

TFIIH controls developmentally-regulated cell cycle progression as a holo-complex

Motomi Matsuno^{1,2,a}, Hiroyuki Kose^{1,b}, Masataka Okabe^{1,2,c} and Yasushi Hiromi^{1,2,3,*}

¹Department of Developmental Genetics, National Institute of Genetics, Yata 1111, Mishima, Shizuoka 411-8540, Japan

²Department of Genetics, SOKENDAI, Yata 1111, Mishima, Shizuoka 411-8540, Japan

³CREST, Japan Science and Technology Agency (JST), Yata 1111, Mishima, Shizuoka 411-8540, Japan

Basal transcription factor, TFIIH, is a multifunctional complex that carries out not only transcription but also DNA repair and cell cycle control. TFIIH is composed of two sub-complexes: core TFIIH and Cdk-activating kinase (CAK). *In vitro* studies suggest that CAK is sufficient for cell cycle regulation, whereas core TFIIH is required for DNA repair. However, the TFIIH complexes that perform these functions *in vivo* have yet to be identified. Here, we perform an *in vivo* dissection of TFIIH activity by characterizing mutations in a core subunit p52 in *Drosophila*. p52 mutants are hypersensitive to UV, suggesting a defect in DNA repair. Nonetheless, mutant cells are able to divide and express a variety of differentiation markers. Although p52 is not essential for cell cycle progression itself, p52 mutant cells in the eye imaginal disc are unable to synchronize their cell cycles and remain arrested at G1. Similar cell cycle phenotypes are observed in mutations in another core subunit XPB and a CAK-component CDK7, suggesting that defects in core TFIIH affect the G1/S transition through modification of CAK activity. We propose that during development the function of TFIIH as a cell cycle regulator is carried out by holo-TFIIH.

Introduction

The temporal and spatial control of differentiation and proliferation in eukaryotes occurs through regulation of fundamental processes including transcription, cell cycle control and DNA repair. Basal transcription factor, TFIIH, is a good candidate for a regulator of differentiation and proliferation since it is a multifunctional complex that links transcription to DNA repair and cell cycle regulation (Egly 2001; Chen *et al.* 2003; Fisher 2005). TFIIH contains several subunits with enzymatic activity including the cdk7 subunit that functions as a kinase, and the XPB and XPD subunits that have helicase/ATPase

activity (Egly 2001). Cdk7 functions both in cell cycle control as the catalytic subunit of a Cdk-activating kinase (CAK) (Fesquet *et al.* 1993; Poon *et al.* 1993; Solomon *et al.* 1993), and in transcription as the TFIIH kinase that phosphorylates the C-terminal domain (CTD) of RNA polymerase II (Roy *et al.* 1994; Makela *et al.* 1995; Serizawa *et al.* 1995; Shiekhattar *et al.* 1995). TFIIH kinase also functions in E2F degradation (Vandel & Kouzarides 1999), and in phosphorylation and activation of nuclear receptors (Bastien *et al.* 2000; Chen *et al.* 2000; Keriell *et al.* 2002). XPB and XPD helicases/ATPases unwind double stranded DNA during transcriptional initiation in a process known as “promoter opening” or “promoter escape,” and during nucleotide excision repair (NER, Guzder *et al.* 1994a,b; Wang *et al.* 1994; Coin *et al.* 1999; Moreland *et al.* 1999; Bradsher *et al.* 2000; Hoeijmakers 2001). Such enzymatic activities are likely employed “on demand,” depending on physiological as well as developmental conditions (Egly 2001).

The subunit requirements for various individual functions of TFIIH have not been clearly elucidated. Biochemical studies have shown that 10 subunits which have been associated with TFIIH can be subdivided in

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*Correspondence: E-mail: yhiromi@lab.nig.ac.jp

^aPresent address: Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183-8526, Japan.

^bPresent address: Division for Animal Research Resources, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan.

^cPresent address: Department of Anatomy, The Jikei University School of Medicine, Tokyo 105-8461, Japan.

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two sub-complexes: core TFIID and CAK. Core TFIID consists of six subunits: p44, XPB, p62, p52, p34 and p8; and CAK is composed of three subunits: Mat1, cdk7 and cyclin H (Giglia-Mari *et al.* 2004). XPD is found in both sub-complexes, suggesting that XPD bridges CAK and core TFIID (Egly 2001). Because each subcomplex contains at least one enzymatic subunit, it is possible that certain TFIID functions are carried out by specific subcomplexes, rather than by the TFIID holo-complex. Indeed, previous experiments suggest that while DNA repair requires core TFIID, CAK is sufficient for cell cycle regulation (Rossignol *et al.* 1997; Yankulov & Bentley 1997; Tirode *et al.* 1999; Araújo *et al.* 2000; Vermeulen *et al.* 2000; Fisher 2005). These *in vitro* experiments, however, do not demonstrate that these subcomplexes are sufficient to perform TFIID function *in vivo*, where TFIID must integrate physiological information from the environment.

One method to determine what function is associated with a particular complex or subcomplex is to examine the consequences of mutations in “structural” subunits of TFIID (p8, p52, p44, p62 and p34, Tirode *et al.* 1999; Giglia-Mari *et al.* 2004). Mutation of a single “structural” subunit can change the stability and function of the TFIID complex that contains the subunit (Seroz *et al.* 2000; Tremeau-Bravard *et al.* 2001; Giglia-Mari *et al.* 2004). One such “structural” subunit that is strongly conserved throughout evolution is p52, a subunit of core TFIID (Feaver *et al.* 1997; Marinoni *et al.* 1997; Lanning & Lafuse 1999). p52 has been proposed to mediate the binding of the XPB helicase/ATPase to other components of the TFIID core complex (Jawhari *et al.* 2002). Loss of the p52 subunit is expected to affect functions performed by core TFIID and the holo-complex, but not those that can be carried out by CAK subcomplex alone. Here, we present a functional analysis of the p52 subunit of TFIID in the *Drosophila* eye imaginal disc, a developing tissue where cell proliferation and cell fate determination are linked and occur simultaneously (Baker 2001).

