



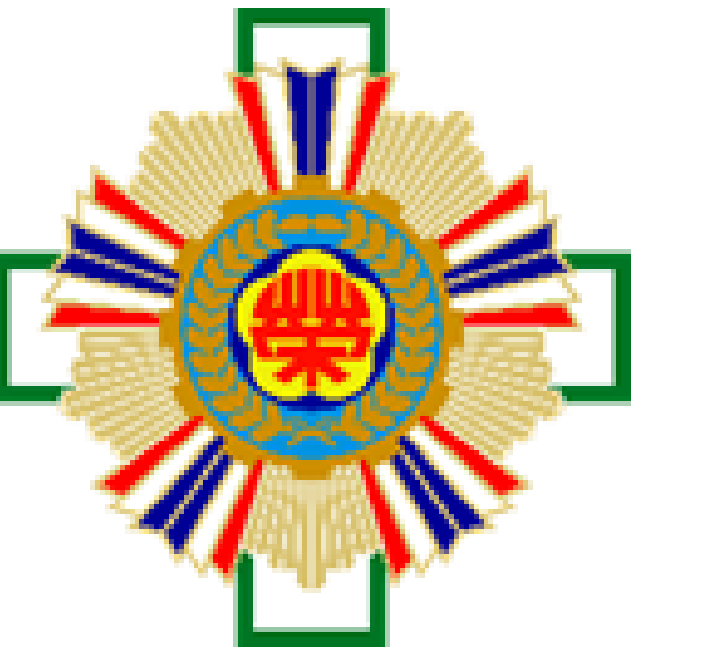
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# Discovery of mechanisms that modulate the hippo pathway and CDK6 expression in CDK4/6 inhibitor resistant breast cancer cells

