Original Article

# Overexpressed *FOXM1* collaborates with MMB to increase *WEE1* inhibitor sensitivity in NSCLC

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**Background:** Non-small cell lung cancer (NSCLC) is a common lung tumor with high mortality. The complex formed by MYB-MuvB complex (MMB) and forkhead box M1 (*FOXM1*) (MMB-*FOXM1*) plays a vital role in cell cycle progression to affect the progression of diseases. The role of the *FOXM1*-MMB complex in Wee1-like protein kinase (*WEE1*) inhibitor sensitivity in NSCLC keeps unclear.

**Methods:** The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to measure the mRNA levels of *FOXM1*, *LIN54*, Replication Protein A (*RPA*), gammaH2AX (*γH2AX*) and Cyclin B (*CCNB*). The western blot was performed to examine the corresponding protein expressions. The Cell Counting Kit-8 (CCK-8) assay was performed to test cell survival.

**Result:** It was demonstrated that after AZD-1775 treatment, the decrease in cell survival mediated by *FOXM1* overexpression (P<0.001) could be reversed by *LIN54* knockdown (P<0.01) and that cell survival in the control group did not differ obviously from that in the pcDNA3.1-FOXM1+siLIN54 group, indicating that the *FOXM1*-MMB complex was necessary for *WEE1* inhibitor sensitivity. Moreover, the mRNA and protein expression levels of *RPA* and *γH2AX* were increased after AZD-1775 treatment and *FOXM1* overexpression (P<0.01), suggesting that *FOXM1* upregulation enhanced DNA replication stress and DNA damage. Finally, we found that the increases in the mRNA and protein expression levels of *CCNB* mediated by *FOXM1* (P<0.01) could be rescued by silencing *LIN54* (P<0.001) and that *CCNB* expression in the control group did not differ obviously from that in the pcDNA3.1-FOXM1+siLIN54 group. These findings revealed that the *FOXM1*-MMB complex activated G2/M checkpoints. In our work, it was discovered that *FOXM1* overexpression increased DNA replication stress, which increased DNA replication and pressure on the *WEE1* checkpoint. On the other hand, *FOXM1* can enhance *CCNB* expression, increase the threshold content of the *CCNB/CDK1* complex, facilitate mitosis, and promote *WEE1* dephosphorylation. Under these two conditions, sensitivity to the *WEE1* inhibitor AZD-1775 is increased, which leads to the accumulation of DNA damage and drives the activation of apoptosis.

**Conclusions:** Overexpressed *FOXM1* collaborates with MMB to increase *WEE1* inhibitor sensitivity in NSCLC. This discovery might highlight the regulatory function of *FOXM1*/MMB in the treatment of NSCLC patients.

**Keywords:** Forkhead box M1 (*FOXM1*); MYB-MuvB complex (MMB); Wee1-like protein kinase (*WEE1*); non-small cell lung cancer (NSCLC)

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## Introduction

More than 80% of all lung cancers are non-small cell lung cancer (NSCLC), and this disease is a major cause of cancer deaths worldwide (1-3). Although great improvements have been made due to progress in treatment strategies and surgical techniques, the 5-year survival rate is only slightly higher than 15% (4,5). And, the prognosis of NSCLC patients is discouraging. Currently, effective treatment strategies such as radiotherapy and chemotherapy are limited. More and more molecular biomarkers have been verified to participate into the progression of NSCLC. Therefore, identifying novel specific molecular biomarkers is of great importance to improve the treatment of NSCLC.

The transcription factor forkhead box M1 (*FOXM1*) is a well-known oncogene and an essential factor for cell proliferation in cancers (6). For example, *FOXM1* facilitates breast cancer cell stemness and migration in a Yes-associated protein 1 (*YAP1*)-dependent manner (7). High expression of *FOXM1* is critical for sustaining cell proliferation in mitochondrial DNA-less liver cancer cells (8). *FOXM1* promotes the migration of ovarian cancer cells through Keratin 5 (*KRT5*) and Keratin 5 (*KRT7*) (9). A novel *FOXM1*-proteasome subunit beta type-4 (*PSMB4*) axis contributes to the proliferation and progression of cervical cancer (10).

