**Title: The R2TP protein complex in human papillomavirus-mediated cervical cancer**

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## Introduction:

Unravelling the interactions and functional dynamics of the proteome is crucial to understanding the biology of cancer. Protein homeostasis and stabilization are essential under normal and stressed conditions. One crucial co chaperone to the Hsp90 chaperone called the PAQosome/the R2TP complex is responsible for bridging proteins and molecular complexes for various cellular processes beyond the transcriptional and translational machinery**.** The PAQosome stands for Particle for Arrangement of Quaternary Structure. (1) It consists of four core proteins - RUVBL1, RUVBL2, PIH1D1 and RPAP3. In humans, R2TP associates with RNA polymerase subunit RPB5, WD40 repeat protein WDR92, and the Unconventional Prefoldin Complex (UPC), comprising of URI1, UXT, PDRG1, PFDN2, PFDN6, and ASDURF. (2) (3) (4) The R2TP/Prefoldin-like complex plays a crucial role in facilitating the assembly and stabilization of various important complexes, including small nucleolar ribonucleoproteins (snoRNPs), RNA polymerase, or PIKK complexes which are related to protein synthesis, cell growth and metabolism, as well as gene expression and genome stability. (5) (6)

RUVBL1 and RUVBL2 are highly conserved AAA+ ATPases (ATPases associated with diverse cellular activities) whose domains senses, binds and hydrolyses ATP. (7) PIH1D1 recognises and bridges proteins to the R2TP-Hsp90 complex. Recent data also shows that the RPAP3 subunit plays the central organizing role by providing a scaffold for the interaction of PIH1D1 with the RUVBL1–RUVBL2 ring and a flexible tether for HSP90, allowing it to interact with a highly diverse set of client proteins and complexes (8)

In this study, we chose to address the role of R2TP in cervical cancer. Cervical cancer is the most common gynaecologic cancer and the leading cause of cancer-related deaths in women worldwide. Approximately ninety five percent of cases are caused by persistent high-risk human papillomavirus (HPV) infection. (9) (10)The association of the major oncoproteins E6 and E7 with critical tumour suppressor pathways of pRb (retinoblastoma) and p53 in the disruption of the cell cycle has been well illustrated yet the contributions of the host and viral oncoproteins together in inducing malignancy is still obscured.

In this work, we studied the expression of R2TP in cervical cancer. We identified PIH1D1 as a novel target of HPV E7, demonstrating in vitro interactions between PIH1D1 and HPV E7 using glutathione S-transferase (GST) pull-down assays, co-immunoprecipitations followed by Western blot analyses. PIH1D1 being a central component of the PAQosome, we uncovered its contribution to the malignancy of cervical cancer cells.

## Objectives:

1. To analyse the immunohistochemical expression of the components of the R2TP components in cervical cancer

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2. To determine the biochemical connection between human papillomavirus oncoprotein E7 and the R2TP complex
3. To study the role of PIH1D1 in Ca Ski cells (HPV16+) by RNAi

## Material and Methods:

### Plasmids and Cell lines

PIH1D1 (NM_017916) Human Tagged ORF Clone (RC200158) was purchased from OriGene Technologies, Inc. Ca Ski and HeLa cell lines were purchased from NCCS, Pune. The cells were maintained in RPMI and DMEM respectively, with 10% Fetal Bovine Serum and 1X penicillin-streptomycin in 5% $CO_2$.

### Cloning of PIH1D1

Specific primers for PIH1D1 were designed to clone into recipient PGEX-2T vector. The digested fragments (amplified PIH1D1 and recipient vector) were ligated with T4 DNA Ligase (5 U/μL), transformed with competent E. coli BL21(DE3) cells and plated on antibiotic-selection LB agar plates. After purifying the plasmid, successful ligations were screened by restriction digestion and 1% AGE.

