**Ubiquitin ligase Fbw7 restricts the replication of hepatitis C virus by targeting
NS5B for ubiquitination and degradation**

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Abstract

The nonstructural protein 5B (NS5B) of hepatitis C virus (HCV) is an RNA-dependent RNA polymerase (RdRp) and responsible for replicating the whole HCV genome with help of viral and cellular proteins. However, how cellular factors influence NS5B and, in turn, regulating HCV replication are still poorly defined. The well known tumor suppressor Fbw7, a component of E3 ubiquitin ligase SCF^{Fbw7}, targets oncoproteins or cellular regulatory proteins for ubiquitin-mediated degradation through a highly conserved binding site called a Cdc4 phosphodegron (CPD). But little is known about whether Fbw7 plays a role in regulation of viral proteins. In this study, we revealed that the conserved CPD is shared by NS5B of almost all genotype of HCV and our data demonstrated that NS5B is a bona fide substrate of Fbw7. Forced expression of Fbw7 promoted the ubiquitination of NS5B and negatively regulated its turnover in the proteasome-dependent manner. We further revealed the interaction between NS5B and Fbw7, which resulted in the relocation of Fbw7 from nucleus to cytoplasm. During HCV replication, ectopic expression of Fbw7 could strongly down-regulate NS5B level and consequently inhibited the virus replication. When endogenous Fbw7 was knocked down, both NS5B protein abundance and HCV replication were remarkably up-regulated. The results provide more insights into the interplay of HCV and cellular factors and shed light on molecular mechanisms of HCV replication and pathogenesis.

Keywords: Fbw7; NS5B; ubiquitination; degradation; HCV replication

Introduction

Hepatitis C virus (HCV) is the major cause of non-A, non-B hepatitis which has infected about 200 million people worldwide. NS5B, one of the non-structural proteins of HCV, was shown to have RdRp activity [1]. In HCV-infected cells, NS5B is anchored to the endoplasmic reticulum (ER) membrane and forms a complex with other nonstructural proteins NS5A, NS2, NS3 and NS4A [2] and undefined cellular proteins [3] to accomplish the HCV replication. HCV replication appears to be restricted in the livers of chronically HCV-infected patients, because the viral RNA and proteins are typically difficult to be detected for their low abundance. It is most likely that HCV RNA and protein expression are regulated by inhibition of synthesis or enhancement of degradation by host cellular factors.

The ubiquitin-proteasome pathway is the major pathway for degradation of proteins. It involves three enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase that recognizes specific substrates for degradation [4]. SCF^{Fbw7} (SKP1-CUL1-F-box protein) is a well characterized E3 ubiquitin ligase, where Fbw7 (F-box and WD repeat domain-containing 7) is the recognition component of SCF^{Fbw7} complex [5]. SCF^{Fbw7} mediated the degradation of several proto-oncoproteins that function in cellular growth and division pathways, including cyclin E [6], c-Myc [7] and c-Jun [8]. Accordingly, Fbw7 acts as an effective tumor suppressor. Numerous cancer types are associated with mutations in Fbw7 or its substrates [5]. Recently researchers further proved that Fbw7 might be used as an independent prognostic marker in hepatocellular carcinoma (HCC) [9]. Almost all the substrates of Fbw7 share the highly conserved Cdc4 phospho-degrons (CPDs) I/L-I/L/P-pT-P-<K/R>4 [5] that are phosphorylated and recognized by Fbw7, leading to the degradation of substrates through ubiquitin-dependent pathway.

Previous studies showed that NS5B can be degraded in ubiquitin-dependent manner, but it still remains unknown what kind of ubiquitin ligase is involved in the process [10]. In this study, we aligned the HCV NS5B sequences of different genotypes and found that they shared a potential CPD domain that may be recognized

by Fbw7. We then revealed that transient expression of Fbw7 α decreased the NS5B protein level through ubiquitin-dependent pathway and, in turn, inhibited the HCV replication. We further demonstrated that Fbw7 α is colocalized with NS5B and knockdown of the endogenous Fbw7 α could promote HCV replication. Together, these results suggest that the SCF^{Fbw7}/ubiquitination system plays a role in the regulation of HCV replication and antiviral defense. To our knowledge, HCV NS5B is the first viral protein identified as a substrate of Fbw7 ubiquitin ligase.

