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Research Article

Misregulated RNA Pol II C-terminal domain phosphorylation results in apoptosis

T. Schauer[a], I. Tombácz[b], A. Ciurciu[b], O. Komonyi[a] and I. M. Boros[a, b, *]

[a] Chromatin Research Group of HAS, Department of Biochemistry and Molecular Biology, University of Szeged, Közép fasor 52, 6726 Szeged (Hungary), Fax: +36-62-544887, e-mail: borosi@bio.u-szeged.hu
[b] Institute of Biochemistry, Biological Research Center, Temesvári krt. 62, 6726 Szeged (Hungary)

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Abstract. Misregulation of the level of RNA polymerase II carboxyl-terminal domain (CTD) phosphatase, Fcp1, in Drosophila results in high level of caspase-mediated apoptosis. Apoptosis induction by Fcp1 misregulation requires the presence of *Drosophila melanogaster* (Dm)p53, but occurs without the transcriptional activation of Dmp53 proapoptotic targets *rpr*, *ark*, and *hid.* Overproduction of a transcription-activation-defective mutant Dmp53 protein increases, while *Dmp53* null background decreases significantly the level of apoptosis in Fcp1-misregulated animals. Generating the apoptotic signal does not require the function of the ATM and Rad3-related kinase (ATR), and no significant level of nucleo-cytoplasmic translocation of Dmp53 is detectable in cells expressing Fcp1 at an abnormal level. Immunostaining of larval salivary gland polytene chromosomes with anti-Dmp53 antibodies indicates Dmp53 localization at several transcriptionally active chromosomal regions in wild-type cells, while in Fcp1-misregulated cells the association of Dmp53 with specific chromosomal sites is decreased.

Keywords. RNA polymerase II, apoptosis, transcription blockage, C-terminal domain, p53, RNA interference, ATM and Rad3-related kinase, polytene chromosome.

Introduction

The C-terminal domain (CTD) of RNA polymerase II (Pol II) consists of tandem repeats of amino acid heptads that undergo phosphorylation-dephosphorylation cycles on serine residues during transcription. A change of the phosphorylated status of RNA Pol II CTD is believed to play diverse regulatory roles in integrating steps of RNA biogenesis. In fact, the signals represented by the status of CTD modification have been proposed to form a so-called CTD code, which is translated into the recruitment of factors to the different steps of RNA synthesis/processing [1]. Reversible modifications are achieved by several kinases and phosphatases at three serine residues at positions 2, 5 and 7, present within most of the 26–52 heptad repeats constituting the CTD (for a review see [2]). TFIIF-interacting CTD phosphatase, Fcp1, is the first CTD-specific phosphatase identified. Fcp1 is the prototype of a family of specific phosphatases sharing a DXDX(TV) signature motif, and it seems to play a major role in CTD dephosphorylation from yeast to mammalians cells [2]. Although Fcp1 function and its interactions with constituents of the transcription and RNA processing machinery are subjects of intense study, many aspects of the *in vivo* role of the protein are still unclear. Fcp1 is essential in yeast, and,

* Corresponding author.

interestingly, recently a partial deficiency of human Fcp1 was linked to the human autosomal recessive developmental disorder, congenital cataracts facial dysmorphism neuropathy (CCFDN) [3]. To facilitate the *in vivo* study of Fcp1 in a multicellular eukaryote, we generated Fcp1 phenocopies in Drosophila by ectopic overexpression of the protein from a transgene, or down-regulating its expression from the chromosomal gene by the use of RNA interference (RNAi) (I. Tombacz, manuscript in preparation). Here we report on our unexpected observation that either up- or down-regulation of Fcp1 level during development induces high levels of inappropriate apoptosis.

When cells experience stress they respond by activating response mechanisms, which often include increased transcription of specific sets of genes. A key transcriptional regulator that accumulates various stress signals and coordinates appropriate cellular responses is the tumor suppressor protein p53. A type of cellular stress, however, can be a blockage of transcription, in which case a transcriptional activation of p53 targets is not feasible. Nonetheless, p53 also plays a guarding role under such conditions, and apoptosis induction through p53-dependent but transcription-independent pathways ensures the elimination of possible genotoxic consequences. The detailed mechanism by which p53 monitors the progress of transcription is unknown. Inhibition of Pol II transcription by α-amanitin or RNAi was reported to result in p53 accumulation and translocation into mitochondria [4]. In addition, microinjection of Pol II-specific antibodies into mammalian cells was found to induce p53 phosphorylation in a replication protein A (RPA)- and ATM and Rad3-related (ATR)-dependent manner [5]. These data, and the recent report by Balakrishnan and Gross [6] on p53 association with elongating Pol II, suggest a mechanism by which p53 senses the status of transcribing Pol II complex and governs responses in accord with the progress of RNA synthesis/processing.

Homologues of the mammalian p53 have been identified in a number of lower eukaryotes and many of the pathways in which p53 plays a role seem to be ancient and evolutionally preserved. The *Drosophila melanogaster* (Dm)p53 was recognized during the course of the genome project [7–9], and subsequent studies have demonstrated its structural and functional similarities to the mammalian protein (for recent reviews see [10, 11]). Dmp53 binds to similar sequences as its mammalian counterparts and plays a role in apoptosis induction, but unlike human p53, Dmp53 has no role in cell cycle arrest [12].

Here we show that in Drosophila, misregulated expression of the CTD-specific phosphatase Fcp1 induces p53-dependent but transcription-independent apoptosis. Furthermore, we present data that in unstressed cells Dmp53 is bound to transcribed regions of the polytene chromosomes. Deregulation of CTD phosphorylation seems to alter Dmp53 localization at chromosomal sites, which might be an indication of the interplay existing between Pol II CTD and p53 during transcription.