### Expression and purification of proteins

A single colony was resuspended in a 10 ml LB growth medium with antibiotics and grown overnight. The following day, 1mL of the overnight culture was added to 100 millilitres of LB medium. This culture was incubated at 200 rpm at 37°C until OD600 reached 0.4–0.8. It was then induced with 1M stock of IPTG to a final concentration of 1mM and induced for 3-5 hours at 37°C. The bacteria were recovered by centrifugation at 4°C, 4000rpm. Cell pellet was resuspended in 10 ml lysis buffer (10mM Tris-HCl, 0.1mM EDTA and 150mM NaCl), sonicated briefly after the addition of 10% Sarkosyl and centrifuged at 4000 rpm at 4°C. To the clarified lysate, 10% Triton X-100 was supplemented and then incubated at 4°C for 2 hours with glutathione-Sepharose 4B (GbioScience). After centrifugation, the beads were washed in 1X PBS (Phosphate Buffer Saline, pH7.6) buffer. 5 microlitres of the eluates were quantified with varying concentrations of BSA protein on a 10% SDS-PAGE. Each step of the purification process was also analysed on an SDS-PAGE and stained with Coomassie Brilliant Blue.

### GST pull-down assay

Briefly, 200ng-1ug of purified GST fusion proteins were incubated with 1 ug of crude lysate of HEK293/Ca Ski/T98G cells, rotated overnight at 4°C. Purified GST protein was used as a control.

### Coimmunoprecipitation

Briefly, cell monolayers were lysed in 1% CHAPS buffer in the presence of protease and phosphatase inhibitors. Crude cell lysates were immunoprecipitated for overnight at 4°C with 1ug of IgG Isotype control and respective antibodies. The antigen-antibody complex was immunoprecipitated with Protein A/G PLUS-Agarose (Santacruz, sc-2003) for 4 hours at 4°C.

### siRNA transfection

$1x10^5$ cells of Ca Ski are seeded in 6-well plate. Cells were then serum starved and transfected with NOP17 siRNA(h) (Santa Cruz, sc-97385) using Lipofectamine RNAiMax reagent (Thermo Fisher,13778030) according to the manufacturer's instructions. Analyses of protein levels were carried out 24-72 hours after transfection. AllStars Negative Control siRNA (5 nmol) (Qiagen, 1027280) was used as control.

**Western Blot**

Total protein was separated by SDS-PAGE (8-12%) at 100 V for 1 h 45 min in SDS running buffer. The separated proteins were transferred onto PVDF membranes (0.45-µm pore size; Merck Millipore) at 300 MA for 1 hour in ice-cold 20% (vol/vol) methanol transfer buffer. The membranes were blocked in 5% non-fat milk powder (Himedia) in TBS-T buffer. Specific proteins were detected with primary antibodies by incubation with membranes overnight at 4°C and with secondary antibodies for 1 h at room temperature. Proteins were visualized using ECL Western blotting detection reagent (GBioscience).

**Wound Healing Assay**

Cells were seeded into 6-well plates. 48 hours after transfection, the monolayer was uniformly wounded using a 200-µL pipette tip. Cells were then incubated in a serum-free medium for 24 h, and images were taken from 5 random fields of view using a microscope. The wound closure percentage was evaluated by comparing the changes in the wound area before and after 8 h.

**Propidium Iodide staining and flow cytometry**

24 to 72 hours after transfection, Ca Ski cells were fixed with 70% ethanol. After washing, cells were incubated with DNA extraction buffer (0.1%Triton X-100 IN 1X PBS) and centrifuged at 400x g. Cells were then incubated with DNA Staining solution (200ug/ml PI, SRL and 200ug/ml RNAse, Thermofisher). Cells were analysed with a flow cytometer.

**Immunofluorescence**

Cells were seeded in a density of 1 x $10^4$ cells/well on clean coated coverslips in 6-well plates. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100-PBS. Blocking was done with 1% BSA (protease free). After washes, cells were incubated with primary antibody 1:200 dilution at 4 degrees overnight. The following day, the cells were washed to remove unbound antibodies and fluorescent dye-labelled secondary antibodies were added and incubated in the dark. Nuclei were counterstained with DAPI Solution (1 mg/mL) Thermo Scientific™. Images were acquired with a confocal microscope and analysed with ImageJ.