Materials and Methods

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen. FuGENE HD was a transfection reagent from Roche. Monoclonal mouse anti-Flag, anti-Myc, anti- β -actin and anti-HA antibodies were purchased from Sigma-Aldrich. The polyclonal rabbit anti-NS5B and polyclonal rabbit anti-Fbw7 antibodies were purchased from Abcam. Polyclonal rabbit anti-ubiquitin antibodies were purchased from Santa Cruz Biotechnology. Cycloheximide(CHX) and MG132 were products of Beyotime. Hoechst was purchased from Sigma-Aldrich. The JFH1 virus titer assay kit was purchased from KHB. Real-time quantitative PCR kit was purchased from Takara.

Cell culture, transfection, Immunoprecipitation, fluorescence,imaging, western blot analysis and virus

Most of these assays were performed as described previously [11,12]. The genotype 2a JFH1 virus and Huh7.5.1 cells were kindly provided by Prof. Hongbin Shu (Wuhan University, Wuhan, China). Huh7.5.1 cells were transfected with JFH1 RNA or infected by the stocked JFH1 viruses, and then the supernatant of the culture was harvested and stored at -80°C for further use. The JFH1 virus copies were monitored by real-time quantitative PCR according to the kit manual. The titer of JFH1 virus used in this study was 10⁴ and 10⁶ focus-forming units (ffu)/mL, and Huh7.5.1 cells were inoculated at a multiplicity of infection (MOI) of 0.5-1. The virus

absorption was carried out in FBS-free DMEM medium for incubation for 2 h at 37°C and then the medium was changed to DMEM containing 10% FBS.

Determination of JFH1 virus titers

Huh7.5.1 cells were seeded at 10^4 /well in 96-well plate with 100 μ L of culture medium. After incubation for 24 h, Huh7.5.1 cells were infected with serial dilutions of JFH1 virus at 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7} . Each well was added with 50 μ L virus solution. After infection for 3 days, virus titers were determined by indirect immunofluorescence staining. The intracellular core antigen was used as the target staining protein. Each fluorescence cluster stained with core antibody was identified as a single infectious focus. To count the number of foci, virus titers were calculated with the formula: virus titers (ffu/mL) = the number of foci $\times$ 20 $\times$ the dilution folds.

RNA analysis

The random hexanucleotide primers were designed for use in reverse transcription. Quantitative forward and reverse primers were 5'-CCACATCAAGTCCGTGTGGA -3' and 5'-AGGCGAGCTGGTTTCTTACC -3', respectively. Fbw7 knockdown DNA oligos were as follows: shFbw7-1 AATGTTTGTAACACTGTCCT, shFbw7-2 AATACTACTGGAGTTCGTGAC. All the knockdown primers were cloned into lentivirus vector pLKO1. For real-time quantitative PCR, *GAPDH* was used as an internal control.

Results

1. Fbw7 negatively regulates the protein level of NS5B

Fbw7 targets substrates for ubiquitin-mediated degradation through a highly conserved CPD domain [5]. This information was used to screen for CPD sequence motif from the viral protein sequences in the database. Interestingly, a CPD consensus motif was identified from HCV NS5B, corresponding to amino acid (aa) position 90-98 (Fig. 1A). Based on the data in Fig. 1A, NS5B of almost all different genotype contains the highly conserved CPD domain. The only exception is subgenotype 3a

whose CPD lacks the central threonine residue (Fig. 1A). Previous studies also showed that NS5B can be negatively regulated in ubiquitination-dependent manner [10]. Taken these clues together, we predicted that NS5B may serve as a potential substrate of Fbw7.