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## Introduction

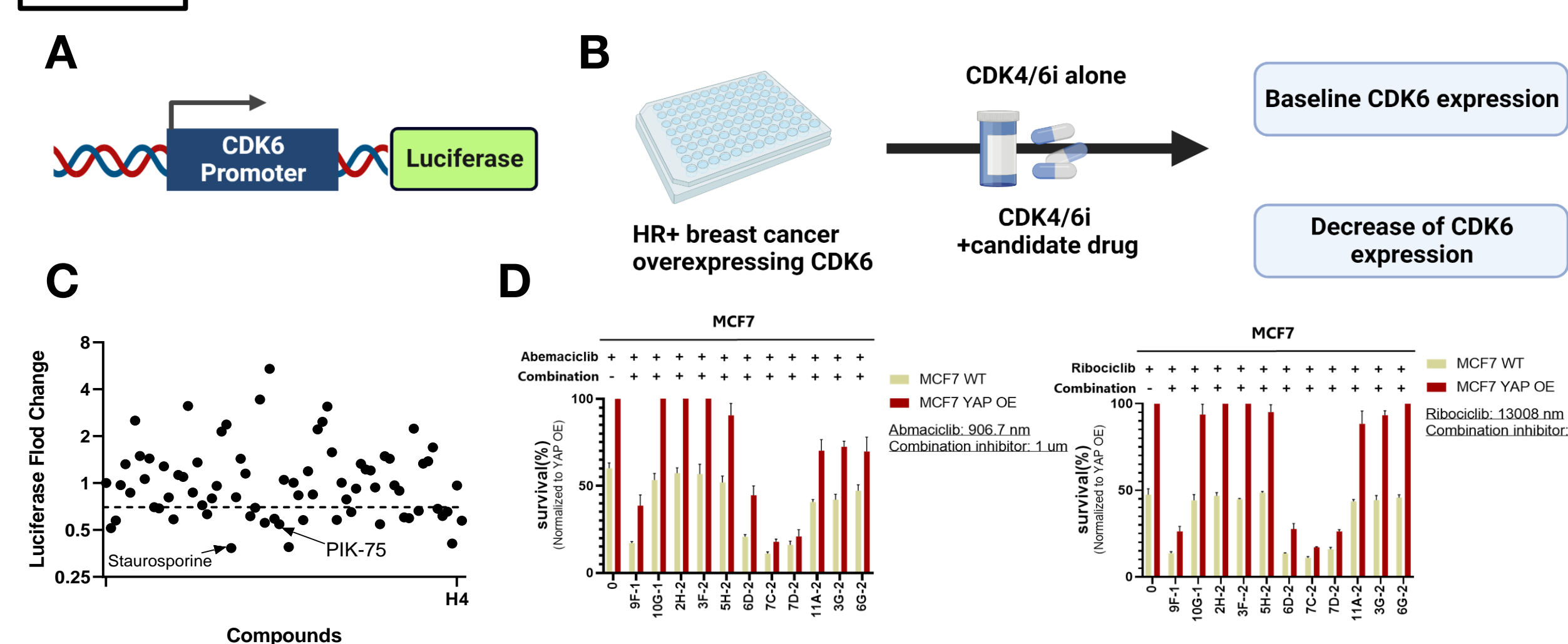
Hormone positive (HR+) is the most common subtype of breast cancer. The current standard of care in advanced HR+ breast cancer includes endocrine therapy (tamoxifen, aromatase inhibitors, etc) and CDK4/6 inhibitors (CDK4/6i). CDK4/6i are now the generally accepted choice of drug; however, most patients ultimately develop resistance. Many mechanisms have been proposed for CDK4/6i resistance, including upregulation of CDK4, CDK6, Cyclin E, and others (1). CDK6 upregulation results in CDK4/6i resistance, and has been shown to be regulated by activation of the Hippo-YAP/TAZ pathway (2-3). In cancer cells with CDK6 upregulation, suppression of CDK6 has been shown to reverse the CDK4/6i resistance (4). We screened a small molecule kinase inhibitor library for inhibitors that downregulate CDK6 expression. Several candidates emerged from the screen, including the PI3K inhibitor PIK-75. We confirmed that PIK-75 downregulated CDK6 expression in HR(+) breast cancer cells and reversed CDK4/6i resistance. We further investigated the underlying mechanisms of PIK-75 induced CDK6 suppression. We discovered that PIK-75 inhibits CDK7, resulting in inhibition of the RNA polymerase II (RNAPII) mediated transcription initiation of YAP, and decreased binding of the YAP promoter by the RNAPII C-terminal domain, leading to CDK6 downregulation. CDK7 inhibition by small hairpin RNA phenocopied the transcriptional effects of PIK-75. In summary, we discovered a transcriptional regulation role of CDK7 in CDK6 overexpression. Our findings implicate a potential role for CDK7 inhibitors in CDK6 amplified cancers.

## Methods

The hormone positive breast cancer cell lines (obtained from ATCC) MCF-7 and T47D are used in this study. Western blotting, real-time PCR, luciferase, ChIP, and cell culture experiments were performed according to standard biology protocols. BRET (bioluminescence resonance energy transfer) was performed according to the protocols of the vendor (Promega). Small molecule inhibitor screening was performed with the Cayman kinase inhibitor library (cat no. 10505). shRNAs were purchased from Academia Sinica in Taiwan.