**Immunohistochemistry**

Immunohistochemistry was done on 5-µm-thick sections from the corresponding formalin-fixed, paraffin-embedded tissue samples which were collected on poly-L-lysine-coated slides. The sections were deparaffinized in xylene and rehydrated in graded series of alcohol. Microwave-mediated antigen retrieval was done using low-pH and high-pH buffers (800 W, 15 min; 480 W, 5 min) as per manufacturer's instruction for the antibodies. The primary anti-bodies anti-RUVBL1, anti-PIH1D1 and anti-RPAP3 incubation was standardized to overnight. The secondary detection was performed using CRF TM Anti-Polyvalent HRP Polymer (DAB) LabPack (Ref. CPP050; Scytech Laboratories) according to the manufacturer protocol. Each

batch was run with an appropriate positive and negative control. Immunohistochemical scoring was done using semiquantitative H-score (stained intensity and positive cell percentage). Staining intensity was graded as 0: no staining, 1: weak staining, 2: moderate staining, and 3: intense staining.

**Antibodies**

For confocal microscopy, proteins were detected using goat anti-rabbit IgG conjugated with TRITC (GeNei, 1120680011730) and goat anti-mouse IgG conjugated to FITC (GeNei, 1120380011730). For Western Blotting, antibodies used were NOP17 (18Y9) (Santa Cruz, sc-101000), beta Actin (C4) (Santa Cruz, sc-47778), RB (BD 554136), RUVBL1 (Protein Tech, 10210-2-AP), RPAP3 (ThermoFisher, PA5-58334), HPV16 E7 (ED17) (Santa Cruz, sc-6981) and HPV18 E7 (F-7) (Santa Cruz, sc-365035).

## Results

### 1. The R2TP protein complex is strongly expressed in cervical carcinoma

We determined the expressions and localizations of three R2TP complex components- RUVBL1, PIH1D1 and RPAP3 in human adjacent-normal and cancer of the cervix. Immunohistochemical analysis was performed by using antibodies against RUVBL1, RPAP3 and PIH1D1.

Immunopositivity was noted as nuclear-cytoplasmic. For all the 33 cases evaluated, 21 cases (63.63%) were strongly stained, and 12 cases (36.36%) had weak staining for RUVBL1. Strong staining was observed in 19 cervical cases (57.57%) and weak staining was observed in 14 cases (42.42%). For RPAP3, out of all the 33 cases of cervical carcinoma, only one case turned out to be positive. Interestingly, out of 10 cases of adjacent tissues which served as a negative control with respect to the cervical cancer, there was no expression or relatively weak staining of RUVBL1, PIH1D1 and RPAP3.

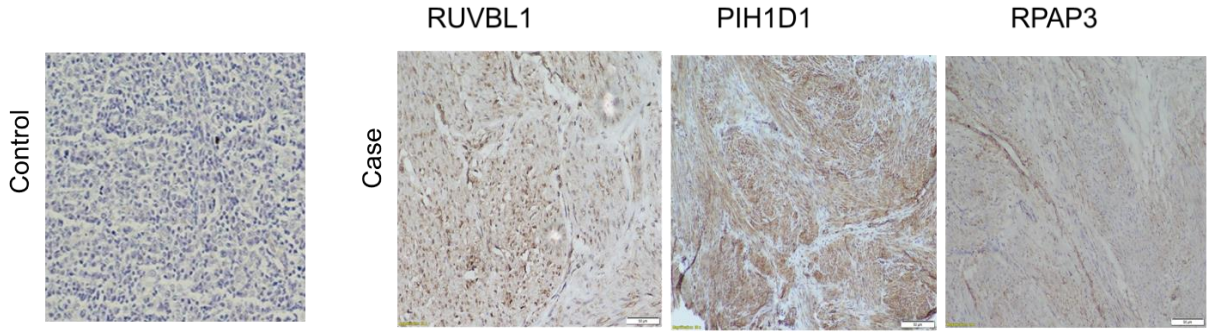



Figure 1: Representative photomicrographs in a control and cervical carcinoma case. RUVBL1, PIH1D1 and RPAP3 shows strong expression patterns in cervical cases but absent in controls. N=30, scale bar=50μm, 10X