The Fbw7 protein has three isoforms Fbw7 α , Fbw7 β and Fbw7 γ (Fig. 1B). In certain cases, all the three isoforms can negatively regulate the same protein, such as c-myc [7], but in other cases, only one of the isoforms is involved in target protein regulation, such as Nrf1 [13]. As NS5B was a potential substrate of Fbw7, we first tested which isoform could negatively regulate NS5B. For this, HEK 293T cells were transfected with NS5B together with each individual Fbw7 isoform or their mutants, respectively. The results showed that only Fbw7 α significantly reduced the protein level of NS5B, while Fbw7 β and Fbw7 γ did not (Fig. 1C). We also generated the ubiquitin ligase-deficient mutants, named as Fbw7 Δ F, where the F-box domain was deleted. As shown in Fig. 1C, Fbw7 α Δ F failed to reduce NS5B in comparison of wild type Fbw7 α , suggesting that the ability of Fbw7 α to negatively regulate NS5B is ubiquitin ligase-dependent. To directly follow the degradation of NS5B mediated by Fbw7 α , we created a fused EGFP-NS5B protein. When EGFP-NS5B was co-expressed with Flag-Fbw7 α or Flag-Fbw7 α Δ F in HEK 293T cells, we found that Fbw7 α could remarkably reduce the green-fluorescence of EGFP-NS5B compared with Fbw7 α Δ F (Fig. 1D). Western blotting results were also consistent with the fluorescence analysis (Fig. 1E). To reveal whether NS5B was affected by Fbw7 α at mRNA level, NS5B was co-transfected with Fbw7 α or Fbw7 α Δ F into HEK 293T cells. We found that there was no obvious difference between the two samples (Fig. 1F). This showed that Fbw7 negatively regulated NS5B at protein level instead of mRNA level.

2. Fbw7 α is relocalized by interacting with NS5B

To determine whether Fbw7 α was involved directly or indirectly with NS5B, Co-immunoprecipitation assay was carried out. HEK 293T cells were co-transfected with Myc-NS5B and Flag-Fbw7 α or vector control in the presence of proteasome

inhibitor MG132 to prevent degradation of NS5B by Fbw7 α . Fbw7 α was immunoprecipitated with anti-Flag antibody from the cell lysates. Subsequently, NS5B was probed by anti-Myc antibody. Western blotting revealed that NS5B and Fbw7 α could be co-precipitated readily (Fig. 2A). This result suggested that there was a direct interaction between Fbw7 α and NS5B.

It has been demonstrated that Fbw7 α is located in the nucleoplasm [14] whereas NS5B resides in endoplasmic reticulum (ER) [15]. Therefore, we predicted that either Fbw7 α or NS5B should be relocalized for their interaction in vivo. To confirm this assumption, immunostaining was performed to check whether there was co-localization between Fbw7 α and NS5B. We transfected EGFP-Fbw7 α and DsRed-NS5B into Huh7 cells individually or together and observed that Fbw7 α was located in nucleoplasm in the absence of NS5B but a fraction of Fbw7 α was relocalized from the nucleus to the cytoplasm and co-localized with NS5B in the presence of NS5B (Fig. 2B). Together, these data suggested that NS5B and Fbw7 α can interact with each other in the cytoplasm.

3. Fbw7 mediates NS5B turnover by ubiquitin-proteasome-dependent pathway

Next we asked whether Fbw7-driven NS5B turnover was dependent on ubiquitin-proteasome. Firstly, HEK 293T cells were transfected with HA-NS5B and treated with CHX. As time went on, NS5B was degraded gradually, which implied that NS5B could be negatively regulated in cells (Fig. 3A). Secondly, we examined the effect of proteasome inhibitor on the turnover of NS5B. HEK 293T cells were transfected with HA-NS5B and treated with MG132. We found that the abundance of NS5B remained unchanged (Fig. 3A). These results indicated that NS5B could be degraded through proteasome pathway. Thirdly, to confirm that NS5B was reduced by Fbw7-mediated proteolysis, HEK 293T cells were co-transfected with NS5B and Fbw7 α and treated with MG132. The results showed that NS5B was decreased notably by Fbw7 α . However, the reduction of NS5B could be reversed by MG132 (Fig. 3B). These data suggested that Fbw7 α -mediated degradation of NS5B took place in a proteasome-dependent manner. In addition, the reduction of NS5B by Fbw7 α was

in a dose-dependent manner (Fig. 3C).

