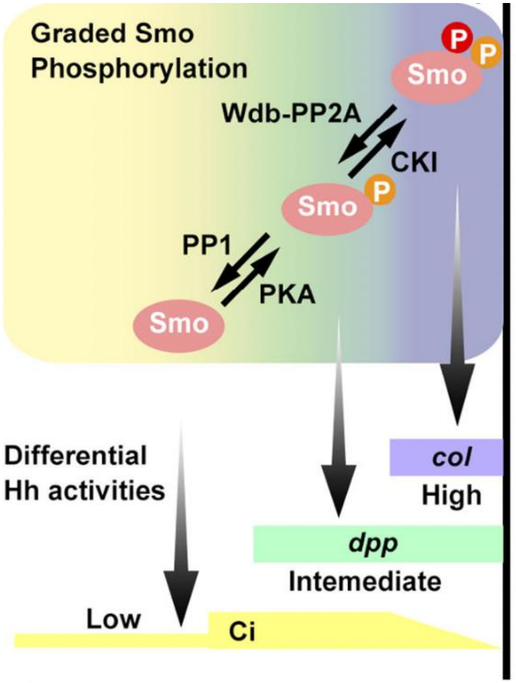
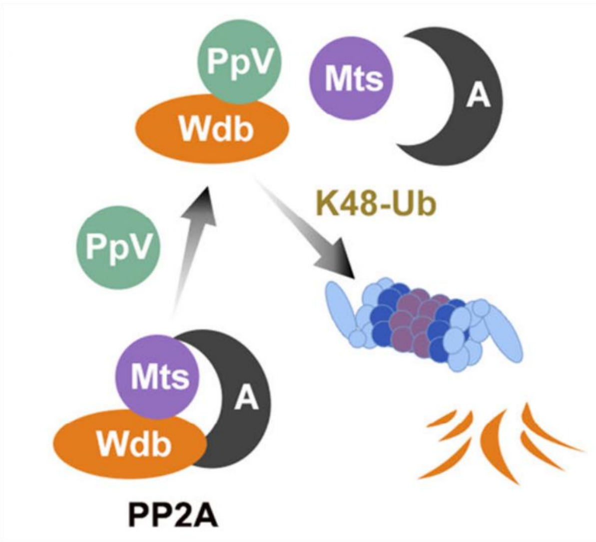
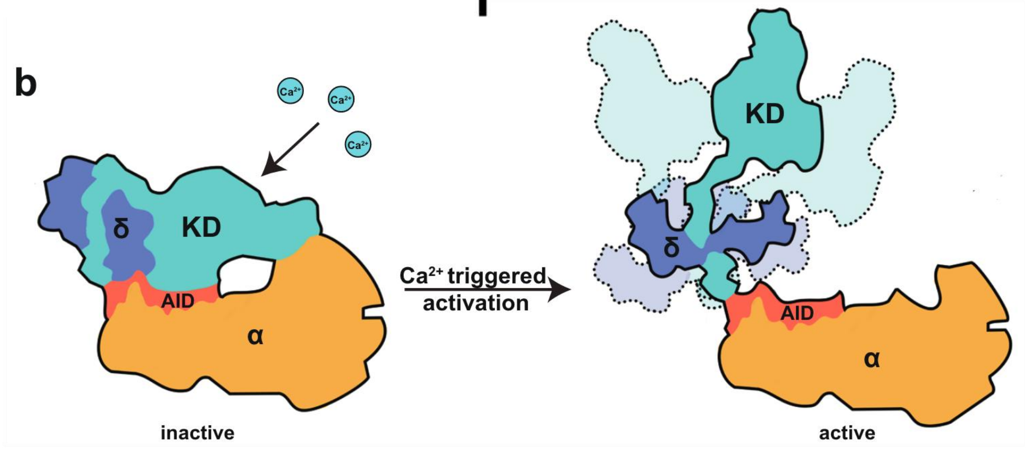
BIOCHEM JC TOPIC 4