Materials and methods

Generation of Fcp1 transgenes and Drosophila genetics. A detailed description of the construction of UAST-fcp1 and WIZ-fcp1i transgenes used for deregulating Fcp1 cellular level is in preparation (I. Tombacz). In short, a full-length *D. melanogaster* Fcp1 cDNA was generated by RT-PCR amplification on a total embryonic RNA template, using primers: Fcp1Fw: GCGAGAATTCATGCAGAACATACCGGACGA, and Fcp1Rev: CGAGTCGACGGTGTTCACAGATGCTACAGA. The obtained cDNA fragment was inserted into a pUAST insertional vector using restriction sites present in the primers to generate pUAST-fcp1. For the construction of pWIZ-fcp1i, a 900-bp fragment from the coding region of *fcp1* was amplified by PCR using primers: Fcp1RiFw: CTGAGCTAGCGCGATGCCAAGGATCCAGAG, and Fcp1RiRev: CTGAGCTAGCGGTGAAGACCAGCAGCGGAT, and inserted into pWIZ [13] in two copies, in a head-to-head orientation. The structure of the transgene constructs was verified by sequencing, and transgenic lines were established by embryo microinjection following a standard protocol.

Driver stocks were obtained from the Bloomington Drosophila Stock Center. $Dmp53^{5A\text{-}1\text{-}4}$ line [14] was a generous gift from Yikang S. Rong (NIH, Bethesda, USA). The *P[UAS-p53]* and $P[UAS\text{-}p53^{H159N}]$ strains were from Michael W. Young [8]. The ATR mutant strain $mei\text{-}41^{RT1}$ (BL-4169) was obtained from the Bloomington Stock Center.

Detailed descriptions of genetic crosses performed to study the effect of *fcp1* misregulation and genetic interaction of fcp1 transgenes with *p53* and *ATR* alleles are available upon request.

Immunostaining of imaginal discs and polytene chromosomes. Acridine orange (AO) staining of imaginal discs of third instar larvae was performed as described [15]. For the detection of caspase activation, imaginal discs of larvae expressing a GFP transgene under the control of en-GAL4 driver were stained with cleaved caspase-3 (Asp175)-specific antibody (Cell Signaling, antibody 9661) used in 1:250 dilution. Polytene

chromosome spreads were prepared as described [15, 16]. Antibodies specific for the large subunit of Pol II [mouse anti-Pol II (7G5), were provided by Dr. Tora (IGBMC, Strasbourg, France)]. Anti-Ser5 phosphorylated Pol II (H14) antibodies were from Covance, and used in 1:50 dilution. Anti-Dmp53 (d-200 sc-25767) antibodies were from Santa Cruz Biotechnology, and used in 1:250 dilutions for staining imaginal discs, polytene tissues and chromosomes. The Pol3/3 monoclonal antibody, which recognizes an evolutionary conserved Pol II large subunit epitope outside the CTD [17], was kindly provided by O. Bensuade (Pasteur Institute, Paris). Secondary antibodies were Alexa Fluor488-conjugated goat anti-mouse IgG and AlexaFluor555-conjugated goat anti-rabbit IgG (Molecular Probes), used in 1:500 dilutions.

Western blot and real-time RT-PCR detection of p53-target gene expression. RNA polymerase large subunit CTD modification was detected on immunoblots of total protein extracts obtained from wild-type and transgene carrier third instar larvae. Protein concentrations were determined by the Bradford method and 50 μg protein was loaded per well onto 6% SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membrane by electroblotting and immunoblots were developed using Pol II large subunit-specific (7G5) primary and rabbit-anti-mouse-horseradish peroxidase-conjugated (DAKO) secondary antibodies. For the detection of mRNA levels of *fcp1*, *p53*, and p53-target genes *ark*, *hid* and *rpr*, total RNA was isolated with RNeasy Mini Kit (Qiagen) from L3 larvae. First-strand cDNA was synthesized from 1 μg RNA using TaqMan reverse transcription reagent (ABI) and quantitative PCR was performed using ABI, 7500 Real Time PCR System as described [18], where the specific primers for *p53, ark*, *hid* and *rpr* are also described. Fcp1-specific mRNA was detected using primers: Fcp1PvuDn: ACATCCTCGCGATCGTCAAT, and Fcp1R251Gfw: CACGCGCCTGGGCCCCGGCACCG.

Results

Altered DmFcp1 level induces apoptosis. We modified the *in vivo* level of RNA Pol II carboxyl-terminal phosphatase, Fcp1 in *Drosophila melanogaster* by overexpressing the protein or silencing its expression from the chromosomal gene by GAL4-regulated transgenes. UAST-fcp1 and WIZ-fcp1i direct the synthesis of the authentic Fcp1 protein and Fcp1-specific double-stranded inhibitory RNA, respectively. As expected, both transgenes caused changes in the Fcp1 mRNA level, and in the phosphorylation status of RNA Pol II CTD. Ectopic expression of Fcp1 from UAST-fcp1 with an act5C-GAL4 or da-GAL4 driver increased the level of the specific message five- to sevenfold, while silencing the gene by WIZ-fcp1i expression with the same drivers decreased the mRNA level to 50–60% of that detected in control animals (Fig. 1A). Immunoblots of total protein extract of UAST-fcp1 and WIZ-fcp1i transgene carrier animals developed by Pol II large subunit-specific, but CTD phosphorylation-insensitive, antibodies also revealed altered phosphorylation levels of RPB1 (Fig. 1B). The proportion of hyperphosphorylated (Pol IIo) and hypophosphorylated (Pol IIa) forms of RPB1 was altered in WIZ-fcp1i and UAST-fcp1 extracts compared to wild type, as expected upon down- and up-regulation of the phosphatase. Polytene chromosomes of WIZ-fcp1i L3 larvae also displayed an increased level of Ser5-phosphorylated CTD signal (Fig. 1C, D). In contrast, we did not observe a significant change in Pol II-specific signal intensity between polytene chromosomes of wild-type and WIZ-fcp1i larvae stained with antibodies insensitive to CTD phosphorylation (Fig. 1D). Up- or down-regulation of the level of Fcp1 also had a significant effect on the phenotype. Expression of either UAST-fcp1 or WIZ-fcp1i with the help of ubiquitous strong drivers such as *da-*, *act5C-*, *tub-GAL4* resulted in lethality in late L3 or at earlier stages of development (data not shown). On the other hand, if the two transgenes were co-expressed no alterations were observed in the phenotype, indicating that the lethality was a result of the misregulated Fcp1 level.