The *Drosophila* eye imaginal disc is an excellent system to examine various functions of TFIID, because cell proliferation and cell fate determination are under spatiotemporal control and cells at varying stages of development can be identified based on their position along the anterior–posterior axis of the disc epithelium (Baker 2001). Cells divide asynchronously until the third instar larva (the first mitotic wave), but then arrest prior to differentiation at the G1 stage in a strip of cells called the morphogenetic furrow. Posterior to the morphogenetic furrow, a minority of cells enter a differentiation program to become specific photoreceptor neurons, whereas the remaining majority of cells undergo synchronous cell

division to produce a pool of uncommitted cells (the second mitotic wave, Baker 2001). Thus, all cells in this region must choose either to initiate photoreceptor differentiation or to re-enter a final mitosis. These features make the eye imaginal disc an ideal system for performing genetic analyses of p52 to unravel its role in TFIID functions.

We assayed various TFIID-dependent cellular functions in a clonal population of cells, where p52 levels were reduced. p52 mutant cells were capable of expressing various differentiation and cell fate markers, but had defects in DNA repair, and progression through a developmentally-regulated cell cycle event. Furthermore, we determined that both p52 and Cdk7 are required for the G1/S transition, a first demonstration in multicellular organisms. Cell cycle defects similar to that observed in our p52 mutants were also observed in mutants of another TFIID core subunit, XPB. Our data indicate that the integrity of the TFIID core is required to regulate cell cycle progression, implying that regulation of the cell cycle by TFIID requires an intact holo-complex.

Results

The p52 subunit of TFIID is essential for viability and tissue growth

We generated loss-of-function mutations in the gene encoding p52 using line ep(3)0572, in which a P-transposable element is inserted 5 bp downstream of the translation initiation site of the p52 open reading frame (Fig. 1A). Excision lines were generated from which we identified line ex9-2, in which 989 bp of the genomic DNA (position +6 to +994, where +1 is the first nucleotide of the translation initiation codon) were deleted and replaced with 24 bp of unrelated sequence. These result in an early in-frame stop codon and suggests that line ex9-2 is likely to contain a null allele of p52. We also found that mutations in a previously identified locus, *marionette* (*mrrn*, Fuller 1986), fail to complement the lethality of ex9-2. Examination of the p52 genomic DNA of six of these *mrrn* alleles revealed that all six alleles had changes within the p52 coding region: *mrrn*², *mrrn*³, *mrrn*⁴ and *mrrn*⁵ had nonsense mutations, *mrrn*⁶ contained a 207 bp deletion including the translation initiation codon, and *mrrn*¹ carried two mis-sense mutations within the open reading frame (Fig. 1A). Thus, the p52 subunit of TFIID is encoded by the *mrrn* locus, and we will refer to ex9-2 as *mrrn*⁹. While this work was in preparation Fregoso *et al.* (2007) also reported that the *mrrn* gene encodes the p52 subunit. In contrast to their report, we found two mis-sense mutations in *mrrn*¹: A318P and E340K.

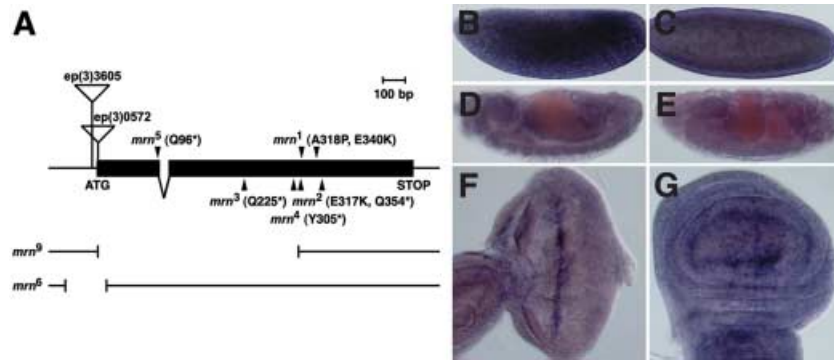


Figure 1 Molecular characterization and expression analysis of *Drosophila* p52. (A) Physical map of the *mrn* region. Two exons are separated by a 55-bp intron. The positions of two P-element insertions are indicated by open triangles. The extents of the deleted areas in the excision alleles *mrn*⁹ (989 bp) and *mrn*⁶ (207 bp) are shown below the map. The positions of mutations in *mrn*², *mrn*³, *mrn*⁴, *mrn*⁵ (nonsense mutations) and *mrn*¹ (two mis-sense mutations) are indicated by arrowheads. (B–G) Expression of *mrn* mRNA in embryos (B–E, anterior to the left) and imaginal discs (F, G). Stages 2–3 embryos contain a high level of maternally supplied *mrn* mRNA (B). Maternal mRNA levels are reduced by stage 5 (C). At stage 14 (D) or 16 (E), zygotic expression of *mrn* is ubiquitous but highest in the brain, midgut and Malpighian tubules. In third instar larval eye imaginal discs (F), *mrn* mRNA is highest in the morphogenetic furrow, where cells are arrested in G1. Anterior is to the left. In a larval wing imaginal disc (G), the expression of *mrn* is especially high in the wing pouch.

All alleles of *mrn* are zygotic lethal, dying before the third instar larval stage. Although *mrn* mutant animals survive for 5 days, which corresponds temporally to the third instar larval stage in normal animals, mutant individuals never grow to sizes larger than normal first instar larvae. This suggests that *mrn* mutants have a defect in tissue growth. Since *mrn*¹ survives to the third instar larval stage in a transheterozygote with a null allele, *mrn*¹ likely retains some p52/MRN activity.

mrn is highly expressed in proliferating cells

We next characterized the expression pattern of *mrn* during development by *in situ* hybridization (Fig. 1B–G). Early embryos contain maternally supplied *mrn* mRNA. As development proceeds, expression becomes more uneven and is highest in the proliferating cells of the nervous system, Malpighian tubules and midgut (Fig. 1D,E). At larval stages, *mrn* is highly expressed in restricted regions of the imaginal discs, including the wing pouch in the wing imaginal disc, and the morphogenetic furrow in the eye imaginal disc, an indentation in the disc epithelium that acts as a moving front of differentiation (Fig. 1F,G). During eye development, cells become arrested in G1 within the morphogenetic furrow prior to differentiation (Baker 2001). The high expression levels of *mrn* in the morphogenetic furrow suggest that the requirement for p52/MRN is particularly high in this region.

mrn is required for cell proliferation in the developing eye

Because *mrn* mutant animals die before the function of the p52 subunit during eye development can be analyzed, we generated homozygous somatic clones of *mrn*⁹ and *mrn*¹ alleles in otherwise heterozygous animals. Mitotic recombination was induced by the FRT/FLP technique using *ey-FLP* which targets the recombination event that generates a homozygous mutant founder cell to the eye imaginal disc (Fig. 2A–F). Proliferation of this founder cell produces a clonal population of mutant cells, which cannot produce MRN mRNA. Therefore, p52 subunit present in the *mrn*^{+/-} mother cell should be serially diluted at each cell division.