Regulation of the cell cycle is critical for normal development; cell cycle disruption causes a number of diseases, especially cancers (11,12). *FOXM1* is a pivotal modulator of periodic gene transcription in the G2-M phase of the cell cycle, and thus, it seems to be connected with the increased cell proliferation ability of tumors (13-15). MuvB is an evolutionarily conserved multisubunit complex, and it can modulate gene expression during the cell cycle (16,17). During S phase, the interaction between the MuvB core and p130/E2F4/DP1 is abolished, and MuvB then interacts with the *B-MYB* (V-Myb avian myeloblastosis viral oncogene homolog-like 2, *MYBL2*) transcription factor to form the MYB-MuvB complex (also called MMB) (18,19).

The complex formed by MMB and *FOXM1* (MMB-*FOXM1*) plays a vital role in cell cycle progression by modulating the transcription of genes needed for cytokinesis and mitosis (20). In the cell cycle, the E2F-dependent wave of gene expression at the G1/S transition accelerates DNA replication, and the MMB-*FOXM1* complex drives the second wave of gene expression at the G2/M transition, subsequently boosting mitosis (21,22). *FOXM1* is then recruited to the promoters of these genes through an MMB-dependent pattern (19). This recruitment is consistent with the increased levels of hundreds of G2/M cell cycle genes, such as Cell Division Cycle Protein 25B (*CDC25B*), Cyclin A2 (*CCNA2*), Polo-like Kinase 1 (*PLK1*) and Cyclin B1 (*CCNB1*) (23,24). According to one study, MMB-*FOXM1*-driven premature mitosis is necessary for sensitivity to checkpoint kinase 1 (*CHK1*) inhibitors (25). However, the regulatory role of the MMB-*FOXM1* complex in NSCLC keeps unclear, and needs more exploration in NSCLC.

Wee1-like protein kinase (*WEE1*) negatively modulates *CDK1*, and inhibition of *WEE1* eliminates inhibitory phosphorylation of *CDK1*, thereby inducing tumor cell apoptosis (26). This regulation of *WEE1* inhibitor also exist in NSCLC, that Wee1 inhibitor MK1775 strengthens sorafenib sensibility in KRAS mutated NSCLC cells (27). In addition, *WEE1* inhibitor AZD1775 has improvement effects on LKB1-deficient NSCLC (28). Besides, *WEE1* inhibitor enhances chemosensitivity in EGFR-TKIs resistant NSCLC (29). However, the relationship between MMB-*FOXM1* complex and *WEE1* inhibitor in NSCLC remain vague.

This study aimed to explore the relationship between *FOXM1* overexpression and *WEE1* inhibitor sensitivity in NSCLC. Our findings revealed that overexpressed *FOXM1* collaborates with MMB to increase *WEE1* inhibitor sensitivity in NSCLC. This discovery might provide novel insight into identifying new biomarkers for NSCLC treatment. We present this article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-750/rc).

### Highlight box

**Key findings**

- The *FOXM1*-MMB complex was found to be necessary for *WEE1* inhibitor sensitivity.
- *FOXM1* upregulation enhanced DNA replication stress.
- The *FOXM1*-MMB complex activated G2/M checkpoints.

**What is known and what is new?**

- According to one study, MMB-*FOXM1*-driven premature mitosis is necessary for sensitivity to *CHK1* inhibitors.
- This is the first study to discover that overexpressed *FOXM1* collaborates with MMB to increase *WEE1* inhibitor sensitivity in NSCLC.

**What is the implication, and what should change now?**

- This work revealed that the complex formed by overexpressed *FOXM1* and MMB might be used as a potential therapeutic biomarker for NSCLC.

## Methods

### *Cell lines and cell culture*

The NSCLC cell lines H1650 (TCHu152) and H2228 (SCSP-5001) were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (C11995500BT, DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (SH30068.03, FBS, HyClone, Logan, UT, USA), penicillin (100 units/mL) and streptomycin (100 mg/mL) at 37 ℃ with 5% $CO_2$.

### *Transfection*

The *FOXM1* overexpression vector (pcDNA3.1-FOXM1) and small interfering RNAs (siRNAs) targeting *FOXM1* and MMB (siFOXM1-1, siFOXM1-2, siFOXM1-3, siLIN54-1, siLIN54-2, siLIN54-3) were obtained from GenePharma (Shanghai, China). These vectors were transfected into NSCLC cells with Lipofectamine 2000 (Invitrogen, USA). AZD-1775 (the *WEE1* inhibitor, 0.5 μM, SC6677) was purchased from Beyotime (Shanghai, China).