An *in vivo* ubiquitination assay was done to test whether Fbw7 promotes NS5B ubiquitination. In this experiment, HEK 293T cells were transfected with Myc-NS5B and HA-ubiquitin in the presence or absence of Flag-Fbw7. The transfected cells were treated with MG132 for 6 h to stabilize the ubiquitinated protein before harvesting of the cells. Myc-NS5B was immunoprecipitated with anti-Myc antibody and detected with anti-ubiquitin antibody. The results showed that MG132 could prevent the ubiquitylated NS5B from degradation (Fig. 3D, lanes 1 and 2). In the presence of exogenous Fbw7 α , the ubiquitylated NS5B was degraded by proteasome when not treated with MG132. When cotransfected with Fbw7 $\alpha\Delta F$, ubiquitination of NS5B was still detectable (Fig. 3D, lanes 3 and 4), and the weak ubiquitination signal may be caused by the endogenous E3 ligase in cells. This indicated that Fbw7 α could enhance the NS5B mult-ubiquitination more effectively than Fbw7 $\alpha\Delta F$. Without transfection of NS5B in cells, there were no any bands in immunoprecipitation no matter whether the cells were treated with or without MG132 (Fig. 3D, lanes 5 and 6). This indicated that the detected ubiquitylated protein was ubiquitylated NS5B. Taken these results together, it could be concluded that Fbw7 α promoted NS5B ubiquitination and degradation by proteasome-dependent pathway.

4. *Fbw7 regulates the JFH1 replication by targeting the NS5B of JFH1*

To test whether Fbw7 can promote degradation of NS5B in HCV infection, the three isoforms Fbw7 α , Fbw7 β and Fbw7 γ and their dead mutants were transfected in Huh7.5.1 cells. Then the cells were infected by HCV strain JFH1. After 72 h, the cells were harvested for analysis of NS5B. The result showed that NS5B generated by JFH1 virus decreased markedly in cells transfected with Fbw7 α in comparison with Fbw7 $\alpha\Delta F$ (Fig. 4A). These results were consistent with that of expression of NS5B in HEK 293T cells.

To test whether the HCV replication and virus growth were affected by ectopic expression of Fbw7 α , we analyzed the intracellular HCV RNA and that of the released viral particles by real-time quantitative PCR. We found that intracellular RNA

decreased about 40% when Fbw7 α was transfected, compared with that of Fbw7 $\alpha\Delta F$ (Fig. 4B). The virus particles in the supernatant decreased nearly about 50 compared with that in the cells transfected with Fbw7 $\alpha\Delta F$ (Fig. 4C). All these data indicated that Fbw7 α could negatively regulate HCV replication by targeting NS5B for degradation.

To further determine whether Fbw7 promoted degradation of the NS5B encoded by JFH1 virus through proteasome-dependent way, the cells were treated with MG132 for 6 h before harvesting. The results showed that the reduction of virus NS5B was reversed after the cells were treated by MG132. The above data revealed that Fbw7 could accelerate degradation of virus NS5B by proteasome-dependent pathway and thereby inhibit HCV replication (Fig. 4D)

Because overexpression of Fbw7 reduced HCV NS5B and suppressed the HCV propagation, we speculated that downregulation of Fbw7 might be beneficial to HCV replication. To test this hypothesis, two different shRNA constructs (shFbw7) were used to knock down Fbw7 in Huh7.5.1 cells. Our data showed that the two shFbw7 shRNAs could decrease expression of Fbw7 efficiently (Fig. 4E). Correspondingly, the amount of NS5B increased in cells when the endogenous Fbw7 was knocked down, compared with control shGFP (Fig. 4E). The copy number of the HCV RNA released from the HCV-infected Huh7.5.1 cells was significantly increased in the Fbw7 α knockdown cells in comparison with that of control cells (Fig. 4F). These results indicated that knockdown of Fbw7 α improved HCV replication and thus further confirmed that Fbw7 α could be involved in the regulation of HCV replication in natural infection.

Discussion

Fbw7 has been known to regulate many oncoproteins and cellular regulatory proteins such as G-CSFR[16], C/EBP Δ [17], Nrf1 [13], Mcl-1[18], KLF5[19], Notch [20], c-myc [7] and cyclin E [6]. In this study, we identified a viral protein, the HCV NS5B, as a substrate of Fbw7. We revealed that Fbw7 negatively regulated NS5B at posttranslational level, and knockdown of endogenous Fbw7 could facilitate replication of HCV. This suggests that Fbw7 may play a physiological role in HCV

replication. Infection of HCV can cause chronic hepatitis, cirrhosis and hepatocarcinoma and Fbw7 mutations lead to loss its function as a tumor suppressor in many caners [21,22]. Therefore, it can be envisaged that Fbw7 may plays a role in the restriction of HCV replication in normal tissues while Fbw7 deficiency in hepatocarcinoma may promote replication of HCV, thus further exacerbate liver carcinogenesis.