Whereas control *mrn*⁺ clones made in this manner covered almost the entire eye, homozygous *mrn* mutant clones were hardly detected in the adult eye. We hypothesized that *mrn* mutant clones may have a strong growth or proliferation defect compared to wild-type cells. Thus, we reasoned that *mrn* mutant clones might be more easily seen in animals with a dominant *Minute* (*M*) mutation (reviewed by Lawrence *et al.* 1986; Ashburner 1989). Since *M*^{-/+} cells have reduced growth rate, *mrn* mutant cells are less likely to be competed out even if they have defects in proliferation. Under these experimental conditions *mrn* mutant clones were recovered in the eye imaginal disc, although their sizes were much smaller compared to *mrn*⁺ control clones (Fig. 2A–C). The clone

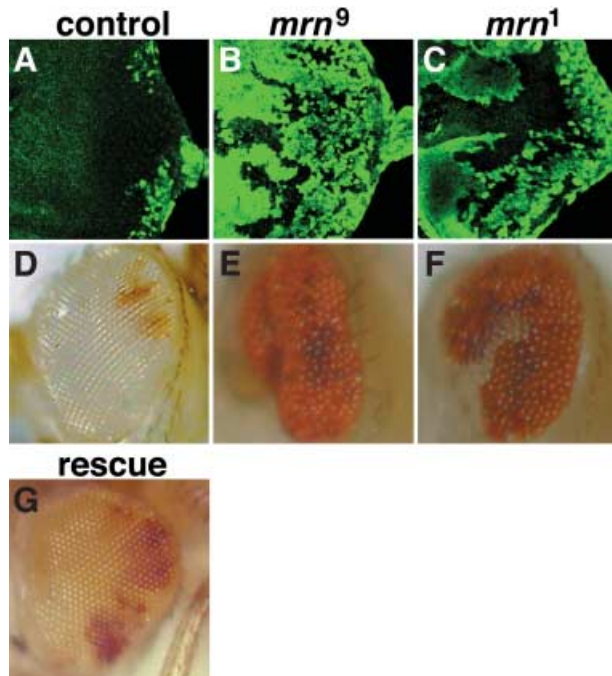


Figure 2 *mrn* mutation affects cell proliferation. *mrn* mutant and control clones were generated in the compound eye by somatic recombination induced by ey-FLP in a *Minute* (*M*) background. Clones were detected by the lack of GFP marker (green) in the third instar larval eye imaginal disc (A–C, Anterior is to the left), or by the lack of red pigments in the adult eye (D–F). (A, D) Control clones consisting of *mrn*⁺ cells. (B, E) *mrn*⁹ clones. (C, F) *mrn*¹ clones. Homozygous *mrn* mutant cells were much less abundant than in controls (A–C), suggesting a defect in cell proliferation. The cell proliferation defect of a homozygous *mrn*⁹ clone was rescued by over-expression of full-length *mrn* (G). The rescue transgene carries a mini-*white* marker and thus the mutant clone exhibits pale orange eye color.

size of *mrn*¹ mutants was larger than that of *mrn*⁹ mutants, suggesting that *mrn*¹ is a hypomorphic allele. The small size of *mrn* mutant clones is likely due to a reduced rate of cell division, since only a small number of TUNEL positive cells are observed in *mrn* clones in larval discs (see below). These results suggest that p52/MRN is required for cell proliferation. When full-length *mrn* is expressed under ey-GAL4 control, normal clonal sizes are restored (Fig. 2G).

Transcription and translation can occur in *mrn* mutant clones

The defects in cell proliferation that we observed in *mrn* mutant clones could be a secondary consequence of defective transcription. To test this possibility, we first

checked transcription in *mrn* mutant clones generated in the eye using a *lacZ* reporter gene driven by transcription factor Glass. Glass is expressed in all cells within the eye morphogenetic field where cell fate determination and differentiation is taking place (Ellis *et al.* 1993). In the third larval instar eye imaginal disc this reporter gene was expressed normally within *mrn* mutant clones (Fig. 3A–C). Furthermore, multiple markers for differentiation of specific cell types could be detected within *mrn* mutant clones (Figs 3D–O and 6A–C, G–I). These results suggest that even under conditions where a core p52 subunit of TFIIF is severely reduced (and possibly not present), cells can still transcribe a wide range of genes. In addition, they further suggest that defects in proliferation may not be caused by defective transcription.

mrn mutant cells are hypersensitive to UV light

TFIIF is involved in a DNA repair mechanism known as NER (Hoeijmakers 2001), and loss of function of yeast and human p52 is associated with defective NER (Feaver *et al.* 1997; Marinoni *et al.* 1997). NER-deficient cells are generally hypersensitive to UV light and UV irradiation triggers apoptosis. In order to test whether *mrn* is required for the NER activity, we examined the UV sensitivity of *mrn* mutant cells in the eye imaginal disc. When larvae are irradiated with 500 J/m² of UV light and dissected 4 h later, massive apoptosis is detected in *mrn* mutant clones (Fig. 4E,F), but not in normal cells (Fig. 4D). The degree of apoptosis increases in proportion to the UV dose. Thus, cells with reduced p52 subunit are hypersensitive to the UV light in *Drosophila*, as in other organisms. Despite this severe phenotype upon UV irradiation, only a small number of TUNEL positive cells are observed in *mrn* mutant clones in the absence of irradiation (Fig. 4B,C). Furthermore, mutations in other NER components show almost normal development and DNA synthesis (Boyd *et al.* 1982; Friedberg & Meira 2000; Calleja *et al.* 2001), implying that under normal environmental conditions a defect in DNA repair function does not necessarily lead to a cell growth phenotype. It is thus likely that the proliferation defect of *mrn* clones is not due to a failure to repair spontaneous DNA damage.