Expression of UAST-fcp1 or WIZ-fcp1i by the help of GAL4 drivers providing tissue-specific expression, such as vestigial-, escargot-, engrailed- and GMR-GAL4, resulted in phenotypic features (ruptured, wrinkled and nicked wings, rough eyes, damaged tergites), suggesting inappropriate apoptosis during the development of affected body parts (Fig. 2A). In general, we observed a similar penetrance of phenotypic traits, suggesting apoptosis in animals expressing a UAST-fcp1 or a WIZ-fcp1i transgene, although we also noticed that with combinations of selected drivers the two forms of Fcp1 misregulation have somewhat different effects. We believe this reflects the different sensitivity of specific tissues or developmental stages in which a given driver causes altered Fcp1 levels. AO staining of imaginal discs of third instar larvae clearly revealed an extensive level of cell death in both Fcp1 up- or down-regulated animals, while the driver expression alone did not increase the number of AO-positive cells (Fig. 2B). In accordance with the high level apoptosis, an activated caspase-3 signal was detected in the regions of imaginal discs where an en-

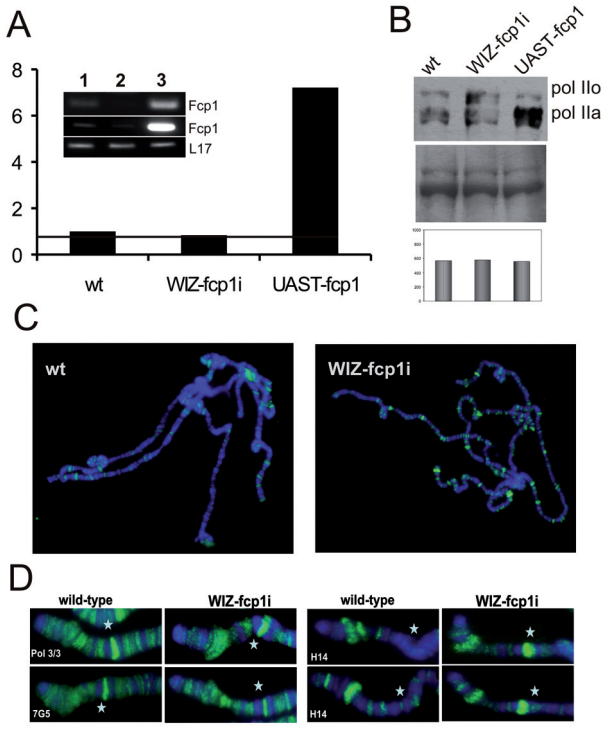

Figure 1. Dmfcp1 phenocopies result in altered Fcp1 mRNA levels and in changes in RNA Pol II phosphorylation. (*A*) The level of Dmfcp1 mRNA is up- and down-regulated in animals expressing UAST-fcp1 or WIZ-fcp1i transgene. Fcp1-specific message levels were determined by real-time RT-PCR in total RNA samples obtained from third instar larvae, which expressed the indicated transgenes under the control of a da-GAL4 driver. Insert: Semi-quantitative RT-PCR amplification of an Fcp1 cDNA fragment on total RNA samples obtained from wt (1), WIZ-fcp1i (2) and UAST-fcp1 (3) L3 larvae that also carry a da-GAL4 (top) or act5C-GAL4 (middle and bottom) driver. (*B*) Western blot indicates altered level of phosphorylated Pol II in animals expressing Fcp1 transgenes. Total protein extract of larvae carrying the indicated transgene and act5C-GAL4 driver were separated by SDS-PAGE, and Western blots were prepared using Pol II large subunit-specific antibodies (7G5). At the immunoblot (top) the position of hyperphosphorylated (pol IIo) and hypophosphorylated (pol IIa) forms of Pol II large subunit are indicated. At the bottom, the same membrane stained with Coomassie Brilliant Blue is shown. (*C*) An increase in the chromosomal localization of Ser5-phosphorylated Pol II is detectable on polytene chromosomes of WIZ-fcp1i animals. Polytene chromosome spreads of wild-type (wt) and WIZ-fcp1i expresser larvae were stained with DAPI (blue) and with antibody (H14) specific for Ser5-phosphorylated Pol II (green). (*D*) Immunostaining of polytene chromosomes with antibodies insensitive to the phosphorylation status of CTD, such as Pol3/3 and 7G5, do no indicate a significant difference in the staining of wild-type and WIZ-fcp1i chromosomes. In contrast, the Ser5-hyperphosphorylated CTD-specific H14 antibody (shown on the left) staining displays an enhanced signal intensity at specific locations. On the segments of X chromosomes shown, a star indicates the position of a band stained by H14 antibody preferentially on WIZ-fcp1i but not on wild-type chromosomes.