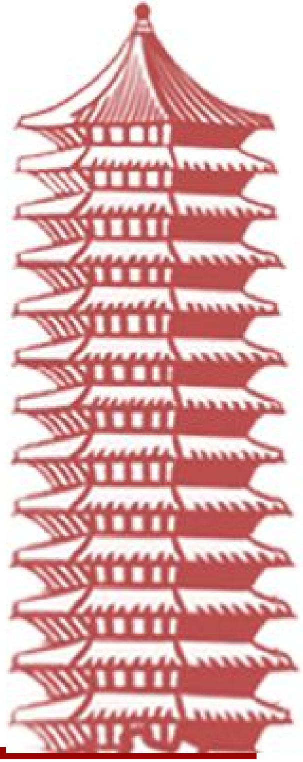
北京大学
PEKING UNIVERSITY

Modern Research on Protein Phosphorylation

主讲人 刘家希

2024年4月17日

CONTENTS



01

Introduction

1 Introduction

1.1 Author

JCB Journal of Cell Biology

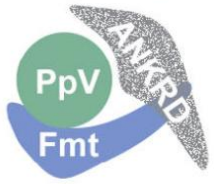
ARTICLE

Competition between two phosphatases fine-tunes Hedgehog signaling

Min Liu[1,2]*, Aiguo Liu[1,2]*, Jie Wang[1]*, Yansong Zhang[1], Yajuan Li[1], Ying Su[3], and Alan Jian Zhu[1,2]

"We identify ***PpV****, the catalytic subunit of PP6, as a direct target of Hedgehog signaling to negatively regulates* ***Wdb****, the regulatory subunit of PP2A. PpV functions independent of its phosphatase activity to fine-tune Hedgehog signaling activities, thus forming a* ***regulatory circuit*** *to maintain homeostatic Hedgehog signaling."*

PP6 in fly



PP2A

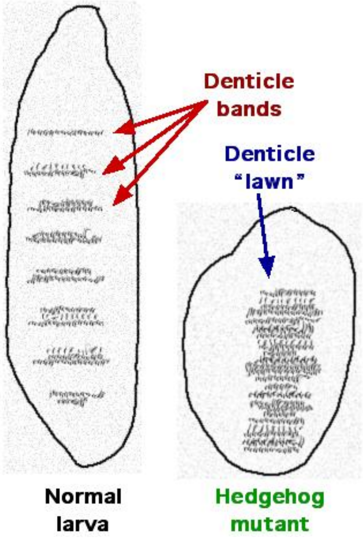
Prof.Alan Jian Zhu

Mice and Drosophila were used as model systems to study the molecular mechanisms of development, aging and metabolism.

1 Introduction

1.2 Hedgehog Signaling in Intertebrates

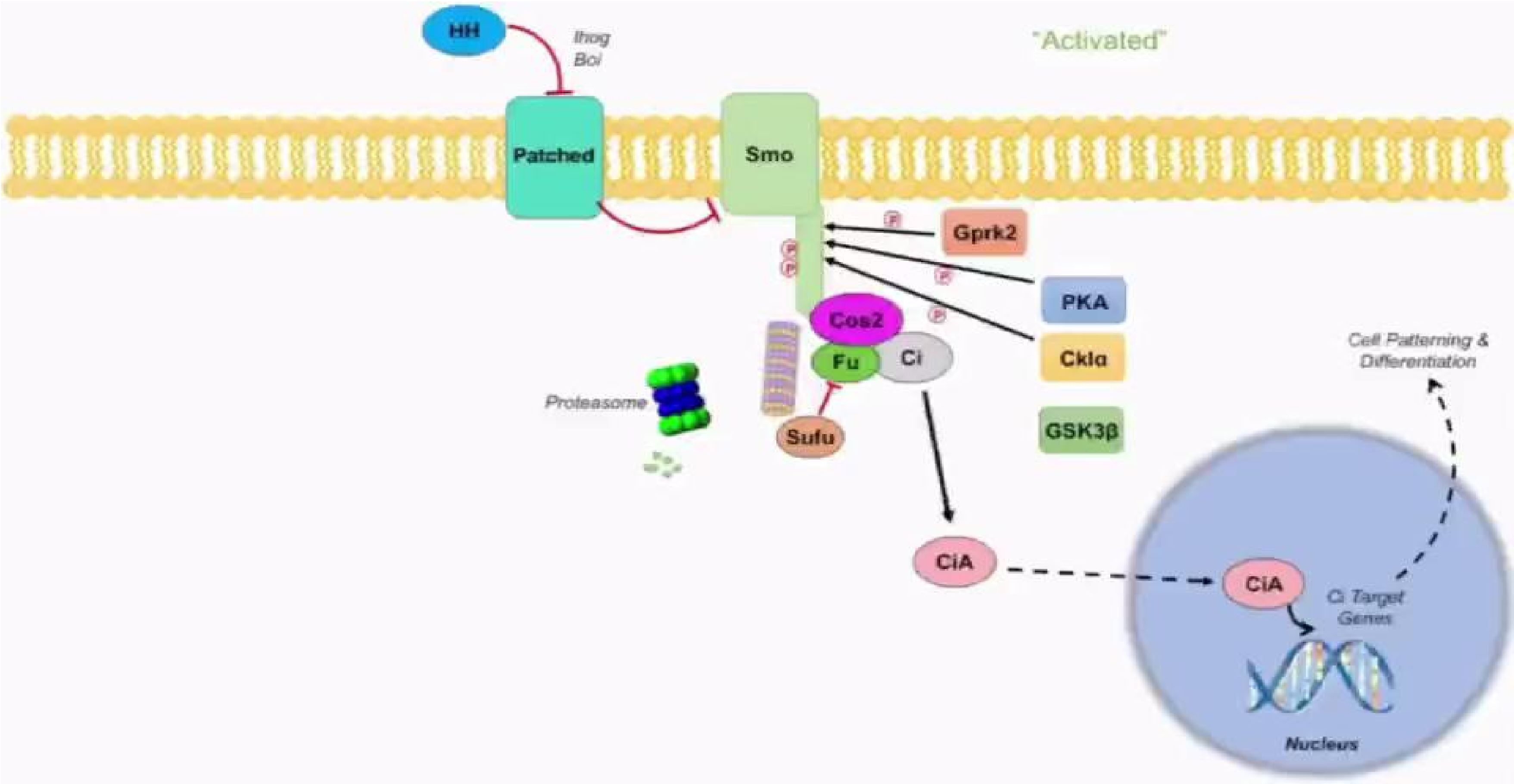
- Involved in embryological development
- Controls cell patterning and differentiation
  - *Ex. Pattern Venation of Wing Discs D. Melanogaster*
- **Graded signaling**
  - Gradient of Hh protein induces different subsets of genes
  - Gene activation at *thresholds*
  - Different concentrations of Hh, different levels of intracellular signaling
  - Ratio of CiR/CiA
- Organ/Tissue-specific gene induction
  - Wing disc
    - *Decapentaplegic(dpp), collier(col) genes etc.*
  - Neural tube
    - *NK6 homeobox 1, oligodendrocyte transcription factor 2 (OLIG2) etc.*