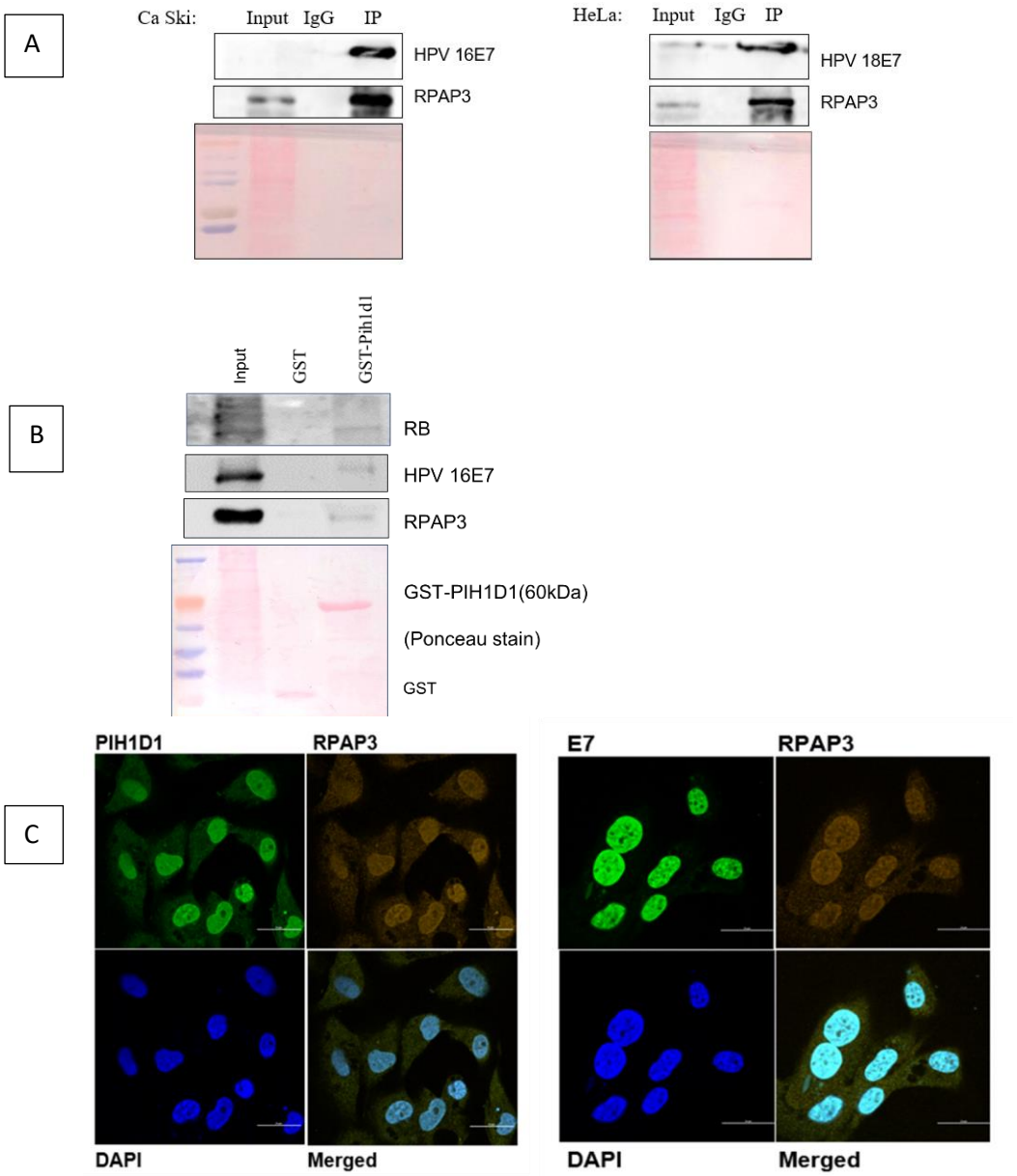
### 2. HPV E7 associates with the R2TP complex

PIH1D1 contains a conserved phospho-peptide binding (Lys57 and Lys64) domain that binds to DpSDD/E motif in bridging proteins, which is phosphorylated by Casein kinase II. (11) This putative site was found present on E7 proteins of HPV. Hence, we performed co immunoprecipitation assay in lysates of Ca Ski (HPV 16+) and HeLa (HPV 18+) by using antibody against PIH1D1 to confirm their physical interaction. Western blotting followed by

co-IP showed PIH1D1, RPAP3 and E7 are associated in Ca Ski and HeLa cells. We also did a GST pull down assay using purified GST-PIH1D1 proteins in Ca Ski cells. Interaction of PIH1D1 with RPAP3 is taken as the positive control.