GAL4 driver ensured the expression of a UAST-fcp1 or a WIZ-fcp1i transgene (Fig. 2C). Since proapoptotic genes rpr, hid, and ark are key regulators of apoptosis in Drosophila, we wondered whether transcription of these genes was changed under conditions in which Dmfcp1 was down-regulated by WIZ-fcp1i expression. Quantitative RT-PCR detection of the level of rpr, ark and hid mRNA in act5C-GAL4;WIZ-fcp1i animals did not indicate a significant change in the level of ark and hid mRNAs and revealed a modest increase in the level of rpr message, as compared to controls carrying only the driver (Fig. 3A). Wild-type animals, displaying similar level of apoptosis following X-ray irradiation, regularly showed a two- to threefold increase in the level of hid and rpr mRNAs, while ark was induced similarly by UVC (data not shown and [18]). On the other hand, the mRNA level of all three proapoptotic genes ark, hid and rpr was drastically decreased in act5C-GAL4;UAST-fcp1 samples (Fig. 3B). Although at present we do not have a satisfactory explanation for the differential response in the transcription of these proapoptotic genes to Fcp1 up- and down-regulation, from these data we conclude that the transcription activator function of Dmp53 is not required for the apoptosis induction resulting from Fcp1 misregulation. In accordance with this, we detected no increase in the level of p53 mRNA by quantitative RT-PCR in Fcp1-misregulated animals (data not shown). Neither did we observe increased levels of p53 by immunostaining of diploid and polytene tissue samples of act5C-GAL4;UAST-fcp1 and act5C-GAL4;WIZ-fcp1i animals; in both cases, as with wild-type samples, p53 staining was hardly detectable.

Fcp1 misregulation-induced apoptosis is p53 dependent. Since p53 is known to play roles in apoptosis in both a transcription-dependent and transcription-independent manner [4] and apoptosis can also take place in the absence of p53 [19], we asked whether the apoptosis observed in animals with misregulated Fcp1 level was dependent on Dmp53. To answer this question, we introduced a GAL4 driver and a WIZ-fcp1i or UAST-fcp1 transgene into p53 mutants. In p53 null background the apoptotic effect of WIZ-fcp1i expression was suppressed, as reflected by the lower number of flies with nicked wings, compared to the number of those observed in wild-type background (Fig. 3E). In contrast, when WIZ-fcp1i was coexpressed with a transcription activation-defective mutant p53 protein ($p53^{H159N}$) [9], we observed an enhancement of the apoptotic phenotype: animals expressing WIZ-fcp1i and UAS-$p53^{H159N}$ under the control of the vg-GAL4 driver displayed stronger aberrant wing phenotype than controls expressing WIZ-fcp1i under the control of the same driver in a wild-type background. Similarly, the expression of WIZ-fcp1i by an en-GAL4 driver resulted in an earlier lethal phase when it was coexpressed with $p53^{H159N}$, as compared to controls expressing wild-type p53