### *Reverse transcription quantitative polymerase chain reaction (RT-qPCR)*

The RT-qPCR was performed to examining mRNA expressions. TRIzol reagent (15596018, Thermo Fisher Scientific) was employed to isolate RNA from NSCLC cells. Reverse transcription reagents (Applied Biosystems, Foster City, USA) were applied for cDNA synthesis by reverse transcription. RT-qPCR was conducted with SYBR Green (Takara Bio, Japan), and *GAPDH* was utilized as an internal control. The relative expression levels were calculated through the $2^{-\Delta\Delta Ct}$ method.

### *Western blot*

The western blot was performed to measuring protein expressions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied for isolation of proteins, which were then transferred to polyvinylidene fluoride (IPVH00010, PVDF, Millipore, Bedford, MA, USA) membranes. After blocking with nonfat milk, the membranes were incubated with primary antibodies at 4 ℃ overnight. After washing, the membrane was incubated with goat anti-rabbit IgG (ab6721, 1:2,000, Abcam) as the secondary antibody, and the bands were visualized with an enhanced chemiluminescence detection system. The primary antibodies included anti-*FOXM1* (ab180710, Abcam), anti-*LIN54* (ab138425, Abcam), anti-*RPA* (ab2175, Abcam), anti-*γH2AX* (ab11175, Abcam), anti-*CCNB* (ab32053, Abcam) and anti-β-*Actin* (ab6276, Abcam).

### *Cell Counting Kit-8 (CCK-8) assay*

The CCK-8 assay was performed to measuring cell survival. NSCLC cells were plated into 96-well plates. The cells were incubated for 24 h and then supplemented with AZD-1775 for 24 h. Next, 10 mL of CCK-8 reagent was mixed into each well. After 2 h, the optical density (OD =450 nm) was assessed with an enzyme immunoassay analyzer (Thermo Fisher Scientific).

### *Statistical analysis*

GraphPad Prism software, version 8.0 (GraphPad Software, La Jolla, CA) was utilized for statistical analysis. All experiments were performed in triplicate with randomization, and data are expressed as the mean ± standard deviation (SD) values. Two-group (or multiple-group) comparisons were carried out with Student's t test [or one-way variance analysis (ANOVA)]. All statistical tests were two-sided, and statistical significance was set at $P<0.05$.

## Results

### *The FOXM1-MMB complex was found to be necessary for WEE1 inhibitor sensitivity*

First, *FOXM1* was overexpressed or silenced using the appropriate vectors to verify the role of *FOXM1* in *WEE1* inhibitor sensitivity. The mRNA and protein expression levels of *FOXM1* were increased by the *FOXM1* overexpression vector (*Figure 1A*,*1B*). Additionally, the mRNA and protein expression levels of *FOXM1* were decreased by the *FOXM1* siRNAs (*Figure 1C*,*1D*). After treatment with AZD-1775 (*WEE1* inhibitor), cell survival was obviously decreased by either overexpression or inhibition of *FOXM1* (*Figure 1E*,*1F*). The MMB complex is formed by *MYBL2* and MuvB, while MuvB is composed of *LIN54*, *LIN9*, *LIN37*, *LIN52* and *RBBP4*. *LIN54* plays a crucial role in the formation of the MMB complex, and the influence of MMB was eliminated by silencing *LIN54* expression. The mRNA and protein expression of *LIN54*

