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# A novel p53 paralogue mediates antioxidant defense of mosquito cells to survive dengue virus replication

Tien-Huang Chen[a], Yi-Jun Wu[b], Jiun-Nan Hou[b], Yi-Hsuan Chiang[a], Chih-Chieh Cheng[b], Eny Sifiyatun[b,c], Cheng-Hsun Chiu[d,e], Lian-Chen Wang[a,b,d,*], Wei-June Chen[a,b,d,*]

[a] Departments of Public Health and Parasitology, Chang Gung University, Kwei-San, Tao-Yuan 33332, Taiwan
[b] Graduate Institute of Biomedical Sciences, Chang Gung University, Kwei-San, Tao-Yuan 33332, Taiwan
[c] Program in Tropical Medical Science, Gadjah Mada University, Yogyakartan, Indonesia
[d] Molecular Infectious Disease Research Center, Chang Gung Memorial Hospital, Kwei-San, Tao-Yuan, Taiwan
[e] Division of Pediatric Infectious Diseases, Department of Pediatrics, Chang Gung Children's Hospital, Chang Gung University College of Medicine, Kwei-San, Tao-Yuan, Taiwan

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ABSTRACT

Mosquito cells allow dengue viruses (DENVs) to undergo replication without causing serious deleterious effects on the cells, leading to advantages for dissemination to other cells. Despite this, increased accumulation of reactive oxygen species (ROS) is usually detected in C6/36 cells with DENV2 infection as shown in mammalian cells. Uniquely, oxidative stress caused by the ROS is alleviated by eliciting antioxidant defense which leads to protection of mosquito cells from the infection. In the present study, a novel p53 paralogue (p53-2) was identified and proved to be regulated in C6/36 cells with DENV2 infection. With a gene-knockdown technique, p53-2 was demonstrated to transcribe catalase which plays a critical role in reducing ROS accumulation and the death rate of infected cells. Ecologically, a higher survival rate of mosquito cells is a prerequisite for prosperous production of viral progeny, allowing infected mosquitoes to remain healthy and active for virus transmission.

## 1. Introduction

Dengue viruses (DENVs) account for 50–100 million dengue infections and around 200,000 deaths each year worldwide (Fredericks and Fernandez-Sesma, 2014), and continue to be a health threat in tropical and subtropical regions (Bhatt et al., 2013). The DENV taxonomically belongs to the family Flaviviridae, the genome of which contains a single-stranded positive-sense RNA of ~ 11 kilobases (kb) in length. Viral RNA is composed of a structure possessing an m7GpppAmp cap at the 5′-end but lacks a poly(A) tail at the 3′-end (Gebhard et al., 2011). When the DENV enters a host cell, viral RNA is directly translated into a single polyprotein that is subsequently cleaved into three structural proteins (C, M, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by the combined actions of host proteases and the trypsin-like viral NS2B/NS3 serine protease (Mukhopadhyay et al., 2005). DENVs can be antigenically divided into four distinct serotypes, although there may be the fifth one (Holmes, 1998; Mustafa et al., 2015). Each serotype of DENVs causes similar clinical symptoms, including frontal headaches, retro-orbital pain, myalgia, joint pain, prostration, a macular rash, and hemorrhagic manifestations in some cases (Gubler, 1998). More-severe forms such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are also observed in a proportion of DENV infections (Guzman et al., 2010). They are hypothesized to occur due to infection by a virulent strain or on the basis of antibody-dependent immune enhancement (Gubler, 2002). In addition, neurological complications of dengue infections were reported in certain cases (Chen et al., 1991; Puccioni-Sohler et al., 2013). DENVs are naturally transmitted between humans by mosquito vectors, primarily *Aedes aegypti* (Yang et al., 2014), meaning that the virus is able to replicate in both human/mammalian and mosquito cells.