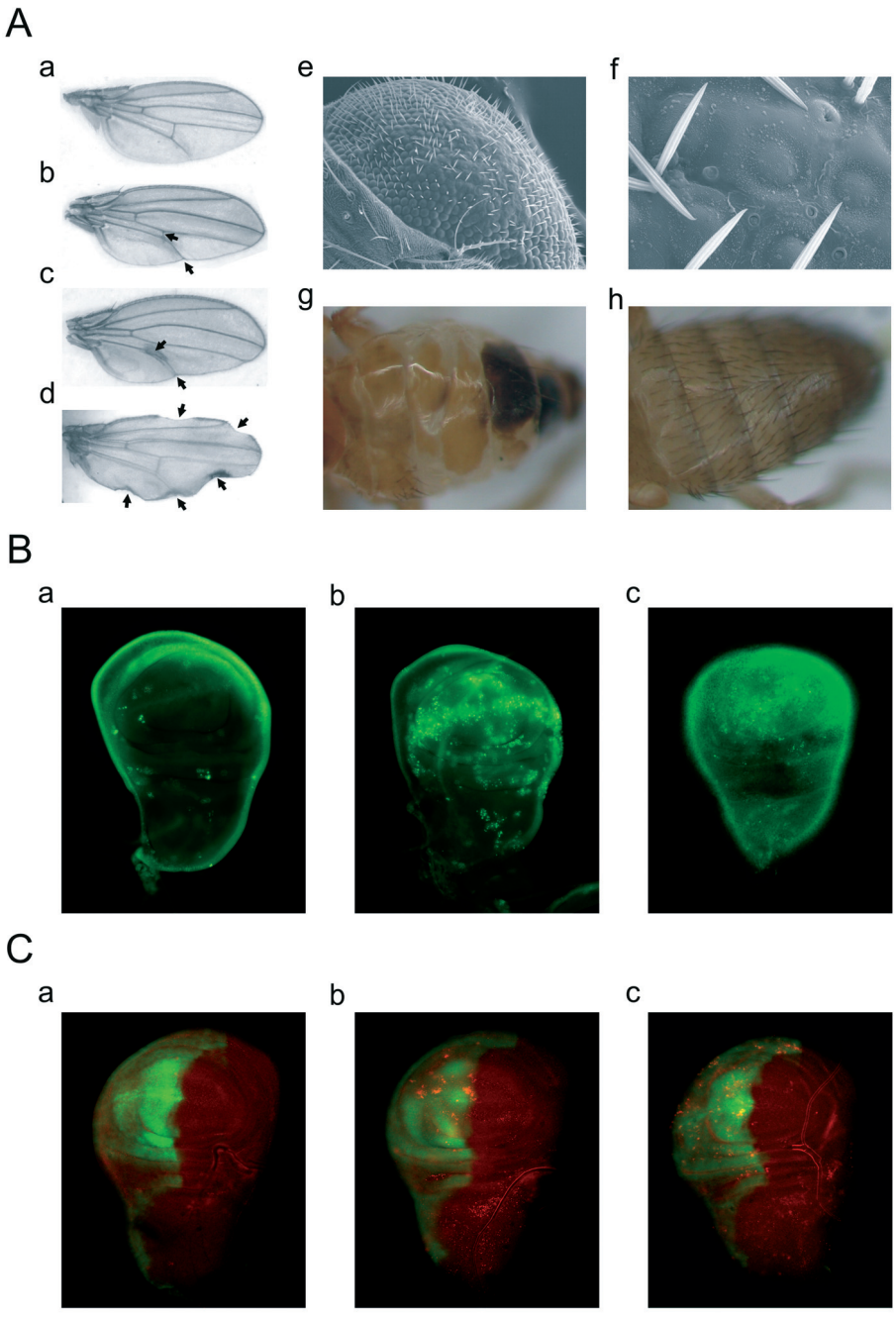

Figure 2. Fcp1 up- or down-regulation results in an increased level of caspase-mediated apoptosis. (*A*) Characteristic phenotypes of animals in which Fcp1 expression is up- or down-regulated by the help of UAST-fcp1 and WIZ-fcp1i transgenes and different drivers are indicated. a–d: Wing phenotypes of wild-type (*a*), en-GAL4;WIZ-fcp1i (*b*), en-GAL4;UAST-fcp1 (*c*), and vg-GAL4;WIZ-fcp1i (*d*) adults. Arrows indicate nicks, notches and damaged veins arising from inappropriate apoptosis during wing development. Images (*e*) and (*f*) show eye phenotype of GMR-GAL4;WIZ-fcp1i animals raised at 29°C. The images were taken by a scanning electron microscope. Images (*g*) and (*h*) show a part of the abdomen of an esc-GAL4;WIZ-fcp1i and an esc-GAL4 adult, respectively. (*B*) Acridine orange staining of imaginal discs of act5C-GAL4 (*a*), act5C-GAL4;WIZ-fcp1i (*b*) and act5C-GAL4; UAST-fcp1 (*c*) third instar larvae. The images were recorded using a green filter. (*C*) Activated caspase-3-specific antibody staining of wing imaginal discs of wild-type (*a*), WIZ-fcp1i (*b*) and UAST-fcp1 (*c*) transgene carrier larvae indicates caspase activation on the segment of the discs where the en-GAL4 driver induced misregulated Fcp1 level and UAS-GFP transgene expression. The intense red caspase-3-positive cells are seen as orange in the green GFP background.

(Fig. 3C). Significantly, the expression of UAS-$p53^{H159N}$ under the control of the en-GAL4 driver in the absence of Fcp1 inhibition had very little effect on viability. We obtained qualitatively identical results by studying the overexpression of Fcp1 in a different p53 background. Coexpression of the dominant negative $p53^{H159N}$ with an en-GAL4-driven UAST-fcp1 resulted in earlier lethality than either transgene alone (Fig. 3D). Similarly, while neither vg-GAL4-driven UAST-$p53^{H159N}$ nor UAST-fcp1 expression caused wing phenotype, coexpression of the two resulted in the appearance of offspring with nicked wings (Fig. 3F). Thus, these data clearly show that apoptosis induction is p53 dependent in cells in which the level of Fcp1 is misregulated, but does not require the transcription activator function of p53.

Next, we wanted to test if a change in the post-translational modification of p53 was detectable as a result of Fcp1 misregulation. Dmp53 has been shown to be modified by phosphorylation [20] and the ATR kinase has recently been shown to phosphorylate p53 following transcription block in mammalian cells [5].