Role of *mrn* in cell cycle progression

Another possible cause for the proliferation defect of *mrn* mutant clones is that p52/*mrn* mutants may have reduced CAK activity, which regulates the cell cycle by phosphorylating CDKs. To address this possibility, we assayed cell cycle progression in *mrn* mutant cells. In the first instar larval CNS and eye imaginal disc, BrdU incorporation of

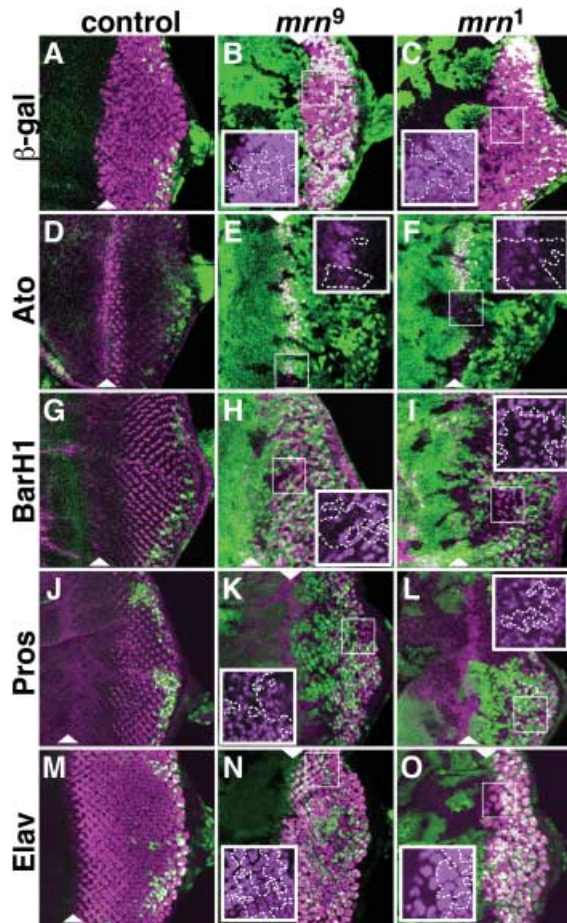


Figure 3 Transcription occurs in *mrn* mutant clones. Antibody staining of various differentiation reporter proteins (magenta) was examined in eye imaginal discs which contain control clones (A, D, G, J, M), *mrn*⁹ clones (B, E, H, K, N), and *mrn*¹ clones (C, F, I, L, O). Because clones lack expression of a GFP marker (green), expression of reporters within a clone are visible as magenta, whereas expression of reporters in non-clonal areas appear white due to the overlap of green and magenta. The morphogenetic furrow is indicated by a white arrowhead. Insets are higher magnification views of the region shown in white frame. Only the magenta channel is shown, where the mutant region is indicated by a dotted contour line. Anterior is to the left. Antibodies to the following proteins were used: (A–C) β -galactosidase which was expressed under control of the Glass transcription factor (GBS–NZ). Glass is expressed in all cells posterior to the morphogenetic furrow. (D–F) Atonal protein which is expressed in R8 neurons. (G–I) BarHI protein which is expressed in R1 and R6 photoreceptor neurons. (J–L) Prospero protein, which is expressed in the R7 neuron and non-neuronal cone cells. (M–O) ELAV protein which is expressed in neurons. We detected expression of all reporters in *mrn* mutant clones, although in many cases the number of positively staining cells was less than normal.

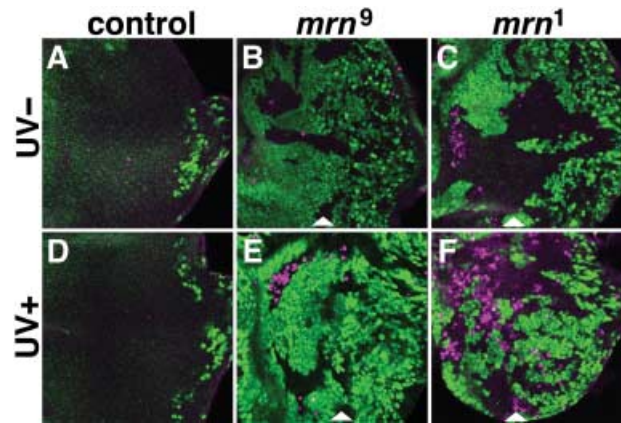


Figure 4 *mrn* mutant cells are hypersensitive to UV-light. Eye imaginal discs from third instar larvae that harbor control clones (A, D), *mrn*⁹ clones (B, E) and *mrn*¹ clones (C, F) were examined for apoptosis using the TUNEL method (magenta). (A–C) Without UV-irradiation. (D–F) 4 h after UV irradiation of 500 J/m². UV irradiation induced massive apoptosis in *mrn* mutant clones (E, F), but not in normal cells (D). Without UV irradiation, little apoptosis occurred in *mrn* mutant clones. White arrowheads indicate the morphogenetic furrow.

mrn^{-/-} individuals is reduced compared to normal animals of the same stage, but not entirely lost (Fig. 5B,D). The lack of a severe phenotype in the first instar stage may be due to the presence of a maternal supply of p52/MRN, and/or the relative insensitivity of cell division at this stage to the reduction in MRN levels.

To test the role of p52/MRN in a developmentally-regulated cell cycle, we chose the third instar larval eye imaginal disc, in which cell fate determination and proliferation are linked and occur simultaneously (Baker 2001). Neuronal development commences at the early third instar larval stage when the morphogenetic furrow forms at the posterior end. Equally-spaced ommatidial clusters form immediately posterior to the furrow, and as the furrow moves from posterior to anterior, new rows of ommatidial clusters are generated throughout the third instar larval stage. Anterior to the morphogenetic furrow cell division occurs randomly, called the first mitotic wave. However, at the morphogenetic furrow, cells are arrested at G1, synchronizing the cell cycle for the onset of ommatidial assembly. After exiting the furrow, cells are recruited into ommatidial clusters for the first round of cell fate specification and neuronal differentiation. While some cells differentiate into neurons, most cells undergo a synchronous cell division called the second mitotic wave, which supplies a pool of uncommitted cells for a second round of recruitment into the ommatidial cluster.

