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NF-Y transcriptionally regulates the *Drosophila p53* gene

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ABSTRACT

The p53 protein is important in multicellular organisms, where it regulates the cell cycle and thus functions as a tumor suppressor that contributes to preventing cancer. However, molecular regulation of *p53* gene expression is not fully understood. NF-YA is a subunit of the NF-Y trimeric complex, a transcription factor that binds to CCAAT motifs in the promoter regions of a variety of genes playing key roles in cell cycle regulation. We have identified four potential *Drosophila* NF-Y (dNF-Y)-binding sites located in the 5'-flanking region of the *Drosophila p53* (*dmp53*) gene. Chromatin immunoprecipitation analyses using anti-dNF-YA antibodies confirmed that dNF-YA binds specifically to the genomic region containing CCAAT boxes in the *dmp53* gene promoter in vivo. Furthermore, the thorax disclosed phenotype of dNF-YA knockdown flies can be enhanced by *dmp53* mutation. In addition, the level of *dmp53* mRNA was found to be decreased in the dNF-YA knockdown cells and transient expression of the *luciferase* gene revealed that wild-type *dmp53* gene promoter activity is much stronger than mutated promoter activity in S2 cells. The requirement of CCAAT boxes for *dmp53* promoter activity was further confirmed by expression of EGFP in various tissues from transgenic flies carrying wild-type and CCAAT box-mutated versions of *dmp53* promoter-*GFP* fusion genes. These results taken together indicate that dNF-Y is necessary for *dmp53* gene promoter activity.

1. Introduction

p53 is a major tumor suppressor whose function is a critical component of the cellular mechanisms that respond to genotoxic stresses and hypoxia to maintain genomic integrity, in part by arresting cell cycle progression or inducing apoptosis (Harris, 1996; Burns and El-Deiry, 1999; Sogame et al., 2003). Mutations in the DNA binding domain of the *p53* gene are found in approximately half of all common human cancers (Somasundaram, 2000; Sharpless et al., 2002; Soussi and Lozano, 2005).

Drosophila p53 (dmp53) has little sequence similarity with human p53 (Brodsky et al., 2000), but structurally and functionally the protein has been proven to be conserved from flies to mammals (Jin et al., 2000; Ollmann et al., 2000). Like mammalian p53, dmp53 binds specifically to human p53-binding sequences in the genome and it is required to maintain normal histone H3-K14 acetylating levels and radiation-induced apoptosis. However, unlike mammalian p53, dmp53 appears unable to induce a G1 cell cycle block when overexpressed (Brodsky et al., 2004; Rebollar et al., 2006). Although the *Drosophila* genome contains only one p53 homolog, recent research has provided new insights into the pathways activated by dmp53 and revealed that it incorporates functions of multiple p53 family members (Fan et al., 2010). Although functions of p53 have been extensively studied, the molecular mechanism that regulates expression of the *p53* is still poorly understood. Since controlling *p53* gene expression is necessary to improve efficiency of establishment of iPS cells (Kawamura et al., 2009), understanding expression control mechanisms is increasingly important. We have reported that the DNA replication-related element (DRE) and DRE-binding factor (DREF) play important roles in *dmp53* gene promoter activity (Trong-Tue et al., 2010), but involvement of other transcription factor(s) is suggested by the only partial decrease of *dmp53* mRNA levels on knockdown of DREF. In the present study, we have identified four potential nuclear factor Y (NF-Y)-binding sites located in the 5'-flanking region of the *dmp53* gene.

NF-Y is a trimetric transcription factor that consists of three subunits, NF-YA, -B and -C, all three of which are essential for functions of NF-Y and for DNA binding (Sinha et al., 1995; Mantovani, 1999; Yoshioka et al., 2007). NF-Y specifically binds to multiple CCAAT boxes in many promoters of eukaryotic genes (Mantovani, 1999), many with key roles in the G1/S and G2/M transitions (Elkon et al., 2003). NF-Y modulates the activity of CCAAT box-containing promoters of the *E2F1*, *cyclin A*, *cyclin B1*, *topo IIα*, and *bsk* genes during different phases of the cell cycle, in response to DNA damage and activation of the *JNK* pathway (Huet et al., 1996; Farina et al., 1999; Adachi et al., 2000; Yoshioka et al., 2008). Among the three subunits, only subunit NF-YA contains a DNA binding domain, participates in the cellular responses to DNA damage

Abbreviation: NF-Y, nuclear factor Y; UAS, upstream activating sequence; pnr, pannier; RT-PCR, real-time polymerase chain reaction; EGFP, enhanced green fluorescence protein.