## Results

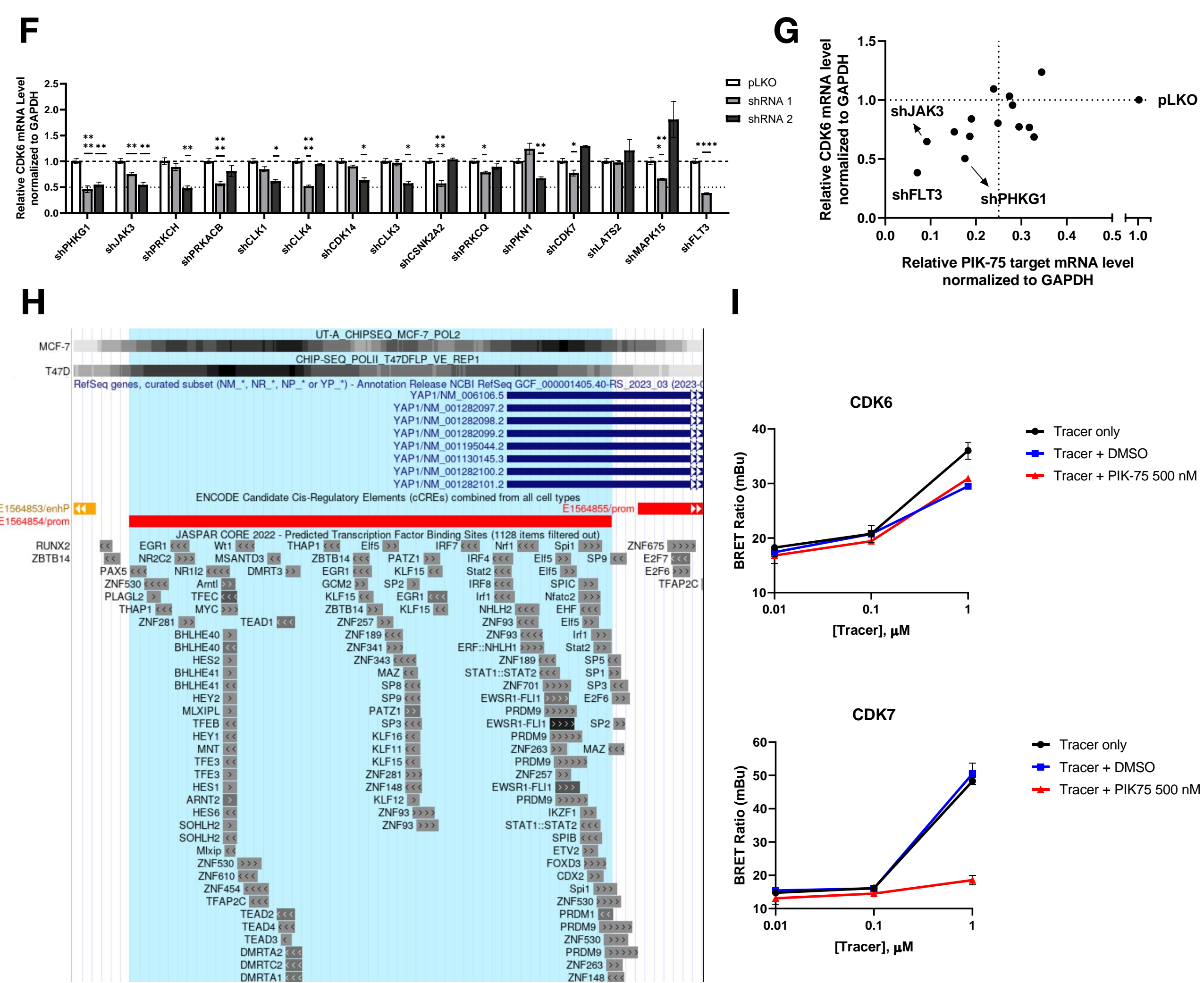