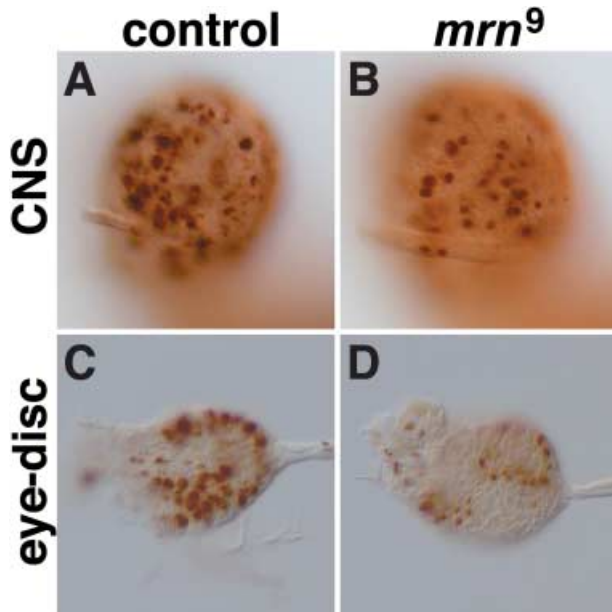


Figure 5 DNA synthesis takes place in *mrn* null first instar larva. DNA synthesis in normal (A, C) and *mrn* null (B, D) first instar larva was examined by BrdU incorporation. In both the CNS (A, B) and eye imaginal discs (C, D), *mrn*⁹ animals were able to incorporate BrdU, although the number of labeled cells was reduced compared to control animals. First instar eye; normal (39.0/eye disc, SE = 3.1, *n* = 5), *mrn*⁹ (24.8/eye disc, SE = 3.2, *n* = 5). First instar brain; normal (39.8/brain lobe, SE = 2.1, *n* = 8), *mrn*⁹ (28.6/brain lobe, SE = 3.9, *n* = 8).

To investigate the consequence of a lack of p52/MRN in this process, we examined the expression of several cell cycle markers in the *mrn* mutant mosaic eye: Cyclin E (CycE), which triggers the G1/S transition, BrdU, which is incorporated at S-phase, Cyclin B (CycB), which is detected in cells that have passed the G2/M transition but have not yet divided, and phospho-H3, which is detected during mitosis. In normal eye imaginal discs these cell cycle markers show a strictly coordinated pattern in the second mitotic wave. CycE is detected just behind the morphogenetic furrow (Fig. 6A), BrdU incorporation begins at column 1 and continues to column 3 (Fig. 6D), CycB is detected from column 3 onwards with a progressive reduction in labeled cells in posterior areas (Fig. 6G), and phospho-H3 is detected in column 4 in cells undergoing mitosis in the second mitotic wave (Fig. 6J).

In the region anterior to the morphogenetic furrow, cell cycle progression is delayed but still occurs in *mrn* mutant clones; large numbers of CycE, CycB and phospho-H3 positive cells are visible (Fig. 6). However,

BrdU staining is reduced (Fig. 6F) possibly accounting for the reduced clonal size of mutants. Since both phospho-H3 and CycB signal is present and mutant cells are larger than surrounding *mrn*⁺ cells, the cell cycle is likely lengthened but not completely arrested (Fig. 6M–O).

Compared to a rather mild phenotype in the anterior region of the eye imaginal disc, posterior to the morphogenetic furrow, cell cycle progression is completely blocked at the G1/S transition; CycE accumulates to abnormally high levels, BrdU incorporation is absent, and CycB and phospho-H3 expression is decreased (Fig. 6). To demonstrate the differential effect of the *mrn* mutation on the first and the second mitotic wave, we counted the number of M-phase cells anterior and posterior to the morphogenetic furrow. This quantitative analysis shows that the posterior region, where the second mitotic wave takes place, is more sensitive to the reduction in MRN activity compared to the anterior region (Fig. 6P). This difference in sensitivity may be due to the difference in the nature of the cell division: only the second mitotic wave is accompanied by developmentally-regulated cell cycle synchronization. Thus, p52/MRN may be necessary for G1/S transitions that occur under specific regulatory control, possibly responding to specific developmental signaling pathways.

CDK7 functions in both G1/S and G2/M transitions

While the CAK subcomplex of TFIID is known to be essential for cell cycle progression, p52 is not a component of this subcomplex but instead is a component of the TFIID core complex (Rossignol *et al.* 1997; Yankulov & Bentley 1997; Tirode *et al.* 1999; Araújo *et al.* 2000; Vermeulen *et al.* 2000; Fisher 2005). Since p52 mutations affect the cell cycle, we reasoned that the absence of p52 may alter the integrity of holo-TFIID, affecting CAK enzymatic activity. To determine whether our *mrn* mutants are similar to CAK mutants, we compared loss-of-function phenotypes of *mrn* to those of *cdk7*, the gene encoding the catalytic subunit of the CAK subcomplex.

To examine the consequence of reduced CAK activity in the eye imaginal disc, we used a temperature sensitive allele of *cdk7* (Larochelle *et al.* 1998). The *cdk7*^{ts} allele encodes a protein that is destabilized at the restrictive temperature. When *cdk7*^{ts} animals are shifted to 29°, the size of the eye imaginal disc becomes smaller in proportion to the time that the animals are kept at this temperature. When maintained at 29 °C for 3 days, abnormalities are seen not only at M-phase but also at S-phase; both phospho-H3 staining and incorporation of BrdU are reduced (Fig. 7E,K). CycE staining is increased, accompanied by