Mohler,1988

1 Introduction

1.2 Hedgehog Signaling in Intertebrates



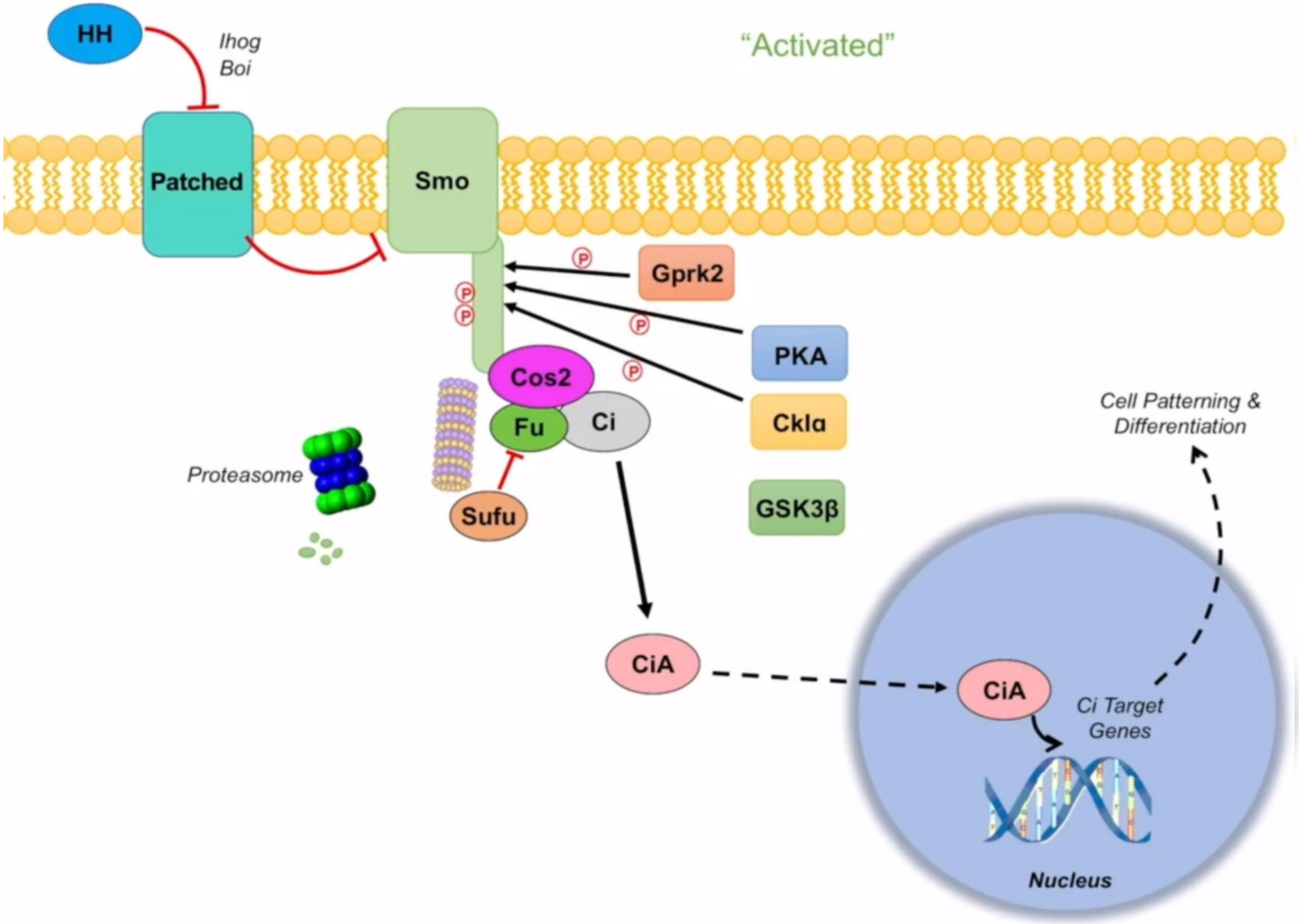
Download From YouTube

Watch the video，and focus on following proteins：

- ***Hh***
- ***Patched(ptc)***
- ***Smo***
- ***Ci***
- ***PKA***
- ***CKIα***

1 Introduction

1.2 Hedgehog Signaling in Intertebrates



Download From YouTube

- Inactivated
- **Ptc** promotes the degradation of **Smo** by proteasome
- Hyperphosphorylation of **CiFL** （Ci155） targets it for proteolytic processing to generate **CiR** (Ci75)