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and is involved in regulation of stress-inducible gene (Jin et al., 2001). The fact that knockout of mouse NF-YA results in early embryonic lethality indicates essential roles in early development (Bhattacharya et al., 2003). In the *Drosophila* model, studies focused on NF-YA have revealed a novel function in regulation of the JNK signal transduction pathway that had not been suggested in mammalian systems (Yoshioka et al., 2008). Moreover, overexpression or knockdown of *Drosophila* NF-YA (dNF-YA) results in specific phenotypes (Yoshioka et al., 2007, 2008) providing useful models for studying NF-Y functions in vivo.

Physical interaction between wild-type p53 or mutant p53 alleles and NF-Y has been reported in mammals (Imbriano et al., 2005), with associations both in vitro and in vivo through the αC helix of NF-YC and a region close to the tetramerization domain of p53. NF-Y trimer interaction with p53 is independent of sequence-specific binding activity and requires C-terminal acetylation of p53 (Imbriano et al., 2005; Di Agostino et al., 2006), suggesting that NF-Y and p53 exhibit strong inherent attraction which might modestly enhance transcriptional activation.

In the present study, we examined the role of dNF-Y in transcriptional regulation of *dmp53* gene expression through analyses of the dNF-YA subunit. This focus was selected since NF-YA plays a major role in DNA-binding and formation of the NF-YA, -YB and -YC heterotrimer. Moreover NF-YA is the most extensively characterized of the three and is essential for early development of the mouse and *Drosophila*. Our results indicate that the dNF-Y is a novel regulatory factor for *dmp53* gene expression.

2. Materials and methods

2.1. Fly strains

Fly strains were maintained at 25 °C on standard food. We used the Canton S fly as the wild-type strain. The *dmp53* null mutant fly line, generated by homologous recombination and homozygous viable (Sogame et al., 2003), was kindly supplied by Dr. M. Abrams. The UAS-dNF-YA line was described earlier (Yoshioka et al., 2007) as well as the transgenic fly line carrying GMR-GAL4 on the X chromosome (Takahashi et al., 1999). All other stocks used in this study were obtained from the Bloomington, Indiana stock center.

2.2. Establishment of transgenic flies

P-element-mediated germ line transformation was carried out as described earlier (Spradling, 1986). F1 transformants were selected on the basis of white eye color rescue (Robertson et al., 1988). The plasmids p5'-*dmp53*wt-EGFP and p5'-*dmp53*mut-EGFP were constructed as described below for establishment (Yoshioka et al., 2008) of the transgenic fly strains listed with their chromosomal linkages in Table 1.

2.3. Oligonucleotides

All oligonucleotides were chemically synthesized. In order to amplify the 5'-flanking region of the *dmp53* gene, the following polymerase chain reaction (PCR) primers were synthesized and used for construction of *dmp53*promoter–luciferase gene fusion or *dmp53*promoter–GFP fusion plasmids.

Table 1
Transformants carrying the EGFP gene fused to the dmp53 gene 5`-flanking region.

P-element plasmids	Strains	Chromosome linkage
5'-dmp53wt-EGFP	8-2	III
	4-1	III
	21-1	II
	14-2	III
5'-dmp53mut-EGFP	3-1	III
	29-2	II
	26-4	II

Dp53XhoIF2	5'-cggctcgaggccgtagctgcctaagctttgtataattcg
Dp53BglIIR2	5'-gttagatctcttagtcggctatatctgatcaagcgatc
P53infusion1	5'-atcagatccGCGGCCGCgccgtagctgcctaagc
P53infusion2	5'-gctcaccatGCGGCCGCcttagtcggctatatctg
GFP.Infusion1	5'-atcagatccGCGGCCGCatggtgagcaagggcgaggag
GFP.Infusion2	5'-acgcaaagcttgggCTGCAGttacttgtacagctcgtcc

For site-directed mutagenesis of CCAAT, the following oligonucleotides were synthesized.