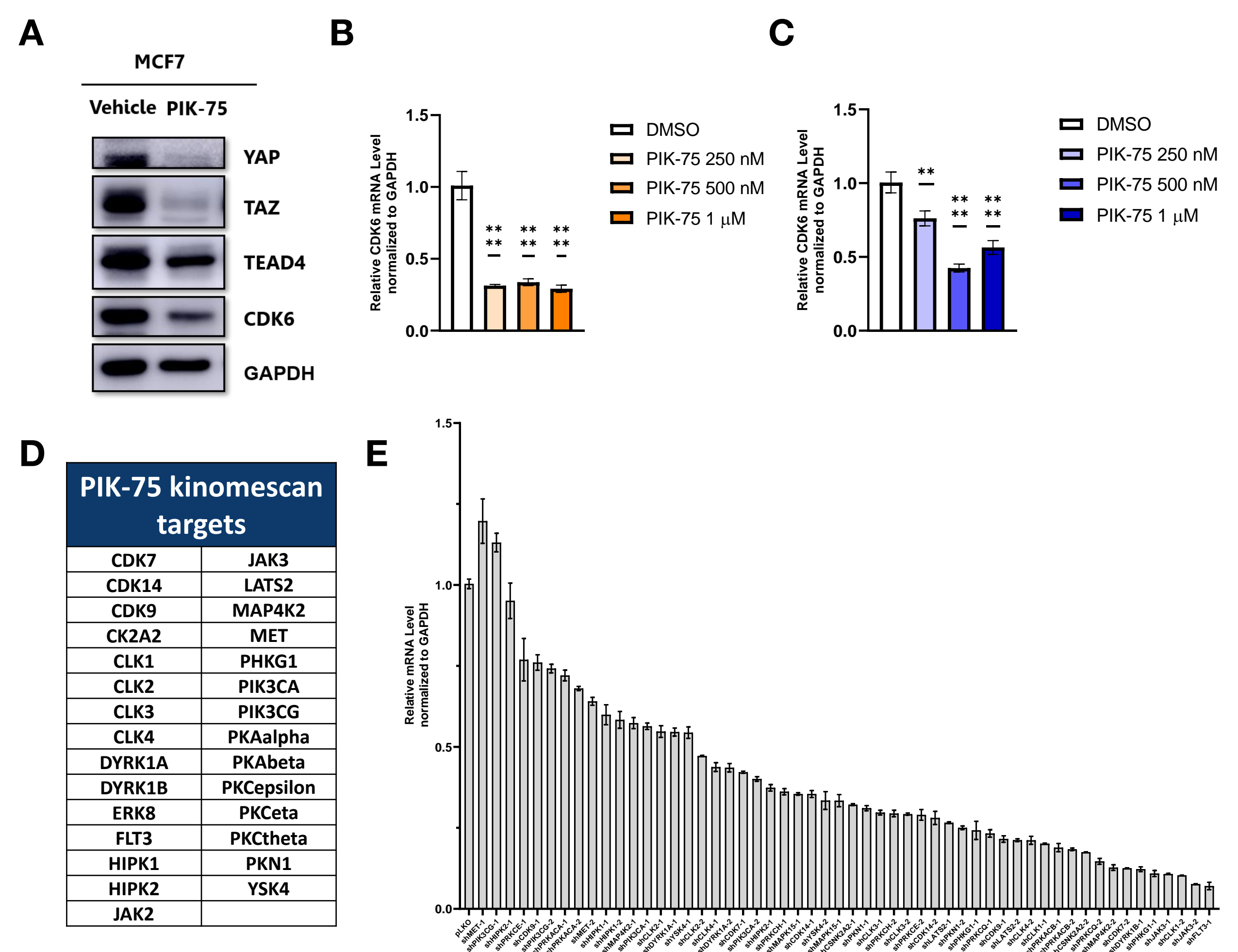
**Fig 1**