Within the human host, the virus injected by the mosquito vector frequently infects and replicates in skin Langerhans cells, monocyte-derived dendritic cells, and monocytes/macrophages (Palucka, 2000; Wu et al., 2000), and perhaps also in megakaryocytes (Noisakran et al., 2010). Panels of host genes being upregulated and downregulated in host cells with DENV infection suggests that selected host factors may be involved in host responses, such as innate immunity (Sessions et al., 2013). Apoptosis is usually the ultimate outcome of cells infected with DENVs (Morchang et al., 2011; Nasirudeen et al., 2008), based on determinants from both the virus and infected cells (Marianneau et al.,

* Corresponding authors at: Departments of Public Health and Parasitology, Chang Gung University, Kwei-San, Tao-Yuan 33332, Taiwan
*E-mail addresses:* wanglc@mail.cgu.edu.te (L.-C. Wang), wjchen@mail.cgu.edu.te (W.-J. Chen).

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1998). According to a study in a p53-deficient cell line, apoptosis became undetectable in response to DENV infection (Nasirudeen et al., 2008). This indicates that DENV-induced cell death is dependent on p53, which is usually upregulated during the infection (Hollstein et al., 1991).

p53 was reported to act as an important transcription factor in mammalian cells, functioning to restrict cellular proliferation via inducing distinct subsets of p53 target genes (Budde and Grummt, 1999; Vousden and Prives, 2009). p53 may be activated by various factors such as stress within cells. p53 is normally activated by low-intensity intracellular stress, facilitating triggering of an antioxidant response. However, a high level of p53 that tends to activate pro-oxidant targets may be induced under high-intensity oxidative stress (Borras et al., 2011). It seems that p53 is a double-edged sword, which can be converted from a killer into a healer based on the intensity of stress-induced ROS levels (Gudkov, 2002). As accumulation of high levels of ROS can cause dysfunction of cells (Mates et al., 1999), p53 may drive cells to a normal physiological or pathological status based on the intensity of the cellular stress (Brady and Attardi, 2010). This indicates that p53 or its downstream genes may be responsible for ROS-regulating activity and subsequent antioxidant defense (Budanov, 2014), by converting itself from a killer into a healer which facilitates DNA repair (Gudkov, 2002).

In addition to mammalian cells, p53 was also identified in insects and other invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Rutkowski et al., 2010), being a structural and functional homologue of that from mammals (Ollmann et al., 2000). The function of *Drosophila* p53 was demonstrated to be similar to that of mammals of triggering apoptosis under stress; this was also observed in *Bombyx mori*-derived cells exposed to $H_2O_2$ (Chen et al., 2015; Liu et al., 2008). Nevertheless, increasing evidence has revealed that invertebrate-derived p53 may function in DNA repair, cell-cycle checkpoint responses, and cell differentiation (Fan et al., 2010), implying that it may be beneficial for cell survival under specific conditions of a cell. Moreover, p53 of *D. melanogaster* was found to consist of two isoforms, A and B, and only isoform A is involved in mediating the apoptotic response to DNA damage (Zhang et al., 2015). Rather recently, two p53 paralogues, p53-1 and p53-2, were identified from mosquitoes and C6/36 cells (Chen et al., 2017).

We previously demonstrated that oxidative stress occurs in DENV2-infected C6/36 cells which usually survive the infection via induction of antioxidant defense and antiapoptotic effects (Chen et al., 2012, 2011). In this study, we continued to further investigate how antioxidant defense is triggered. Herein, we found that the p53-2 paralogue was specifically activated in response to DENV2 infection in C6/36 cells. Its roles of determining the fate of mosquito cells and facilitating viral replication were also studied and are discussed in this report.

## 2. Results

### 2.1. Identification of p53 paralogues from C6/36 cells

Two p53 homologues (p53-1 and p53-2) were previously demonstrated in C6/36 cells which were derived from the *Ae. albopictus* mosquito. Using designed primer pairs from each sequence of the corresponding paralogue, the mRNA level of p53-1 was shown to have not significantly changed throughout the period of observation, i.e., 48 h of infection (< 1.32-fold increase) compared to that in mock-infected cells (Student's *t*-test; $p > 0.05$). In contrast, p53-2 remained near the baseline (a 1.13-fold increase) at 12 h post-inoculation (hpi); however, it had significantly increased to 2.27-fold at 24 and 2.37-fold at 36 hpi, and eventually reached 2.98-fold at 48 hpi (Fig. 1). This revealed that p53-2, but not p53-1, was responsive to DENV2 infection in C6/36 cells.