Region1a.mut.F	5'-cacgcgctCCAATCGATAaacgcTGGctacggcg
Region1a.mut.R	5'-cgccgtagCCAgcgttTATCGATTGGagcgcgtg
Region1b.mut.F	5'-cacgcgctagAATCGATAaacATTGGctacggcg
Region1b.mut.R	5'-cgccgtagCCAATgttTATCGATTctagcgcgtg
Region1a.1b.mut.F	5'-cacgcgctagAATCGATAaacgcTGGctacggcg
Region1a.1b.mut.R	5'-cgccgtagCCAgcgttTATCGATTctagcgcgtg

For chromatin immunoprecipitation assays, the following oligonucleotides were synthesized.

p53.region2.ChIP-F	5'-cggtacattgctcgaccattcaaagggc
p53.region2.ChIP-R	5'-cgcggacttgtgaagactcatggctgtgcactc
p53.region1.ChIP-F	5'-gcaacaaattcgtctgccacaaggggtgagc
p53.region1.ChIP-R	5'-cttagtcggctatatctgatcaagcgat
bsk.F	5'-gcggcactttcgcatgagaataattg
bsk.R	5'-tcgattggctgactttagccgtttct
RP49-chipF	5'-agcgcaccaagcacttcatc
RP49-chipR	5'-cgttctcttgagaacgcagg

For quantitative real-time PCR, the following oligonucleotides were synthesized.

P53RT.F3	5'-gtcgtggcacaaagaaagcact
P53RT.R3	5'-gggcggctcatccagaaccatgc
NF-YA.RT.F3	5'-cgccaagaaagtgtaagtgt
NF-YA.RT.R	5'-aatcataattggccagcagccacag
RPLP0-F	5'-agctgctaccccacatcaag
RPLP0-R	5'-tgttcccttggaaattttgg
RP49-RT.F	5'-gcttctggtttccggcaagcttcaag
RP49-RT.R	5'-gacctccagctcgcgcacgttgtgcaccaggaac

2.4. Plasmid construction

The promoter region of the *dmp53* gene (−944 to +96 with respect to the transcription initiation site) was amplified by PCR using *Drosophila* genomic DNA. To construct the plasmid p5'-*dmp53*wt-luc, the primer set Dp53XhoIF2 and Dp53BglIIR2 was used to amplify a 1040 bp fragment, PCR products being digested with *Xho*I and *Bgl*II and inserted into plasmid pGVB (Toyo Ink). For site-directed mutagenesis, PCR was carried out using a QuickChangeSite-directed mutagenesis kit (Stratagene), with plasmid p5'-*dmp53*wt-luc applied as a DNA template to make a series of pGVBs carrying mutations in CCAAT boxes for analysis of *dmp53* promoter activity. The introduction of each mutation was confirmed by nucleotide sequencing.

To construct the plasmids p5'-*dmp53*wt-EGFP and p5'-*dmp53*mut-EGFP to produce transgenic flies, primers p53infusion1 and p53infusion2 containing *Not*I sites were employed for PCR to amplify the p53 promoter using p5'-*dmp53*wt-luc and p5'-*dmp53*mut-luc as templates. PCR products were combined with the pOBP plasmid (Galindo and Smith, 2001) in *Not*I site of the p.p53 plasmid with the aid of a In-Fusion PCR cloning kit (Clontech, US).

The EGFP gene was amplified from the pEGFP-C1 vector using GFP. Infusion1 and GFP.Infusion2 primers carrying *Not*I and *Pst*I sites respectively and then the PCR products were combined between *Not*I and *Pst*I sites of the p.p53 plasmid using an In-Fusion PCR Cloning Kit (Clontech, US).