**Fig 1 Small molecule screen identifies candidates that decrease CDK6 expression** (A) A luciferase reporter screening platform by fusing the CDK6 promoter to the pMIR-report luciferase plasmid (Promega). This allowed a high throughput screening for agents that modulated CDK6 expression.

(B) Rationale for screening (C) We used a kinase inhibitor library of 160 kinase inhibitors to select for kinase inhibitors that decreased CDK6 expression. A portion of the screening result is shown here (screen repeated three times). (D) Validation of screening results. The PKC inhibitor staurosporine and PIK-75 decreased CDK6 expression (data not shown) and reversed CDK4/6i resistance (i.e. MCF7-YAP overexpression was resistant towards ribociclib or abemaciclib, but was sensitive with the combination of CDK4/6i + staurosporine or PIK-75)

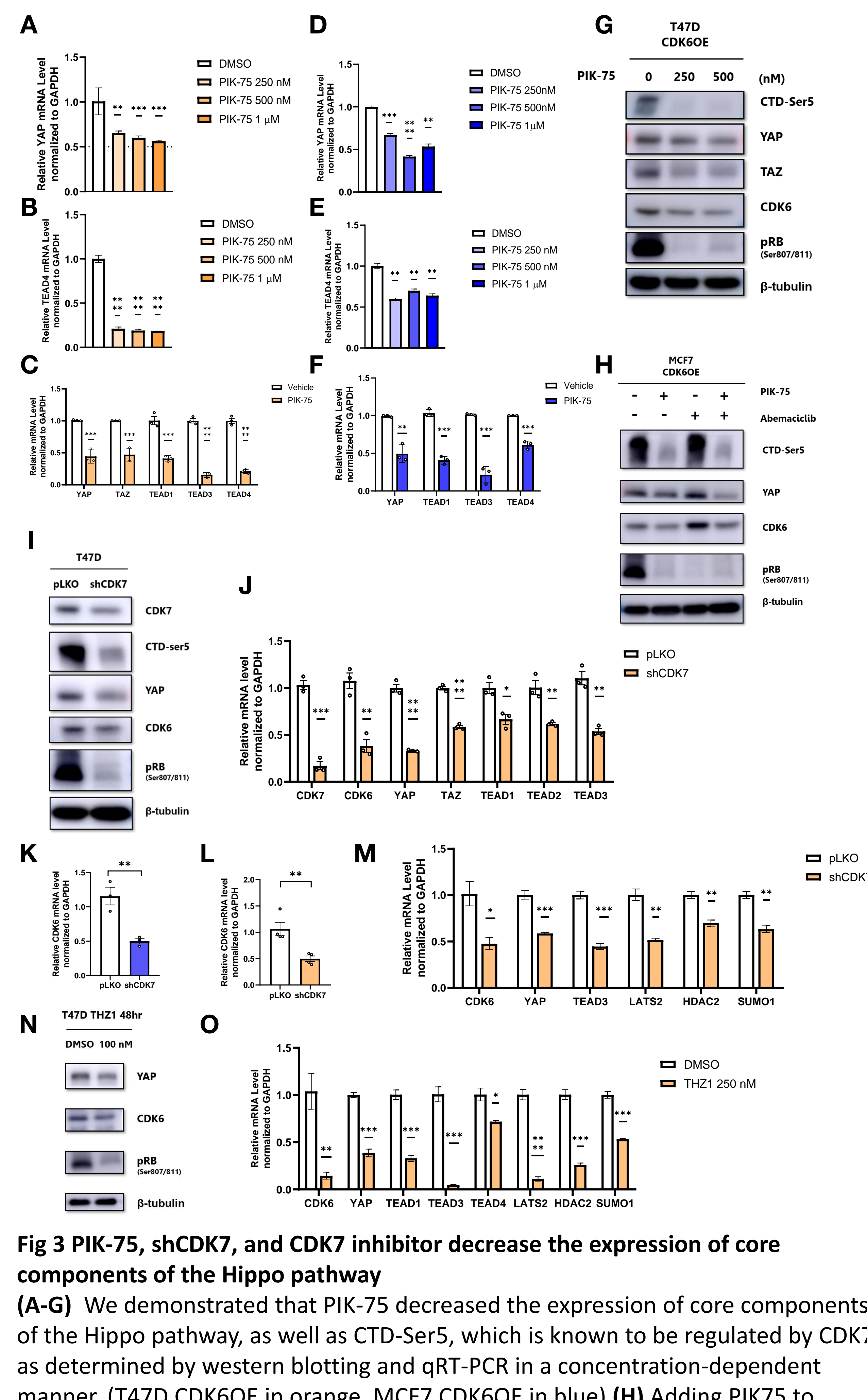
**Fig 2**



**Fig 2 PIK-75 downregulates CDK6 may be mediated by CDK7** (A-C) PIK-75 decreased CDK6 by western blotting and in a concentration-dependent manner by qRT-PCR. (T47D CDK6OE in orange, MCF7 CDK6OE in blue)

(D) The KINOMEScan analysis of PIK-75 revealed the involvement of 29 targets (data retrieved from Walczak, Cell Death & Differentiation 2014, PMID: 24362439)(5). (E-G) shRNA screen by individual silencing of PIK-75 targets for candidates that downregulate CDK6 (H) Query of the UCSC genome browser analysis revealed no PIK-75 KINOMEScan targets on the YAP promoter, but RNAPII, a target of CDK7, was observed. (I) BRET (bioluminescence resonance energy transfer) assay to verify the binding affinity of PIK-75 to CDK7, but not CDK6.