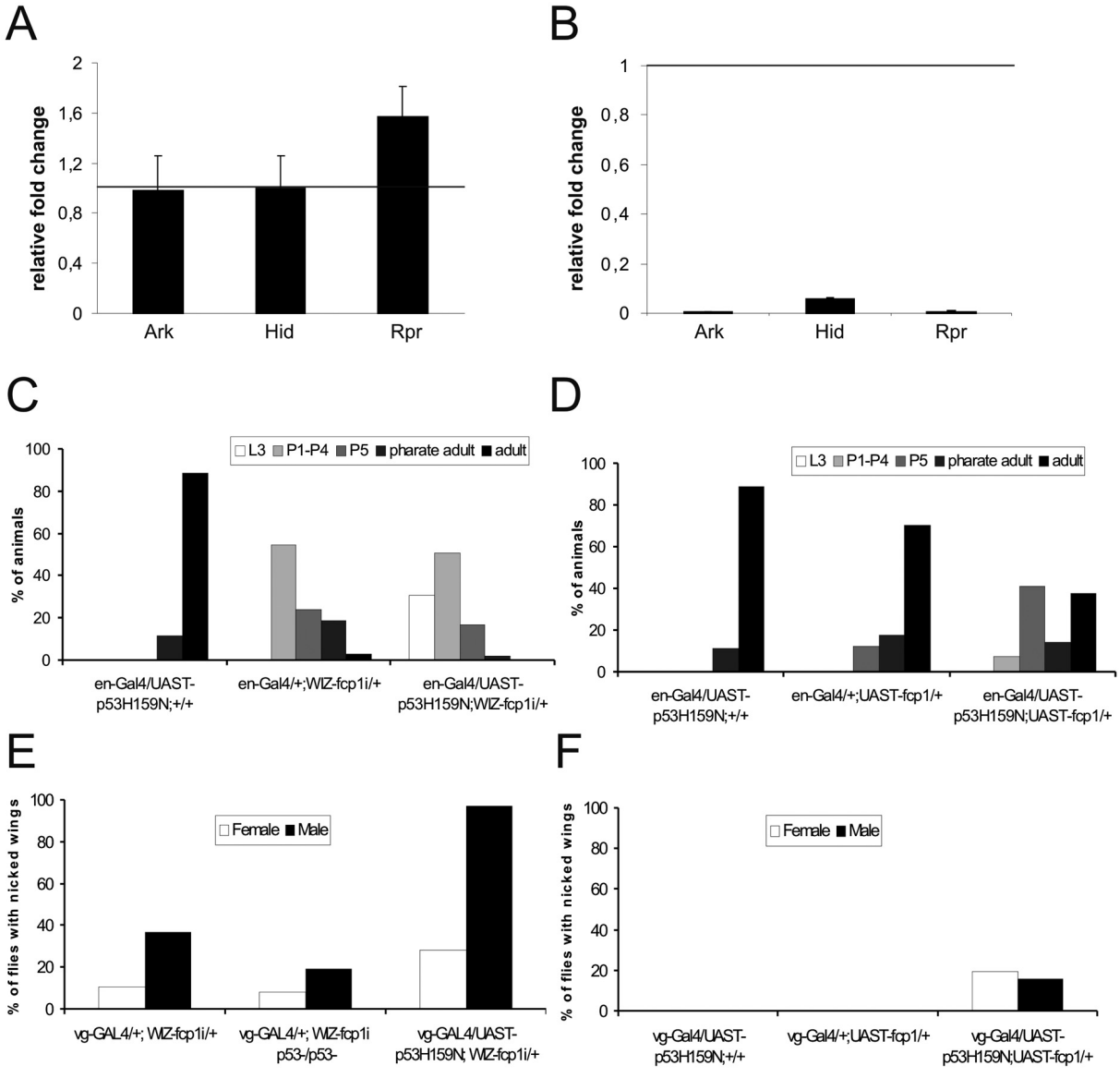

Figure 3. Misregulated Fcp1 expression does not induce transcription activation of p53 target genes, but the strength of phenotypes arising from Fcp1 misregulation depends on the p53 background. (A) The relative levels of *ark*, *hid*, and *rpr* mRNAs in act5C-GAL4;WIZ-fcp1i compared to act5C-GAL4 3rd instar larvae. (B) The relative level of the same messages in act5C-GAL4;UAST-fcp1 compared to act5C-GAL4 animals. The mRNA levels were determined in four independently obtained total RNA samples by real-time RT-PCR as described [18]. (C, D) The effect of p53 background on the viability of animals expressing WIZ-fcp1i and UAST-fcp1, respectively, under the control of an en-GAL4 driver. The percentages of animals reaching a specific developmental stage are shown. (E, F) The frequency of nicked wing phenotype in animals carrying vg-GAL4 together with WIZ-fcp1i or UAST-fcp1 transgenes, respectively, in wild-type and modified p53 backgrounds (wild type, p53 null or $p53^{H159N}$ overexpressed, as indicated).

We wondered whether ATR function was required in Drosophila for apoptosis induction by Fcp1 misregulation. A comparison of the wing phenotype of animals expressing an en-GAL4-driven UAST-fcp1 transgene in wild-type and ATR null (*mei-41*^{RT1}) background revealed no differences (Fig. 4A). Similarly, no differences were observed in the lethality phase of animals expressing UAST-fcp1 *via* a da-GAL4 driver in ATR null and wild-type backgrounds. Furthermore, the ATR background had no effect on the phase of lethality caused by Fcp1 down-regulation, such as WIZ-fcp1i expression under the control of en-GAL4 or da-GAL4 (data not shown). In summary, these results suggested that the apoptosis observed in Fcp1-misregulated cells did not require p53 modification by ATR kinase.

Fcp1 misregulation alters Dmp53 localization to polytene chromosome regions. Next, we wanted to test if Fcp1 misregulation affected the cellular local-