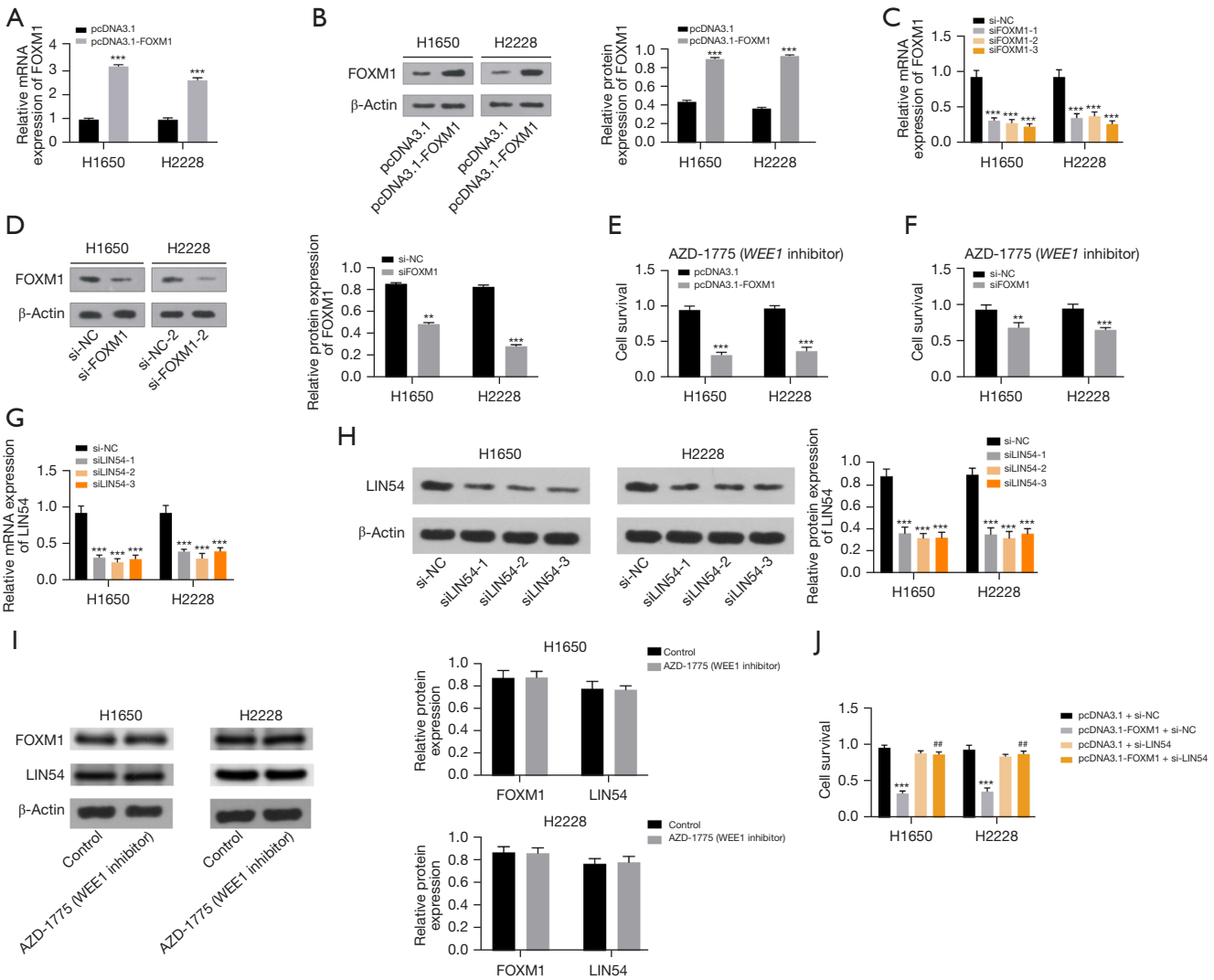


**Figure 1** The *FOXM1*-MYB-MuvB complex (MMB) was found to be necessary for Wee1-like protein kinase (*WEE1*) inhibitor sensitivity. (A,B) The mRNA and protein expression levels of *FOXM1* were measured after overexpressing *FOXM1* through reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blotting. (C,D) The mRNA and protein expression levels of *FOXM1* were measured after inhibiting *FOXM1* through RT-qPCR and western blotting. (E,F) Cell survival was measured after AZD-1775 (*WEE1* inhibitor) treatment and *FOXM1* overexpression (or inhibition) through Cell Counting Kit-8 (CCK-8) assay. (G) The mRNA expression of *LIN54* was detected after silencing *LIN54*, as determined through RT-qPCR. (H) The protein expression of LIN54 was detected after silencing LIN54 was examined through western blot. (I)The protein expressions of FOXM1 and LIN54 were measured through western blot. (J) Cell survival was evaluated after overexpressing *FOXM1* and silencing MMB through a CCK-8 assay. **, P<0.01; ***, P<0.001; ##, P<0.01 *vs.* pcDNA3.1-FOXM1+si-NC.