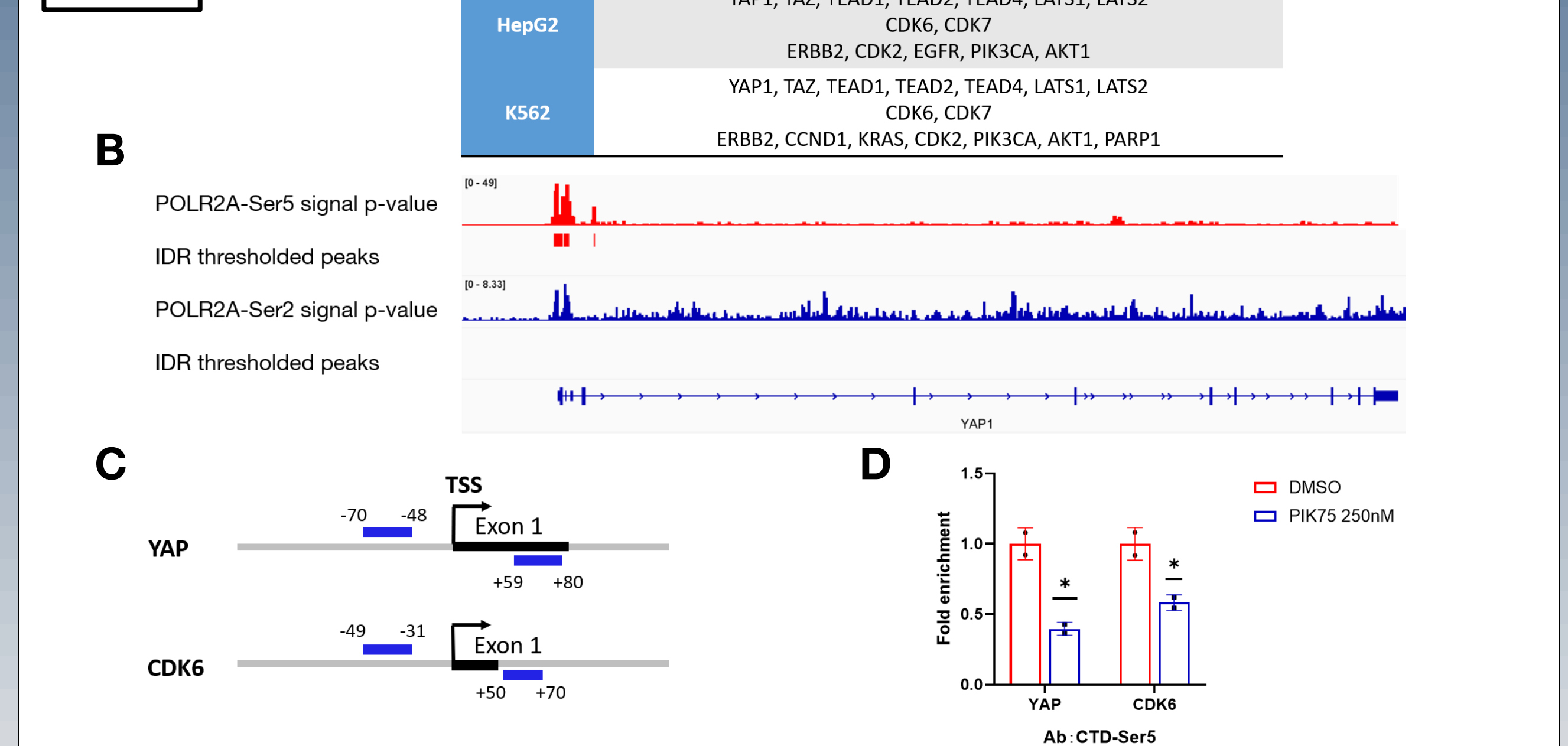
**Fig 3**



**Fig 3 PIK-75, shCDK7, and CDK7 inhibitor decrease the expression of core components of the Hippo pathway** (A-G) We demonstrated that PIK-75 decreased the expression of core components of the Hippo pathway, as well as CTD-Ser5, which is known to be regulated by CDK7, as determined by western blotting and qRT-PCR in a concentration-dependent manner. (T47D CDK6OE in orange, MCF7 CDK6OE in blue) (H) Adding PIK75 to CDK4/6 inhibitor in CDK6 overexpression cells decreased YAP, CDK6, and CTD-Ser5, which is known to be regulated by CDK7. (I-J) To further confirm the mechanism of CDK7 regulates CDK6 and YAP, we showed that shCDK7 decreased CDK6 and other core components of the Hippo pathway by western blotting and qRT-PCR.

