +, (C) *GMR-GAL4*; *UAS-p53*/+; *crb*^{1}/+. Arrows indicate the position of the morphogenetic furrow (MF). The anterior of the discs is on the left.

The enhanced apoptosis may be due to the increased p53 level and the half-dose reduction of the *crumbs* gene may enhance *p53* gene expression or the stability of p53 protein. To address this point, the expression level of p53 in the wild-type and the mutant flies was examined. Immunostaining with anti-p53 antibody revealed that p53 protein was expressed in cells posterior to the furrow in the eye imaginal discs and the p53 levels were comparable between wild-type flies and flies heterozygous for the *crumbs* mutation or the *shotgun* mutation (Fig. 6). These data indicate that mutations in at least these two genes do not affect the level of p53, suggesting that the increased p53-induced apoptosis may be mediated by some downstream events in p53-signaling.

Discussion

Increased levels of p53 following DNA damage can lead to cell cycle arrest or apoptosis. In this way, p53 is thought to prevent the excessive accumulation of mutations and harmful cells that could give rise to malignancies. However, tumor suppressor functions may not be the primary role of p53. Although p53-deficient mice exhibit only a marginal defect during development, it is reported that the absence of p53 function in mice increases the prevalence of defective embryos and fetuses, particularly after exposure to drugs or radiation.[30, 31] Furthermore, the p73-deficient mice exhibit defects in the central nervous system[13] and the p63-deficient mice exhibit severe defects in limb formation and epithelial tissues.[14, 15] Therefore, p53 and/or its family proteins likely play a critical role in the organization of various tissues and organs during development.

It is well known that signaling pathways derived from the cell surface plays a critical role during development and oncogenesis. The adherens junction is a multiprotein complex that attaches one cell to another in an epithelial layer.[25, 26] The junction is not spread randomly between the cells, but is found in a belt-like, zonular structure encircling the apical side of the cell.[32] Placement of the adherens junction is critical because it signals to the cell which side is out and which side is in, preventing the mixing of apical cell membrane tissue with the biochemically distinct basal cell membrane, and thereby assuring cell polarity.[32] Crumbs protein is essential for the biogenesis of the zonula adherens junction in epithelial cells,[21–24] and both Shotgun and Armadillo proteins are major constituents of the adherens junction.

As described in the present study, a half-dose reduction of the gene for Crumbs, Shotgun or Armadillo strongly enhanced the p53-induced rough eye phenotype through induction of the p53-dependent apoptosis in the eye imaginal disc. Mutations of the *crumbs*, *shotgun* or *armadillo* gene leads to widespread defects in the development of epithelial tissues, followed by massive cell death during embryogenesis.[20] Although flies heterozygous for either *crumbs*, *shotgun* or *armadillo* mutations develop apparently normally, overexpressed human p53 in the eye disc may sense a subtle abnormality at the adherens junction or in signals derived from the junction and induce apoptosis to remove abnormal cells from the tissue. Our observations