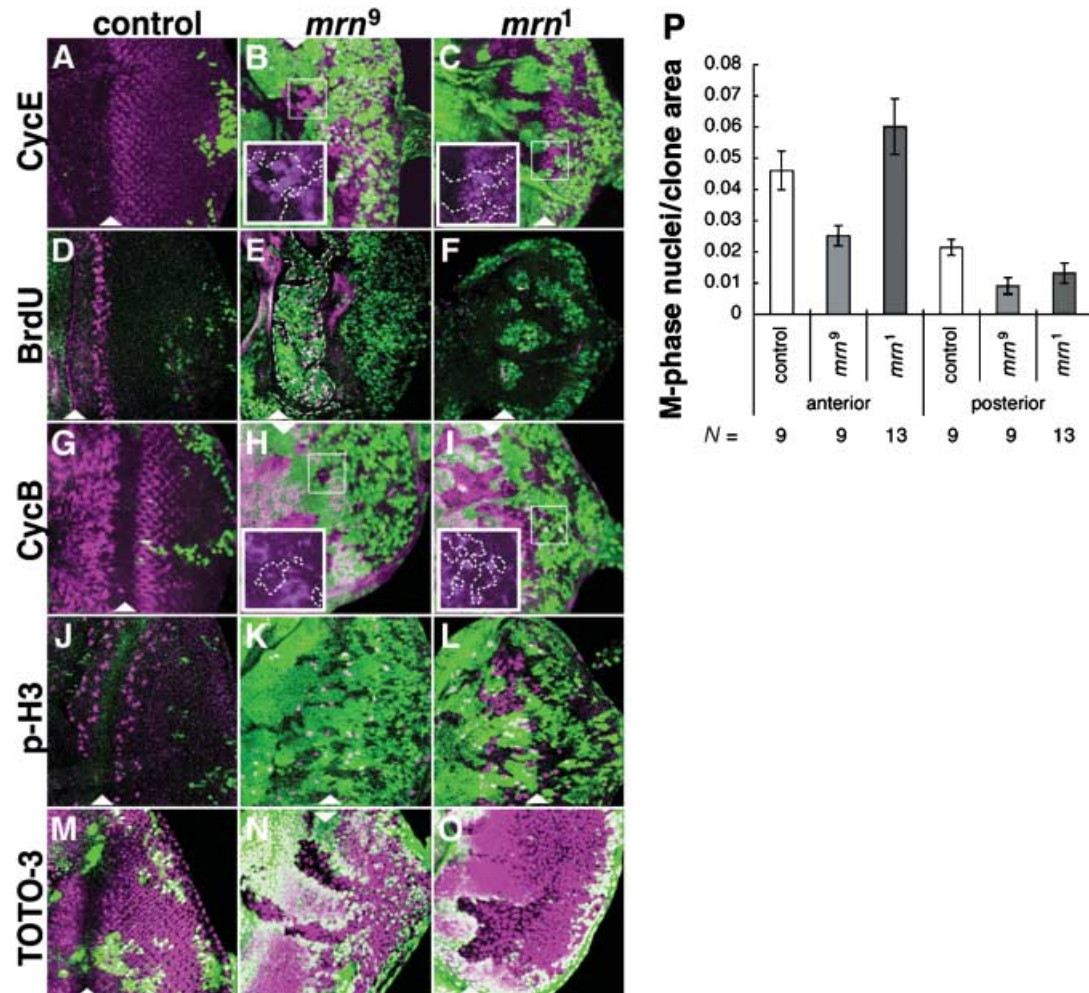


Figure 6 *mrn* mutant clones exhibit G1 arrest at the second mitotic wave. Clones homozygous for *mrn⁹* (B, E, H, K, N), *mrn¹* (C, F, I, L, O), as well as control clones (A, D, G, J, M) were made in the eye imaginal disc, and cell cycle progression was examined at the late third instar larval stage. Cells within the clone can be recognized by the lack of the GFP signal (green). The position of the morphogenetic furrow is indicated by a white arrowhead. Markers used are indicated on the left and shown in magenta. Insets in B, C, H, I are higher magnification views (magenta channel) of the region shown in white frame, and the mutant region is indicated by a dotted contour line. In E the mutant clone region is indicated by a dotted contour line. DNA was labeled in M, N, O by staining with TOTO-3. *mrn* mutant clones exhibit lower nuclear density. Cells in *mrn* mutant clones contain high levels of Cyclin E (B, C), but fail to incorporate BrdU (E, F) at the second mitotic wave (arrow). Cyclin B levels are high in the anterior region of the disc, but are lost posterior to the morphogenetic furrow (H, I). Phospho-H3 signal is decreased in the posterior region of the morphogenetic furrow (K, L). The number of phospho-H3 signal per unit area (P; number of positive nuclei/pixels). In the hypomorphic allele *mrn¹*, the reduction in the mitotic signal is specific to the posterior region. Mitotic index per nuclei also exhibits anterior vs. posterior difference in *mrn¹* discs.

a reduction in CycB (Fig. 7B,H). When animals are returned to 22° for 24 h after a 3-day shift to 29°, cells in G2 remain arrested (CycB accumulation and small number of phospho-H3 cells), while G1 arrested cells are able to enter S-phase (Fig. 7C,F,I,L). Thus, a CAK functions at the G1/S transition in *Drosophila*, suggesting that the cell cycle defects seen in *mrn* mutants may be caused by defective CAK activity.

Disruption of the XPB activity leads a cell cycle defect

If p52/MRN is required for CAK activity, other subunits of the TFIIH core may also be required for *in vivo* CAK activity and/or cell cycle regulation. In order to determine whether a helicase/ATPase subunit participates in cell cycle regulation, we assayed for cell cycle progression in

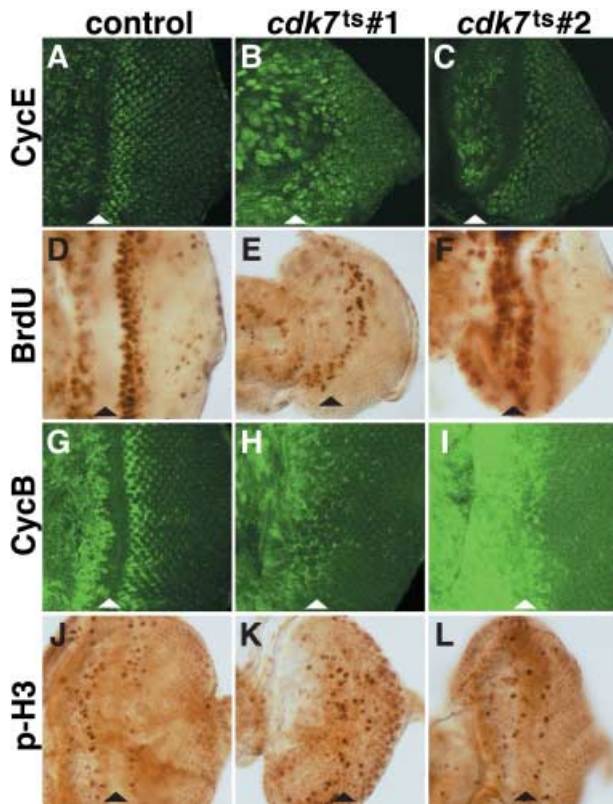


Figure 7 CDK7 subunit is required for the entry to the S-phase. Cell cycle progression in control (A, D, G, J) and *cdk7^{ts}* (B, C, E, F, H, I, K, L) animals. *cdk7^{ts}* animals were shifted to 29 °C for 3 days and fixed either immediately (B, E, H, K) or after a 24 h recovery period at 22 °C (C, F, I, L). The position of the morphogenetic furrow is indicated by a triangle. Markers used are indicated on the left. At 29 °C *cdk7^{ts}* animals exhibited similar phenotypes to *mrn* mutant clones. When animals are returned to the permissive temperature for 24 h, BrdU incorporation recovers indicating re-entry to the S-phase, but G2 arrested cells fail to resume mitosis.