Thus, the in vitro binding assays confirm the interaction of E7, PIH1D1, RPAP3 and RB proteins and association as a complex in Ca Ski and HeLa cells. We also performed immunofluorescence staining to localize E7, PIH1D1 and RPAP3. PIH1D1 and RPAP3 are present in the nucleus and the cytoplasm. E7 is localized to the nucleus.

Figure 2: E7 binds to PIH1D1 and RPAP3. (A) Western blot analysis of the co-IPed proteins by using an anti-PIH1D1 antibody in Ca Ski and HeLa cell lysate respectively. IgG isotype control was used as a negative control for detecting non-specific protein binding. (B) Western Blot analysis of GST pull-down assay from Ca Ski cells, showing interaction of E7 and PIH1D1. Incubation of GST is a negative control. (C) Immunofluorescence images of PIH1D1 or E7 (FITC-green) and RPAP3 (TRITC-orange) in Ca Ski cells. The nucleus is stained by DAPI (blue). Scale bar=25µm, 60X



### 3. Knockdown of PIH1D1 results in defects in proliferation & cell cycle

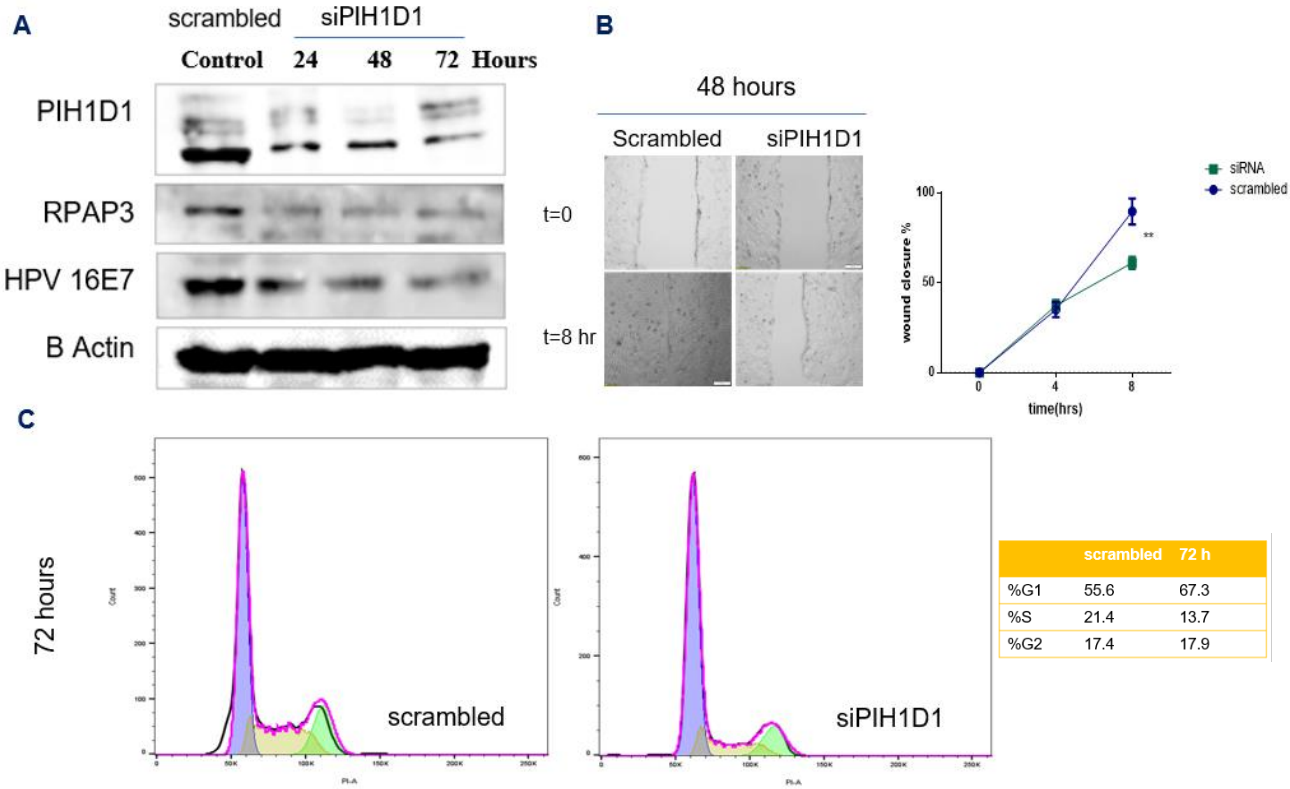



Figure 4: siRNA interference on PIH1D1 in Ca Ski cells A. Western Blot analysis of the siRNA transfected Ca Ski cell lysates in a time-course B. In vitro wound healing assay on siRNA transfected cells at 48 hours. Data were expressed as mean ± S D (n=3). Error bars represent SD. (**P<0.01, scale bar=200µm) C. Cell cycle analysis by PI staining using FlowJo software

We transfected Ca Ski cells with 100nM of siRNA against PIH1D1 for 24 to 72 hours and analysed the protein levels of RPAP3 and E7 by Western Blotting. RPAP3 and E7 protein levels decreased during this time course experiment. At 48 hours after transfection, in vitro wound healing also shows decrease in proliferation of Ca Ski cell as shown in 4(B). The relative residual wound area at 8 h after scratched is shown. Cell cycle analysis by PIH1D1 shows a decrease in S phase and an increase in G0-G1 phase respectively throughout the time course. Shown in 4(C) is a representative image at 72 hours after transfection. Thus, these results show that PIH1D1 contributes to the stabilization of E7 and consequently, the proliferation of Ca Ski cells.

**Statistical analysis**

Paired T test was calculated to analyse in vitro wound healing assay.

**Discussion**

This study identified and characterized R2TP protein complex in cervical carcinoma and revealed that PIH1D1, which is one of the core subunits of R2TP, contributes to the proliferation of Ca Ski cells by stabilizing E7 oncoprotein.

First, we analysed the expression of the R2TP complex – RUVBL1, PIH1D1 and RPAP3 in normal epithelia and cervical carcinoma. We observed that these proteins are strongly expressed in the malignant tissues. These observations suggests that the increased expression