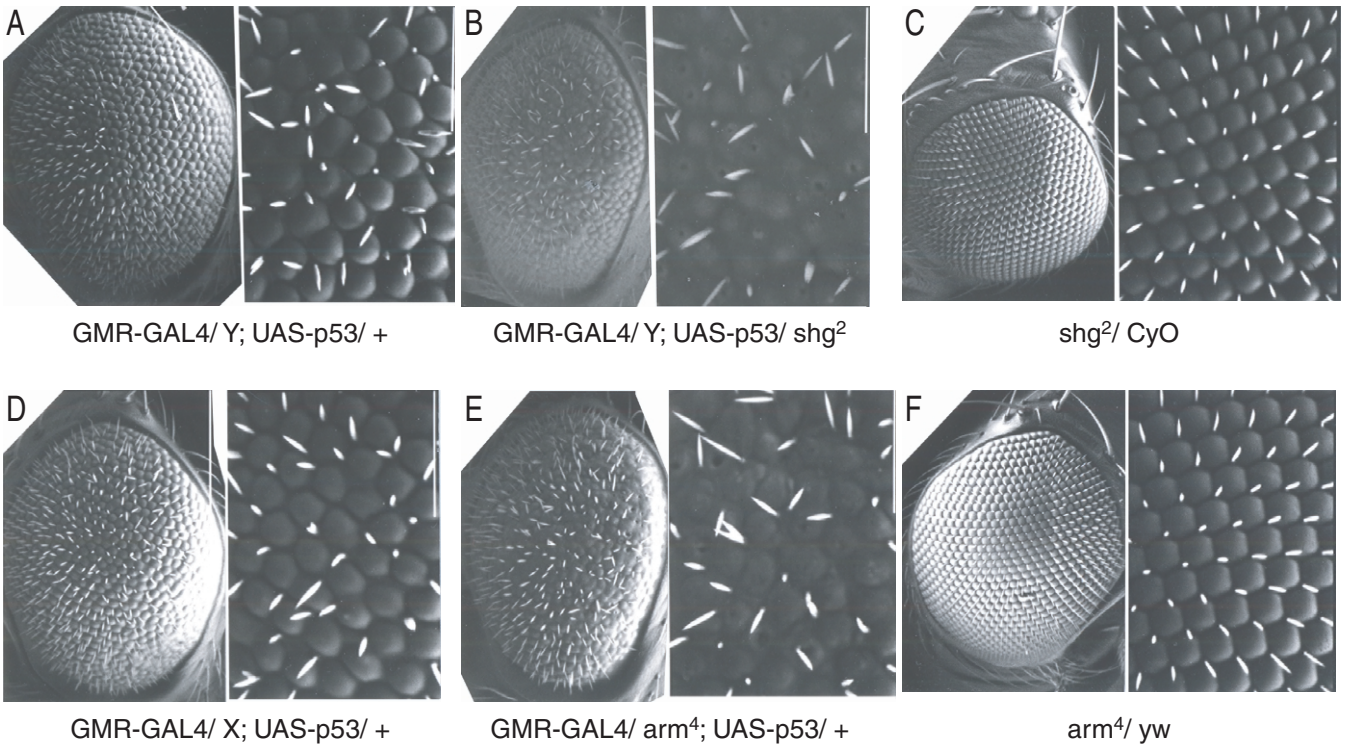

Fig. 5. Scanning electron micrographs of adult compound eyes. (A) *GMR-GAL4/Y; UAS-p53/+; +*, (B) *GMR-GAL4/Y; UAS-p53/shg²; +*, (C) *shg²*/CyO, (D) *GMR-GAL4/X; UAS-p53/+; +*, (E) *GMR-GAL4/arm⁴; UAS-p53/+; +*, (F) *arm⁴/yw*. The rough eye phenotype is enhanced in panels B and D. Scale bars for 50 μm (right panel) are indicated.

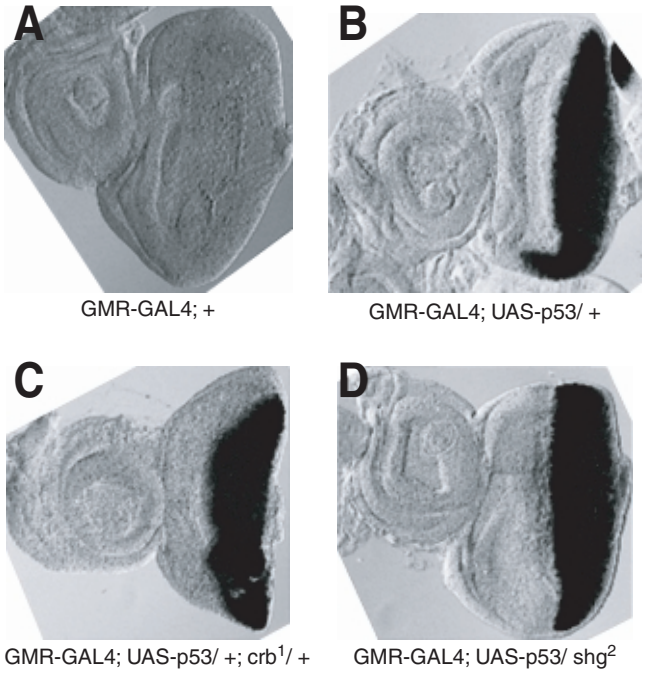

Fig. 6. Immunostaining of eye imaginal discs with anti-human p53 antibodies. (A) *GMR-GAL4; +*, (B) *GMR-GAL4; UAS-p53/+*, (C) *GMR-GAL4; UAS-p53/+; crb¹/+*, (D) *GMR-GAL4; UAS-p53/shg²*. The dissected eye imaginal discs were immunostained with the anti-human p53 monoclonal antibody DO-7. The anterior of the discs is on the left.

suggest that p53 plays a role as a guardian of the tissue by not only sensing damaged DNA, but also sensing signals from the adherens junction. It is noteworthy that Wnt-1-signaling inhibits apoptosis by activating β-catenin/T cell factor-mediated transcription in mammalian cells.[33] It would be interesting to determine whether this apoptosis depends on p53 function or not. In addition, it is reported that mutations in a human homologue of *Drosophila crumbs* cause retinitis pigmentosa.[34] Retinitis pigmentosa patients suffer from a progressive degeneration of the photoreceptors, resulting in severe visual impairment. Our observations raise the possibility that human p53 may be involved in the degenerative process of retinal pigment epithelium in retinitis pigmentosa.

Despite relatively low amino acid sequence conservation between human and *Drosophila* p53 proteins, overexpression of human or *Drosophila* p53 in the eye imaginal disc exhibits similar effects on eye development.[18, 19] We do not yet know whether *Drosophila* p53 genetically interacts with *crumbs*, *shotgun* or *armadillo* genes or not, and this awaits further studies.

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