mutant clones of TFIIF XPB, encoded by the *haywire* (*hay*) gene (Mounkes *et al.* 1992). We used two nonsense mutations of *hay*: *hay^{rv3}* and *hay^{rv7}*, both of which have reduced levels of *hay* mRNA (Mounkes & Fuller 1999). Since these alleles are homozygous lethal, *hay* mutant clones were generated in eye imaginal discs using somatic recombination. For both alleles, only small clones were obtained in the third instar larval eye disc (Fig. 8A–C), suggesting that *hay*, like *mrn*, is essential for cell proliferation. As in *mrn* mutant clones, CycE accumulated to high levels in *hay* mutant clones (Fig. 8E,F), consistent with the idea that XPB is also required for cell cycle regulation. Similarities in phenotypes among *cdk7*, *mrn* and *hay* suggest that *in vivo*, an intact TFIIF core complex is required for cell cycle regulation.

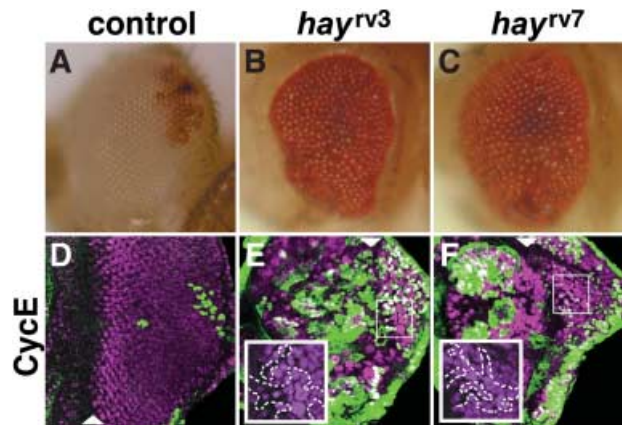


Figure 8 Mutations in the XPB helicase/ATPase subunit have cell cycle defects similar to *mrn* mutants. Mutations in *hay*, a gene encoding the XPB helicase/ATPase subunit, were examined for defects in cell cycle progression. Clones homozygous for *hay^{rv3}* (B, E) and *hay^{rv7}* (C, F) were made in the eye imaginal disc by mitotic recombination, using ey-FLP in a *Minute* background. A, D carry control clones. Clones were identified in the adult eye by the lack of *white⁺* pigments (A–C, white area), or in the third instar larval eye imaginal disc by the lack of the GFP signal (D–F green). White arrowheads indicate the morphogenetic furrow. D–F show Cyclin E expression in magenta. Insets are higher magnification views of the region shown in white frame, showing higher Cyclin E levels in mutant regions (indicated by dotted contour lines).

Discussion

TFIIF has various enzymatic activities that can potentially be regulated by external developmental signals or physiological inputs. Because most previous analyses of TFIIF function have employed reconstitution experiments using recombinant proteins to focus on combinations of subunits that are sufficient to exhibit biochemical activities of TFIIF *in vitro*, and not much attention has been made on the structure of the TFIIF complex that performs these functions in the context of the whole organism. In order to probe the nature of the TFIIF complex *in vivo*, we carried out a genetic dissection of a *Drosophila* TFIIF core subunit p52, encoded by the *mrn* locus. Previous mutational and inhibitory-antibody studies of p52 in yeast, human fibroblast cell lines and *Drosophila* had shown that this subunit is required for NER and transcription (Feaver *et al.* 1997; Marinoni *et al.* 1997; Fregoso *et al.* 2007). Although transcription could still take place in the *Drosophila mrn* mutant clone, we demonstrated that p52/MRN is essential for NER activity as well as cell cycle control *in vivo*. p52 has been shown to interact with XPB, one of the TFIIF helicases/ATPases and functions in unwinding the double strand

DNA in NER, and to anchor it to the TFIIH core (Jawhari *et al.* 2002; Fregoso *et al.* 2007). In the *mrn* clone, it is likely that XPB fails to function fully, causing a block in NER. Since the absence of the CAK subunit CDK7 has no effect on NER, our results confirm the idea that the NER function of TFIIH is carried out by the core complex (Tirode *et al.* 1999; Araújo *et al.* 2000; Vermeulen *et al.* 2000; Merino *et al.* 2002).

TFIIH regulates the cell cycle as a holocomplex

The most striking phenotype observed in *mrn* mutant clones is a defect in cell cycle progression. Two distinct forms of cell divisions take place in normal eye imaginal disc; one occurs anterior to the morphogenetic furrow in which cell divides randomly, and the other occurs posterior to the morphogenetic furrow (second mitotic wave), where the cell cycle is coordinated with morphogenesis and ommatidial differentiation. In the *mrn* mutant clone, cell cycle progression anterior to the morphogenetic furrow is slower than in wild-type, while at the second mitotic wave, *mrn* mutant cells are incapable of synchronizing their cell cycles and performing coordinated patterns of cell cycle events. Since similar phenotypes are observed in a *cdk7* mutant, the cell cycle defect observed in *mrn* mutant clones is likely to be a consequence of compromised activity of CDK7, the enzymatic component of CAK.