of R2TP is related to the proliferation activities of the cells. The functional analyses of PIH1D1 showed PIH1D1 is required for cell proliferation of Ca Ski cells, suggesting the contribution of PIH1D1 to the malignant phenotype. In this context, the functional contribution of the other subunits-RPAP3, RUVBL1 and RUVBL2 to cancer development should also be studied. Recently, studies on characterizing the R2TP complex in malignancy have attracted the interest of many cancer researchers. (12) (13) (14) (15)

We also found that PIH1D1 interacts with HPV E7 and RB proteins and is involved in the stabilization of HPV E7 protein. The interaction may likely play a role in the various activities of E7 responsible for tumorigenesis, which warrants further investigations to identified as an effective therapeutic strategy targeting tumours infected by human papillomavirus. Clarifying the cellular function of the PAQosome and the molecular dynamics of all its 12 subunits will require the identification of additional clients as well as the identification of pathways, molecules, and post translational modifications regulating PAQosome activity. Further investigations are needed to provide new insight into this biologically important protein complex

**Impact of the research in the advancement of knowledge or benefit to mankind**

Research on cervical cancer has led to the development of potent vaccines, improvements in prevention strategies, enhancing early detection, advancing treatment options, and promoting health equity. Ongoing research and its translation into practice will continue to play a vital role in reducing the burden of cervical cancer on a global scale. Since its discovery in 2005, biochemical and structural studies highlighted a chaperoning role for the R2TP complex. The R2TP complex's multifaceted roles in protein complex assembly, DNA repair, telomerase regulation, signalling pathways and cellular stress response makes it a significant player in cancer biology. Its involvement in fundamental cellular processes presents opportunities for research and therapeutic interventions aimed at combating cancer and improving patient outcomes. Modulating the function of the R2TP complex could have implications for controlling cancer cell growth and survival. Further research into the precise mechanisms by which the R2TP complex contributes to cancer could provide insights into novel significant strategies for combating cervical cancer.

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