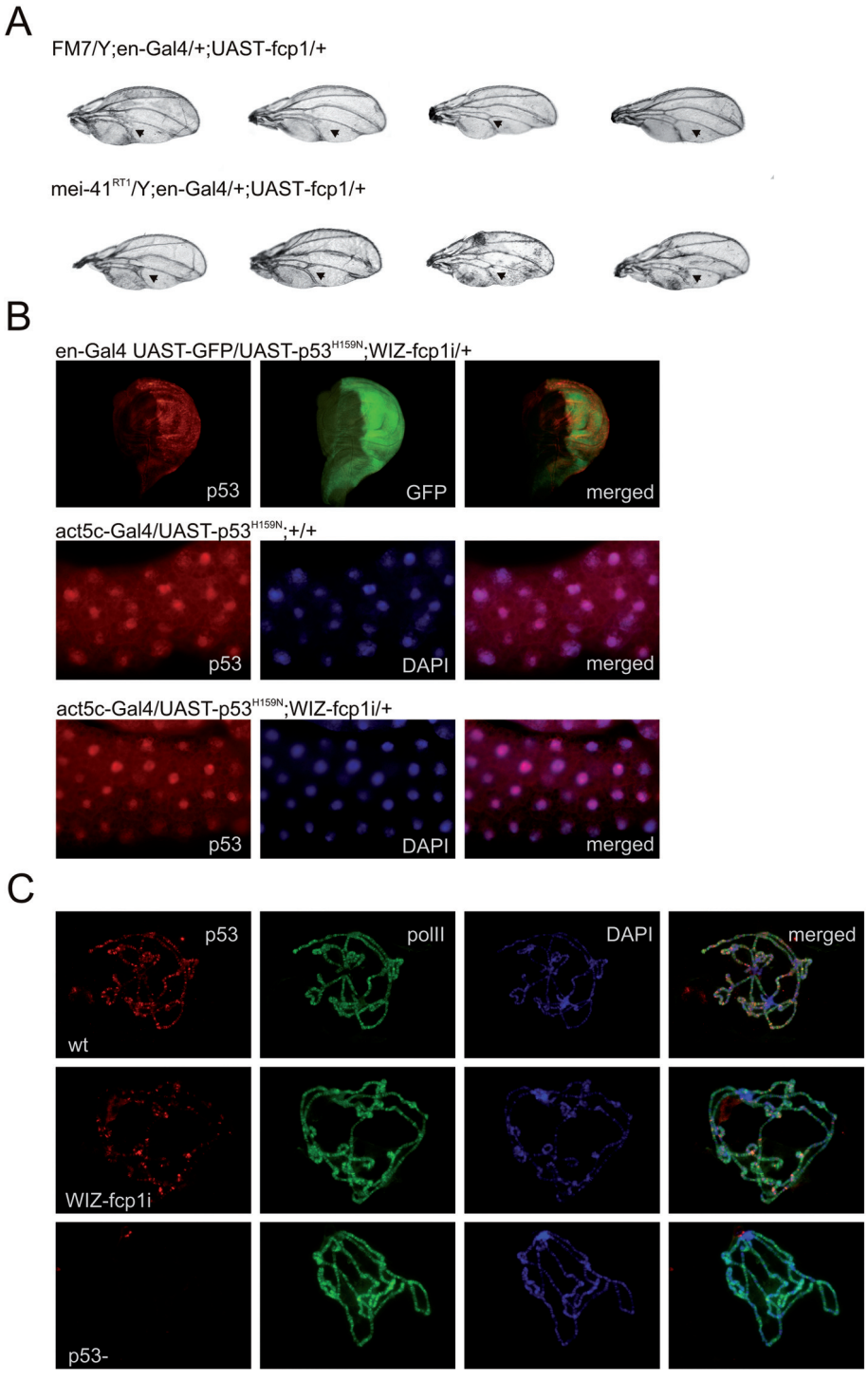

Figure 4. ATR does not affect the phenotype resulting from *fcp1* misregulation, and Dmp53 binds to specific sites of polytene chromosomes. (*A*) Signs of similar abnormal development are seen in the posterior part of wings of animals expressing an UAST-fcp1 transgene under the control of an en-GAL4 driver in wild-type (FM7), or ATR null (*mei-4I*^{RT1}) background. (*B*) Wing disc of an en-GAL4 UAST-GFP/UAST-p53^{H159N};WIZ-fcp1i/+ animal (top row). In the posterior part, the en-GAL4 driver expression results in GFP expression seen as green. No p53-specific signal is detectable in the segment where the driver is inactive, but the overexpressed p53^{H159N} is well detectable in the segment where the driver is active (red). Act5C-GAL4-driven expression of p53^{H159N} in control (+/+) (middle row), and in WIZ-fcp1i fat body cells (bottom row). No change in the localization of p53^{H159N} is observable. DAPI staining (blue) shows the nuclei. (*C*) Images of polytene chromosome spreads obtained from salivary glands of third instar wild-type (wt, top row), WIZ-fcp1i (middle row) and p53 null (p53-, bottom row) larvae co-stained with antibodies specific for Dmp53 (red) and Pol II (7G5, green), and with DAPI (blue). The preparation of chromosomes, staining, visualization of images and data processing were done simultaneously using identical experimental conditions and settings. Each studied genotype also contained the act5C–GAL4 driver.

ization of p53, and for this we immunostained tissue samples and polytene chromosome spreads with a Dmp53-specific polyclonal antibody. Immunostaining of polytene or diploid tissues of animals expressing normal level p53 revealed low-level staining. Nuclear localization of the overexpressed p53^{H159N} was detectable and down-regulation of the Fcp1 level did not change this (Fig. 4B). In contrast, on polytene chromosome spreads of third instar larvae we detected intensive Dmp53-specific signal at selected sites. We observed a well-reproducible specific staining pattern of wild-type chromosomes in numerous independent preparations, while control samples prepared and stained simultaneously from p53 null animals did not display specific staining (Fig. 4C). The p53-positive signal was observable in most cases at interbands

colocalized with the RNA Pol II-specific signal. Curiously, the p53 staining pattern of polytene chromosome spreads obtained from larvae expressing the WIZ-fcp1i transgene was altered. As shown most clearly on the merged images in Figure 4C, overall, in the presence of the WIZ-fcp1i transgene, a weaker p53 staining was detectable. While specific bands at chromosomal regions displaying strong staining were also present in WIZ-fcp1i chromosomes, in general the staining on these chromosomes was less intensive compared to the staining of wild-type chromosomes.