How does the p52 subunit of the TFIIH core affect CAK activity? Because the three subunits of CAK are sufficient to activate Cdks *in vitro*, it has been thought that cell cycle regulation is performed by the trimeric CAK subcomplex, independent of the TFIIH core (Fisher 2005). However, events such as E2F degradation, nuclear receptor activation and phosphorylation of the RNA polymerase CTD require an intact TFIIH holoenzyme (Roy *et al.* 1994; Makela *et al.* 1995; Serizawa *et al.* 1995; Shiekhattar *et al.* 1995; Vandel & Kouzarides 1999; Bastien *et al.* 2000; Chen *et al.* 2000; Keriell *et al.* 2002). Likewise, cell cycle regulation *in vivo* may also be mediated by the TFIIH holocomplex. In fact, the substrate specificity of CAK is altered by the association between Mat1 and the TFIIH core, and Cdk4 is phosphorylated by the TFIIH complex rather than the CAK subcomplex (Rossignol *et al.* 1997; Yankulov & Bentley 1997; Watanabe *et al.* 2000). Our results demonstrate that two of the TFIIH core subunits, p52 and XPB, are required for CDK7-dependent cell cycle regulation strongly suggests that *in vivo*, CAK activity is carried out by the TFIIH holocomplex. This may allow integration of various developmental signals, such as those present at the morphogenetic furrow, to control TFIIH activity and thus cell cycle progression and synchronization.

p52/MRN is necessary for the CAK activity in G1/S transition

Our work on p52/MRN has also revealed a new role of CAK in cell cycle regulation. Two classes of CAKs have so far been identified: monomeric Cak1p from budding yeast and the *cdk7*/cyclin H/MAT1 subcomplex of TFIIH from metazoans. While yeast CAK is important for both G1/S and G2/M transitions, in other metazoans including *Drosophila*, only a role in the G2/M transition had been identified (Kaldis *et al.* 1996; Thuret *et al.* 1996; Sutton & Freiman 1997; Larochelle *et al.* 1998; Saiz & Fisher 2002). Because both p52/*mrn* and *cdk7* mutations cause a block in the G1/S transition (G1 arrest) at the morphogenetic furrow, our results demonstrate that CAK activity of TFIIH is also required for the G1/S transition during a developmentally-regulated cell cycle progression. A recent report also showed the requirement of CAK in the G1/S transition in a human cell line (Larochelle *et al.* 2007). The dual functions of CAK in both G1/S and G2/M transitions may be a conserved feature of TFIIH-mediated cell cycle regulation in metazoans.

Experimental procedures

Genetics and fly stocks

We used the following fly strains: *mrn* mutant lines *mrn*¹, *mrn*², *mrn*³, *mrn*⁴, *mrn*⁵, *mrn*⁶, were gifts from Margaret T. Fuller and are listed in Flybase <<http://flybase.bio.indiana.edu/>>. Df (3 L) BK10, which deletes the *mrn* locus, is described in Leicht & Bonner (1988). *hay*^{rv3} and *hay*^{rv7} (Mounkes & Fuller 1999) were obtained from Margaret T. Fuller. *mrn* mutant clones in the eye were made in the following genotype: *y w ey-Flp: mrn FRT80/P[ub-GFP] P[w⁺70C M (3 L) FRT80*. A line containing the P[y⁺70C FRT80 chromosome was used as a *mrn*⁺ control. The strain that carries a temperature sensitive allele of *cdk7* (Df(1)JB254 P[w⁺snf⁺, dhd⁺];+; P[w⁺ cdk7^{P140S}]) is described in Larochelle *et al.* (1998). GBS-NZ (West 1998; Niwa *et al.* 2004) carries a NLS-*lacZ* fusion gene in the pGMR vector (Hay *et al.* 1994), and expresses nuclear β -galactosidase in all cells posterior to the morphogenetic furrow. *ey-GAL4* (Halder *et al.* 1998) was used for rescue experiments.

Molecular biology

The full-length p52 cDNA was cloned into the pUAS vector for transgenic rescue and over-expression experiments. The P-element in line ep(3) 0572 was excised by crossing to a genomic source of transposase activity, and chromosomes that lost the *white*⁺ marker were screened by PCR. Lines that exhibited amplification fragments smaller than normal size were chosen as potential *mrn* deletion mutants, and their deletion end points were determined

by sequencing. The mutation sites of all *mrn* alleles were determined by sequencing a 2.7 Kbp genomic PCR fragment including the *mrn* coding region.

Histology

In situ hybridization and antibody staining were performed as described (Tomlinson & Ready 1987; Tautz & Pfeifle 1989; O'Neill & Bier 1994) with minor modifications. Antibodies used in this study were rabbit anti-GFP (CLONTECH, Mountain View, CA; used at a 1 : 500 dilution), mouse anti-GFP (CLONTECH, used at a 1 : 500 dilution), mouse anti- β -galactosidase (CPL, West Chester, PA; used at a 1 : 1000 dilution), mouse anti-CycE 8B10 (used at a 1 : 3 dilution), mouse anti-CycB F2F4 (used at a 1 : 5 dilution), rabbit anti-phospho-H3 (Upstate Biotechnology, Lake Placid, NY; used at a 1 : 200 dilution), rat anti-ELAV MAb 7E8A10 (Developmental Studies Hybridoma Bank, developed by G.M. Rubin, used at a 1 : 4 dilution), mouse anti-PROS (Kauffmann *et al.* 1996, used at a 1 : 4 dilution), rabbit anti-ATO (Jarman *et al.* 1995, used at a 1 : 5000 dilution) and rabbit anti-BarH1 (Higashijima *et al.* 1992, used at a 1 : 30 dilution). TOTO-3 (Molecular Probes, Eugene, OR) was used at 0.5 μ M. Mutant clone area was calculated by VH ANALYZER software (Keyence, Osaka, Japan).

BrdU incorporation

Tissue was dissected in S2 medium (SIGMA, St. Louis, MO) and incubated in 80 μ g/mL BrdU in S2 medium at RT for 2 h. The tissue was then fixed with 4% PFA for double staining and Carnoy's fixative for single staining. BrdU was detected by mouse anti-BrdU (Zymed, San Francisco, CA; used at a 1 : 2 dilution), with signal amplification using the TSA Biotin system (Perkin Elmer Life Science, Waltham, MA). All secondary antibodies (FITC or Cy3, HRP conjugated) were used at 1 : 400 dilutions.

TUNEL

Apoptotic signals were detected by TACS 2TdT DAB *in situ* Apoptosis Detection Kit (TREVIGEN, Gaithersburg, MD).

UV sensitivity

UV-C irradiation was performed for third instar larva using a XL-1500 UV cross LINKER (SPECTRONICS Corporation, Westbury, NY). After irradiation, larvae were transferred to yeast food vials and incubated at 25 °C for 4 h before fixation.

Figure preparation

Figures were prepared according to "Barrier-free presentation that is friendly to colorblind people" <<http://jfly.iam.u-tokyo.ac.jp/color/>>.

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