HCV RNA polymerase lacks proofreading activity and therefore the HCV genome mutation rate is very high. HCV can be classified into six major genotypes on the basis of the genome similarity. Despite the genome variance, some domains are highly conserved among NS5B sequences. For instance, the segment from position 91-99, LTPPHSARS, is a highly conserved region[23]. This region contains the typical CPD domain that is similar to that of the substrates of Fbw7 E3 ligase. It is worth mentioning that previous study has shown that the serum HCV RNA titer and genotypes have significant positive correlation[24]. And it has been reported that genotype 3a has higher serum HCV RNA titer than others [24]. Subtype 3a is the only genotype whose NS5B does not contain the typical CPD (Fig. 1A), suggesting that Fbw7 most likely could not mediate the degradation of the NS5B of HCV 3a. It is reasonably to speculate that lack of typical CPD domain in NS5B may contribute to the higher RNA titer of HCV 3a in patient serum.

In this work, we showed that HCV NS5B could associate with and alter the subcellular location of Fbw7 α . Previous studies demonstrated that HCV NS5B can also interact with the intracellular RNA helicase p68 [25] and cyclin-dependent kinase 2-interacting protein (CINP) [26]. HCV replicates in the cytoplasm and NS5B is localized at ER. Interactions of Fbw7 α , p68 and CINP with NS5B lead to redistribution of these proteins mainly distributed in nucleus when cells are not infected by HCV. These functional interaction and redistribution may serve either to regulate virus replication in host cells or affect host cell growth by HCV. Although Fbw7 α is co-localized with NS5B in cytoplasm and mediates NS5B degradation, we still do not know whether Fbw7 α was sequestered in cytoplasm by NS5B or transported from nucleus back to cytoplasm during HCV infection. The detailed

mechanism needs to be further investigated.

In summary, our current study shows that HCV NS5B harbors a CPD motif that can be recognized by the ubiquitin ligase Fbw7 and undergoes K48-type ubiquitination and degradation mediated by the isoform Fbw7 α . Knockdown of Fbw7 promotes HCV replication while exogenous expression of Fbw7 can suppress HCV replication. The results provide new insights into the regulatory mechanisms of HCV replication by cellular factors.

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Figure Legends

Fig. 1 Fbw7 negatively regulates the protein level of NS5B. (A) Alignment of the CPD domains in NS5B of six different genotypes of HCV. (B) The diagram of three isoforms for Fbw7. NLS, nuclear localization signal; TMD, transmembrane domain. (C) Expression of Fbw7 α reduces the protein level of NS5B. HEK 293T cells were transfected with vector alone (vec), or Fbw7 α , Fbw7 β , Fbw7 γ or their mutants Fbw7 $\alpha\Delta$ F, Fbw7 $\beta\Delta$ F, Fbw7 $\gamma\Delta$ F with NS5B. At 30 h after transfection, cells were harvested for western blotting (WB) analysis. All three isoforms of Fbw7 and their mutants carry anti-Flag tags, while NS5B is HA-tagged. (D) & (E) HEK 293T cells were transfected with Flag-Fbw7 α or Flag-Fbw7 $\alpha\Delta$ F with EGFP-NS5B. At 30 h after transfection, the fluorescence of cells was observed (D). In parallel, the cells were harvested for WB analysis (E). (F) The NS5B mRNA was analyzed by real-time PCR. In the graph, column 1 represents the cells co-transfected NS5B and Fbw7 α , and column 2 represents the sample with NS5B and Fbw7 $\alpha\Delta$ F. *, $p < 0.05$, **, $p < 0.01$; the bar graphs show the mean value $\pm$ SD of triplicate samples.