was decreased by silencing *LIN54* (*Figure 1G-1H*). The protein expression of FOXM1 and LIN54 were not changed after AZD-1775 treatment (*Figure 1I*). Furthermore, after AZD-1775 treatment, the decrease in cell survival mediated by *FOXM1* overexpression could be reversed by *LIN54* knockdown (*Figure 1J*). In addition, the cell survival in the control group did not differ obviously from that in the pcDNA3.1-FOXM1+siLIN54 group, indicating that overexpression of *FOXM1* increased *WEE1* inhibitor sensitivity in a manner dependent on the complex formed with MMB. Taken together, these findings indicate that the *FOXM1*-MMB complex is necessary for *WEE1* inhibitor

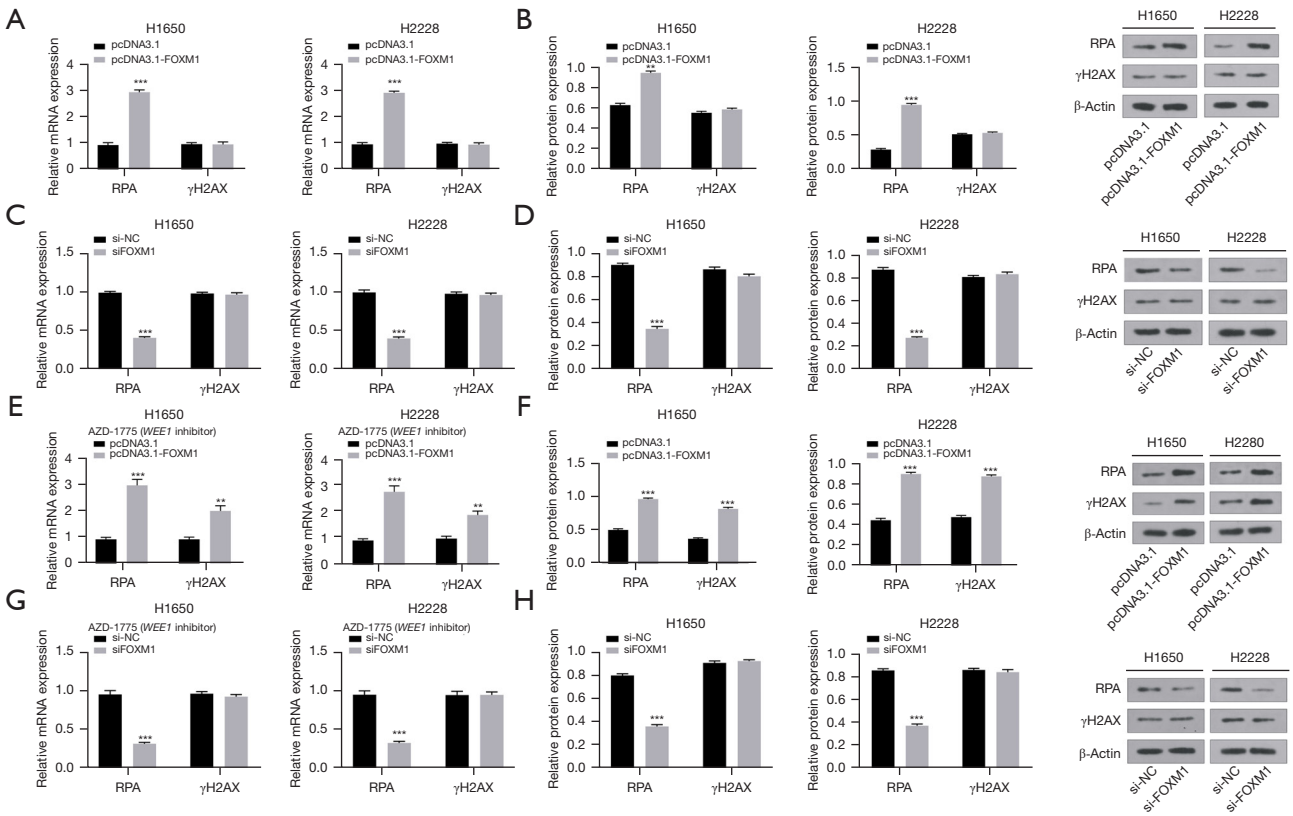


**Figure 2** *FOXM1* upregulation enhanced DNA replication stress. (A,B) The mRNA and protein expression of replication protein A (*RPA*) and gammaH2AX (*γH2AX*) were measured after upregulating *FOXM1* through reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blotting. (C,D) The mRNA and protein expression of *RPA* and *γH2AX* were measured after silencing *FOXM1* through RT-qPCR and western blotting. (E,F) The mRNA and protein expression of *RPA* and *γH2AX* were measured after AZD-1775 (*WEE1* inhibitor) treatment and *FOXM1* overexpression through RT-qPCR and western blotting. (G,H) The mRNA and protein expression of *RPA* and *γH2AX* were measured after AZD-1775 (*WEE1* inhibitor) treatment and *FOXM1* overexpression through RT-qPCR and western blotting. **, P<0.01; ***, P<0.001.

sensitivity.