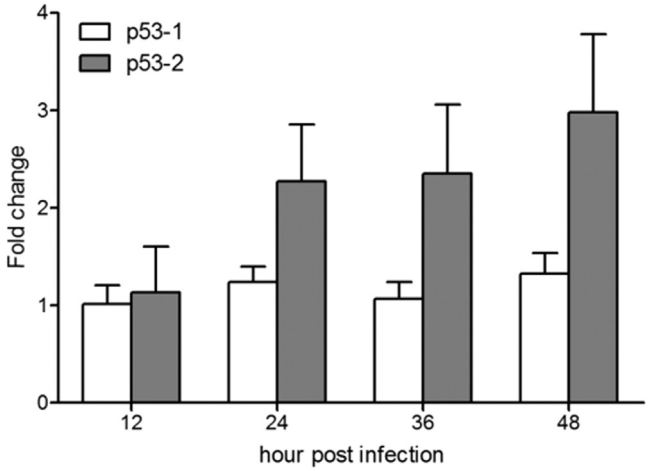


**Fig. 1.** Identification of p53 paralogues from C6/36 cells and measurement of its expression in response to dengue 2 virus (DENV2) infection. Gene expressions of two p53 homologues (p53-1 and p53-2) were evaluated by a real-time RT-PCR in C6/36 cells in response to dengue 2 virus (DENV2) infection. It was shown that the mRNA level of p53-1 did not significantly change even though cells were infected for 48 h (< 1.32-fold increase) (Student *t*-test; $p > 0.05$). Nevertheless, although p53-2 remained at the baseline (1.13-fold increase) at 12 h post-infection (hpi), it had significantly increased by 2.27-, 2.37-, and 2.98-fold by 24, 36, and 48 hpi, respectively (one-way ANOVA, $p < 0.05$). This indicates that only p53-2 was responsive to DENV2 with infection time in C6/36 cells.

### 2.2. The absence of cross effect in knockdown of specific p53 paralogues by using specific dsRNA

To examine mutual effects between the two paralogues of p53, synthesized dsRNAs were used to specifically knock down p53-1 or p53-2, and then their expressions were measured by a real-time RT-PCR. Results revealed that p53-1 was reduced to 21% and 25% of the expression in cells with knockdown of p53-1 at 24 and 48 h, respectively (Student's *t*-test, $p < 0.05$) (Fig. 2a). In contrast, p53-2 showed no significant change in this group with observations at the same time points (Student's *t*-test, $p > 0.05$) (Fig. 2b). On the other hand, only 5% and 1% of p53-2 was left in cells with p53-2-knockdown at 24 and 48 h, respectively, revealing statistically significant decreases (Student's *t*-test, $p < 0.05$) (Fig. 2c). However, a change in p53-1 was not observed in the same group (Student's *t*-test, $p > 0.05$) (Fig. 2d). Results revealed that a significant reduction in expression appeared only in C6/36 cells transfected with the corresponding dsRNA, reflecting that a mutual effect might be absent between the two paralogues of p53.

### 2.3. Association of the cell's fate with p53-2 in C6/36 cells with DENV2 infection

We further evaluated the role of p53-2 in the death of C6/36 cells with DENV2 infection, by measuring the $subG_1$ phase of cell-cycle progression. According to results, the cell death rate remained at ≤ 1% in all groups of cells with knockdown of p53-2, both when infected by the virus and not (Fig. 3a). Low cell death rates (< 1%) were also observed in mock- and DENV2-infected cells without knockdown of p53-2 at 48 hpi, while an obvious increase of cell death rate was seen in cells with p53-2 knockdown at 48 hpi (Fig. 3b). Statistically, an evident increase in the cell death rate was not seen in C6/36 cells at 24 hpi, even though p53-2 had been knocked down. On the other hand, a significant elevation in the cell death rate of up to 11.91% was detected at 48 hpi compared to the control groups (0.58% for cells without transfection and 0.73% for cells transfected with luciferase dsRNA) (Student's *t*-test, $p < 0.01$) (Fig. 3c). This implied that p53-2 indeed plays a role in regulating the fate in C6/36 cells during DENV2