Discussion

Fcp1 is an evolutionally conserved phosphatase, essential for Pol II recycling and initiating new rounds of transcription cycles. We report here the *in vivo* effects resulting from higher, or lower than normal levels of Fcp1 expression. The transgenes we generated increased and decreased the cellular level of Fcp1-specific message, and resulted in a shift in the modification status of CTD. As expected, the RNA inhibition-triggered decrease of Fcp1 level increased the level of Pol II with phosphorylated CTD. In contrast, the transgene-provided overexpression of Fcp1 resulted in a decrease in the level of phosphorylated CTD (Fig. 1B). The increased level of hyperphosphorylated Pol IIo form resulting from Fcp1 down-regulation was also observable by staining of polytene chromosomes with antibodies specific for Ser5-phosphorylated CTD (Fig. 1C, D). This observation is in accordance with reports that Fcp1 preferentially dephosphorylates Ser5 [21], or both Ser2 and Ser5 [22].

The consequences of altered Fcp1 expression on the phenotype suggest an increased level of cell death during development. The cell loss causes lethality, or deformation of specific organs, depending on the driver controlling ectopic Fcp1 expression. AO staining of imaginal tissues and detection of caspase activation in UAST-fcp1 and WIZ-fcp1i carriers indicate that deregulation of DmFcp1 levels indeed induces high level of apoptosis (Fig. 2). This apoptotic program is completed without the need of transcription activation of proapoptotic targets such as *rpr*, *ark*, and *hid*. Our data do not indicate whether apoptosis is initiated and takes place by the same or different mechanisms in Fcp1-up- and -down-regulated cells. The differences in *ark*, *hid* and *rpr* mRNA levels in Fcp1-up- and -down-regulated animals might indicate a difference in the mechanism by which cell death is accomplished. Specifically, the modest increase of *rpr* mRNA in WIZ-fcp1i animals, compared to wild type, can play a role as these animals display a strong or even stronger apoptotic phenotype than UAST-fcp1 transgene carriers, despite a seemingly smaller alteration in the Fcp1 message level.

In the absence of p53, the phenotype caused by Fcp1 up- or down-regulation is suppressed. In contrast, overproduction of a transcriptionally inactive p53 enhances it. This dependence of Fcp1 misregulation-induced apoptosis on a p53 background indicates p53 involvement, although misregulated CTD phosphorylation does not trigger Dmp53-mediated transcription activation. It was shown earlier that ectopic expression of the transcription activation-defective $p53^{H159N}$ suppressed the rough-eye phenotype that resulted from wild-type Dmp53 overexpression [9]. This might seem to be in contradiction with our observation described above. The discrepancy can be resolved, however, assuming that the wild-type p53 overexpression results in the rough-eye phenotype through transcription activation of specific genes, while the apoptosis in Fcp1-deregulated animals is linked to transcription-independent p53 function. In this context it might be significant that dominant negative mutant forms of mammalian p53 did not inhibit stress-induced extranuclear apoptosis [23].

The mechanism by which transcription stress response is activated and leads to apoptosis is complex, and reports suggest that it might involve, among others, nuclear accumulation, ATR-dependent phosphorylation and nucleo-cytoplasmic translocation of p53 [24]. In our study, the absence of ATR kinase did not affect the Fcp1 misregulation-induced apoptosis, nor was it preceded or accompanied by a change in the cellular localization of p53. However, in light of the low p53 level, and the previous observations that stress-triggered translocation of approximately 2 % of p53 to mitochondria can cause apoptosis [25], such a possibility cannot be excluded. To our surprise, we detected specific binding of p53 at a large number of polytene chromosome sites. As this was an unexpected observation, we took care to make sure that the signal we detected on polytene chromosomes was indeed p53 specific. Several lines of evidence suggest this: first, in p53 null mutants, we did not observe specific staining (Fig. 4); in contrast, in animals that overexpressed p53 from a transgene an enhancement of the signal was detectable. Moreover, pre-clearing the antibody by incubating it with chromosome spreads obtained from p53 null animals did not eliminate the signal obtained on wild-type chromosomes. Dmp53 has been shown to be expressed in non-stressed larvae [8], although its level is low and hardly detectable by Western blotting [26]. With the antibody we used to stain polytene chromosomes, we also could not detect p53 in total protein extracts of wild-type cells by Western blots. Immunoblots of extracts of

p53 overexpressers developed with this antibody revealed a single band, indicating a protein migrating at the expected position (data not shown). Therefore, the only explanation we can offer at present is that the p53 epitope recognized by this antibody is more accessible/reactive in the native chromosome-bound protein.

To our knowledge this is the first report on the localization of Dmp53 to polytene chromosomes. However, several laboratories have shown the association of the mammalian protein with chromatin and numerous studies used chromatin immunoprecipitation or similar assays to estimate a high number of p53 binding sites in mammalian genomes [27, 28]. Recently, the physical association of human p53 with the elongating Pol II complex was demonstrated in *Saccharomyces cerevisiae* cells [6]. p53 was found associated preferentially with transcribed genes and the association of p53 and Pol II paralleled the transcriptional activity of the genes.

Blockage of RNA Pol II transcription by the use of different types of inhibitors has been shown to result in transcription-independent, but p53-dependent apoptosis in mammalian cells [4, 5, 24, 29]. It was also shown that RNA synthesis inhibitors blocking the phosphorylation of the Pol II CTD induced rapid nuclear accumulation of p53 [30]. In fact, these observations prompted us to investigate whether association of Dmp53 with stalled RNA Pol II complexes was detectable on polytene chromosomes. The observation that Dmp53 was present at many actively transcribed chromosomal genes makes us consider the possibility that at specific genes Dmp53 is associated with the transcribing Pol II complex. A change in the transcription machinery, which might be related to a change in the CTD phosphorylation status, can thus initiate a p53-dependent response. We expect that further studies will explore the relationship between the observed alteration of p53 staining pattern in Fcp1-deregulated cells and the mechanism by which p53 is activated and initiates apoptosis.

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