### *FOXM1 upregulation enhanced DNA replication stress*

Next, experiments were performed to confirm the role of *FOXM1* in DNA replication stress and DNA damage. Replication protein A (*RPA*) is a marker of replicative stress, and *γH2AX* is a marker of DNA double-strand breaks. As displayed in *Figure 2A,2B*, the mRNA and protein levels of *RPA* were increased by overexpressing *FOXM1*, but that of *γH2AX* did not obviously change. Moreover, *FOXM1* overexpression enhanced the mRNA and protein expression of *RPA* but did not affect the expression of *γH2AX* (*Figure 2C,2D*). We found that the mRNA and protein expression levels of *RPA* and *γH2AX* were increased after AZD-1775 treatment and *FOXM1* overexpression (*Figure 2E,2F*). In addition, the mRNA and protein expression of *RPA* was downregulated and that of *γH2AX* was not changed after AZD-1775 treatment and *FOXM1* knockdown (*Figure 2G,2H*). In summary, *FOXM1* upregulation enhanced DNA replication stress.

### *The FOXM1-MMB complex activated G2/M checkpoints*

*CCNB* and *CDK1* form a complex to regulate the G2/M checkpoint. *FOXM1* can enhance *CCNB* expression and increase the threshold content of the *CCNB-CDK1* complex, facilitating the opening of the G2/M checkpoint. The *CCNB* mRNA and protein expression levels were increased after upregulating *FOXM1* (*Figure 3A,3B*). Additionally,