(K-M) We used different breast cancer cell lines and performed transient transfection of shCDK7, which also showed a decrease in CDK6 expression and other core components of the Hippo pathway. (N-O) THZ1, a CDK7 inhibitor, also showed a decrease in CDK6 expression and other core components of the Hippo pathway in western blotting and qRT-PCR.

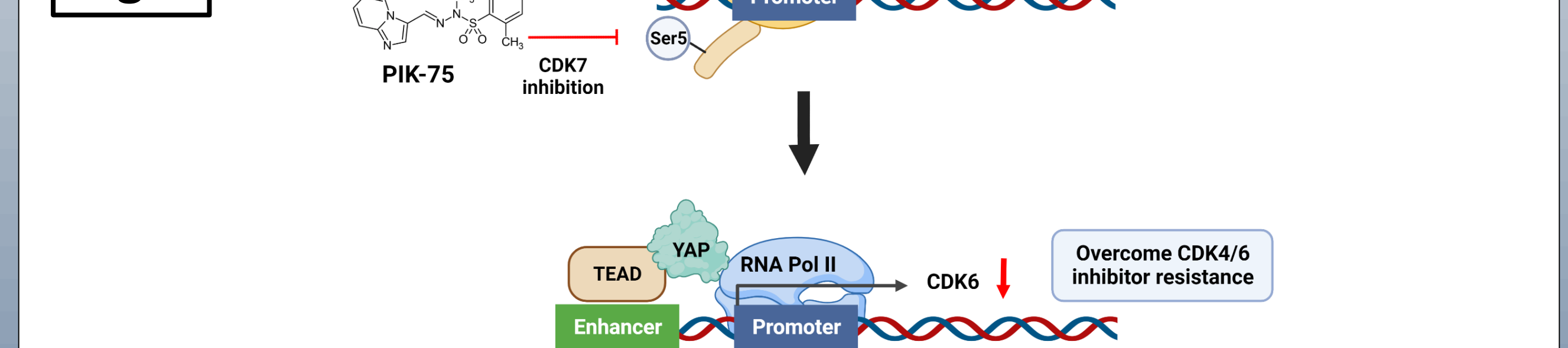
**Fig 4**



**Fig 4 CTD-Ser5 is a key factor that regulates the YAP and CDK6 promoters, rather than CTD-Ser2**

(A) To further validate the role of CTD-Ser5, we collected ChIP-seq data from the ENCODE database regarding POLR2A CTD-Ser5 and CTD-Ser2. The data suggested that CDK6 and core components of the Hippo pathway were primarily regulated by CTD-Ser5 rather than CTD-Ser2. (B) The genomics viewer IGV revealed significant peaks of POLR2A CTD-Ser5 ChIP-seq in the YAP promoter region of HepG2 (ENCSR000BPI), but not in CTD-Ser2 (ENCSR000EDX). (C-D) Chromatin immunoprecipitation (ChIP) revealed PIK-75 affected CTD-Ser5 binding in the YAP and CDK6 promoters.

**Fig 5**



**Fig 5 Proposed Mechanism of Action for PIK-75 overcome CDK4/6 inhibitor resistance**

PIK-75 has the potential to effectively inhibit CDK7 kinase activity, which leads to the prevention of phosphorylation of POLR2A CTD-Ser5, resulting in transcriptional suppression of both YAP and CDK6. Ultimately, this molecular intervention may represent a promising therapeutic strategy for overcoming resistance to CDK4/6 inhibitors.

## Conclusions

CDK7 inhibitors such as PIK-75 and THZ1 decrease CDK6 by regulation of the RNAPII CTD phosphorylation, transcriptional repression of the Hippo pathway protein YAP and resulting in CDK6 downregulation. This novel pathway represents a novel therapeutic opportunity for therapeutic development for candidates that can potentially reverse CDK4/6i resistance.

**References**  
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