Fig. 2 Fbw7 α interacts with NS5B and is relocalized in cytoplasm in the presence of NS5B. (A) The interaction of NS5B and Fbw7 were determined by co-immunoprecipitation (Co-IP) analysis. HEK 293T cells were co-transfected with vec or Flag-Fbw7 and Myc-NS5B. After transfection for 24 h, the cells were treated with 20 μ M of MG132 for 4 h, and then harvested for Co-IP analysis. (B) The localization of Fbw7 α and NS5B was determined in fixed Huh7 cells by confocal microscopy. Huh7 cells were transfected with DsRed-NS5B and EGFP-Fbw7 alone or

co-transfected with DsRed-NS5B and EGFP-Fbw7, and after transfection for 24 h, the cells were treated with 20 μ M of MG132 for 4h, and then observed for fluorescence.

Fig. 3 Fbw7 mediates the degradation of HCV NS5B in ubiquitin-proteasome dependent pathway. (A) Left panel: The half life of NS5B was tested. CHX: 100 μ g/mL. Right panel: The degradation of NS5B was proteasome-dependent. MG132: 20 μ M (B) The decrease of the amount of NS5B induced by Fbw7 expression could be reversed by MG132 treatment. HEK 293T cells were transfected with NS5B and vec or Fbw7 α , and after transfection for 24 h, the cells were treated with MG132 (20 μ M) for 6 h, and then harvested for WB analysis. (C) Fbw7 α decreased the amount of NS5B protein in a dose-dependent manner. HEK 293T cells were co-transfected with 1 μ g NS5B and vec or Fbw7 α with increasing amount. After transfection for 30 h, cells were harvested for WB analysis. (D) The ubiquitination of NS5B was analysed in the presence of Fbw7 α in vivo. HEK 293T cells were co-transfected with plasmids as indicated in the figure. myc-antibody was used for immunoprecipitation and anti-HA antibody was used for IB analysis. Cells used for lanes 2, 4 and 6 were treated with MG132 (20 μ M) for 4 h before harvesting.

Fig. 4 Fbw7 regulates the JFH1 replication by targeting the NS5B of JFH1.

(A) Fbw7 α reduces the protein level of NS5B coded by JFH1. Huh7.5.1 cells were transfected with Fbw7 isoforms and their mutants, and after transfection for 6 h, the cells were infected with JFH1 for three days, and then harvested for analyzing the amount of NS5B. Fbw7 and NS5B were detected with anti-Flag antibody and anti-NS5B antibody, respectively. The asterisk represents the non-specific band. (B) The HCV RNA was analyzed by real-time PCR. Sample 1 represents the Huh7.5.1 cells transfected with Fbw7 α and infected with JFH1. Sample 2 represents the Huh7.5.1 cells transfected with Fbw7 α Δ F and infected with JFH1. (C) Huh7.5.1 cells were transfected with Fbw7 α and Fbw7 α Δ F, and after transfection for 6 h, the cells were infected with JFH1 for three days, and then the supernatant was collected for measuring the copies of JFH1 virus with KHB kit. Sample 1 represents the

425 supernatant from Huh7.5.1 cells transfected with Fbw7 α and infected with JFH1.
426 Sample 2 represents the supernatant from Huh7.5.1 cells transfected with Fbw7 $\alpha\Delta F$
427 and infected with JFH1. (D) The decreased protein level of NS5B could be recovered
428 by treatment with MG132. Huh7.5.1 cells were transfected with vec or Fbw7 α , and
429 after transfection for 6 h, the cells were infected by JFH1 for three days, and treated
430 with MG132 for 6 h before harvesting. Anti-Flag antibody was used to detect Fbw7 α ,
431 and anti-NS5B antibody was used to detect NS5B encoded by JFH1 virus. (E) The
432 effects of Fbw7 knockdown on virus NS5B. shGFP was used as a control. Fbw7 and
433 NS5B were detected with anti-Fbw7 and anti-NS5B antibodies respectively. (F) The
434 effects of Fbw7 knockdown on JFH1 replication. The JFH1 virus copies in the
435 supernatant were measured with KHB kit. *, $p < 0.05$, **, $p < 0.01$; the bar graphs
436 show the mean value $\pm$ SD of triplicate samples.

Figure1

Figure2

Figure3

Figure4