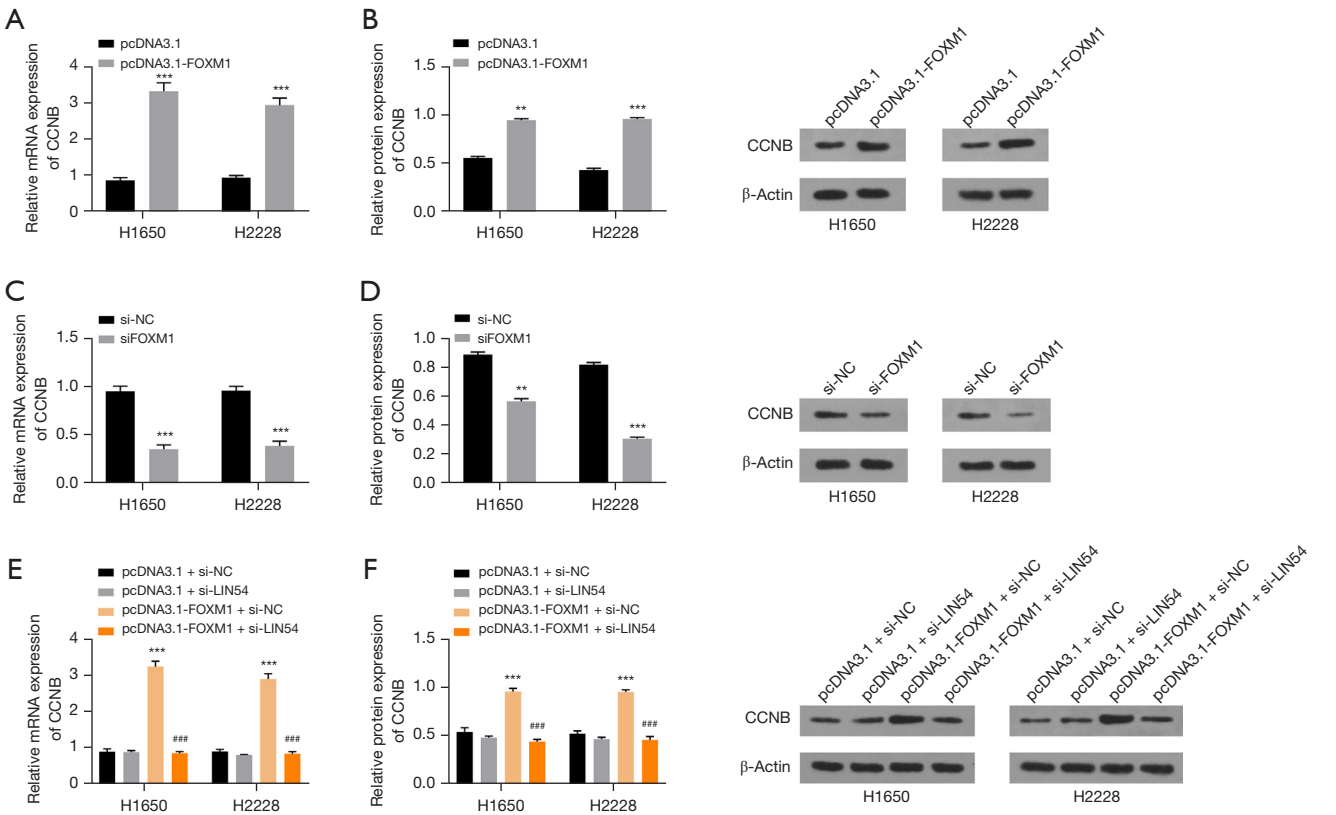


**Figure 3** The *FOXM1*-MYB-MuvB complex (MMB) activated G2/M checkpoints. (A,B) The mRNA and protein expression levels of Cyclin B (*CCNB*) were measured after overexpressing *FOXM1* through reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blotting. (C,D) The mRNA and protein expression levels of *CCNB* were measured after suppressing *FOXM1* through RT-qPCR and western blotting. (E,F) The mRNA and protein expression levels of *CCNB* were measured in the pcDNA3.1+si-NC, pcDNA3.1-FOXM1+si-NC, pcDNA3.1+siLIN54 and pcDNA3.1-FOXM1+siLIN54 groups through RT-qPCR and western blotting. **, P<0.01; ***, P<0.001; ###, P<0.001 *vs.* pcDNA3.1-FOXM1+si-NC.

the *CCNB* mRNA and protein expression levels were decreased after silencing *FOXM1* (*Figure 3C,3D*). This finding suggested that overexpression of *FOXM1* enhances *CCNB* expression and increases the threshold content of the *CCNB-CDK1* complex. Finally, it was demonstrated that the increased mRNA and protein expression of *CCNB* mediated by *FOXM1* could be rescued by silencing *LIN54* (*Figure 3E,3F*). Furthermore, *CCNB* expression in the pcDNA3.1+si-NC group showed no obvious difference from that in the pcDNA3.1-FOXM1+siLIN54 group, indicating that *FOXM1* and MMB form a complex to regulate the *CCNB* expression level. These findings indicated that the *FOXM1*-MMB complex activated G2/M checkpoints.

## Discussion

The regulatory mechanisms of cell cycle checkpoints and DNA repair have been the focus of many studies (30,31). However, clinical translation of the findings has been unsatisfactory. A critical challenge is the lack of validated predictive biotargets. These biotargets can easily identify tumors that may respond to DNA repair pathway inhibitors, particularly inhibitors of DNA replication stress (32,33).

The G2/M cell cycle checkpoint plays a key role in the cell cycle to guarantee that DNA repair is finished before entry into mitosis (34). Cyclin dependent kinase 1 (*CDK1*) is a crucial regulatory factor of the G2/M transition, and its phosphorylation controls whether cells enter mitosis (35).

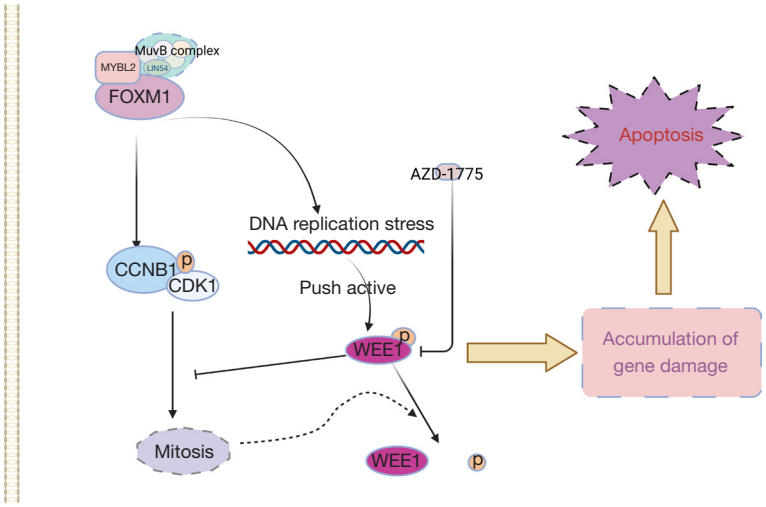


**Figure 4** Diagram of the mechanism of action. The regulatory mechanism of the *FOXM1*/MYB-MuvB complex (MMB) axis in non-small cell lung cancer (NSCLC) progression.

*WEE1* serves as a negative modulator of *CDK1*, and *WEE1* suppression eliminates inhibitory phosphorylation of *CDK1* (36). When cells enter mitosis, the continuous accumulation of damaged DNA induces apoptosis, leading to tumor cell death (37,38).

One inhibitor, AZD1775, targets *WEE1* kinase, which is an important regulator of the G2/M checkpoint (39,40). AZD1775 negatively modulates mitosis by inactivating *CDK1*, inducing G2/M arrest and permitting DNA repair (41,42). Thus, cells with DNA replication stress are susceptible to *WEE1* kinase inhibition (43). In view of the complex modulation of cell cycle checkpoints, it is necessary to identify alternative pathways and biomarkers that can help predict the response to *WEE1* kinase inhibition.

*Cyclin E* upregulation in triple-negative breast cancer enhances sensitivity to *WEE1* inhibitors (44). Is there any association between *FOXM1* expression and *WEE1* inhibitor sensitivity in NSCLC? The MMB complex is formed by MYBL2 and MuvB, while MuvB is composed of LIN54, LIN9, LIN37, LIN52, and RBBP4 (16,45). LIN54 plays a crucial role in the formation of the MMB complex, and silencing of LIN54 can reduce the effect of MMB. Our work demonstrated that after AZD-1775 treatment, the decrease in cell survival mediated by *FOXM1* overexpression could be reversed by *LIN54* knockdown and that cell survival in the control group did not differ obviously from that in the pcDNA3.1-FOXM1+siLIN54 group, indicating that the *FOXM1*-MMB complex was necessary for *WEE1* inhibitor sensitivity. Moreover, the mRNA and protein expression of replicative stress marker-*RPA* and DNA double-strand break marker-*γH2AX* were increased after AZD-1775 treatment and *FOXM1* overexpression, suggesting that *FOXM1* upregulation enhanced DNA replication stress and DNA damage. Finally, we found that the increased mRNA and protein expression of *CCNB* mediated by *FOXM1* could be rescued by silencing *LIN54* and that *CCNB* expression in the control group did not differ obviously from that in the pcDNA3.1-FOXM1+siLIN54 group. These findings revealed that the *FOXM1*-MMB complex activated G2/M checkpoints.

As shown in *Figure 4*, *FOXM1* overexpression increased DNA replication stress, which increased DNA replication and pressure on the *WEE1* checkpoint. On the other hand, *FOXM1* can enhance *CCNB* expression, increase the threshold content of the *CCNB*/*CDK1* complex, facilitate mitosis, and promote *WEE1* dephosphorylation. Under these two effects, sensitivity to the *WEE1* inhibitor AZD-1775 is increased, which leads to the accumulation of DNA damage and drives the activation of apoptosis.

## Conclusions

In this study, we verified that overexpression of *FOXM1* induces DNA replication stress and stimulates DNA repair responses, thereby sensitizing cells to AZD1775. Thus, this work revealed that the complex formed by overexpressed *FOXM1* and MMB might be used as a potential therapeutic biomarker for NSCLC, making NSCLC treatment

decisions more accurate.

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## Footnote

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-750/rc

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*Peer Review File:* Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-750/prf

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.



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