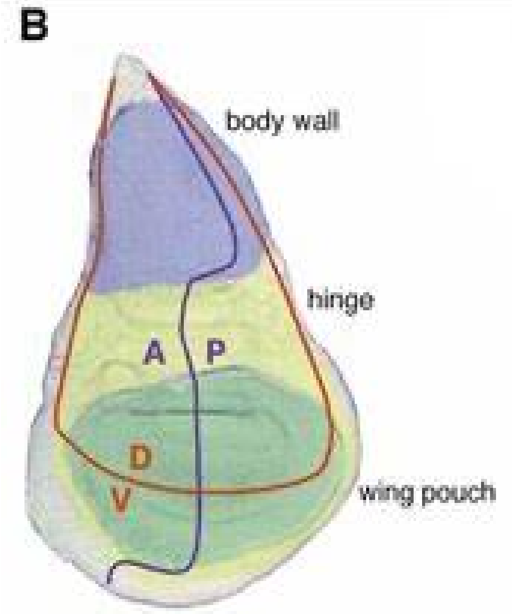
- Activated
- **Hh** alleviates the inhibition of **Smo** by **ptc** and allows it to insert into the plasma membrane and be Sequential phosphorylated by **PKA**, **CKI** and etc.
- High levels of **Hh** induce maturation of **CiFL** into a labile, hyperactive form-**CiA**

1 Introduction

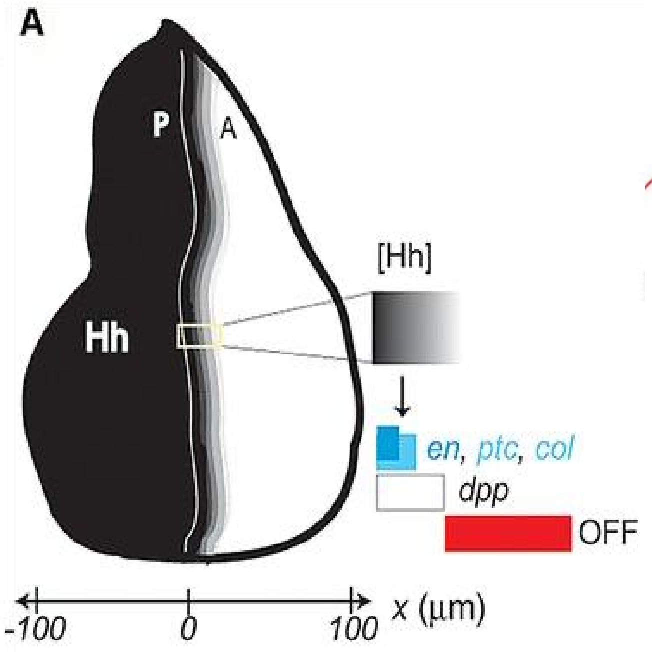
1.3 Grade Hh Signaling in Wing Discs

Hh signaling is produced only at posterior(p) and form an Hh gradient in anterior(a)

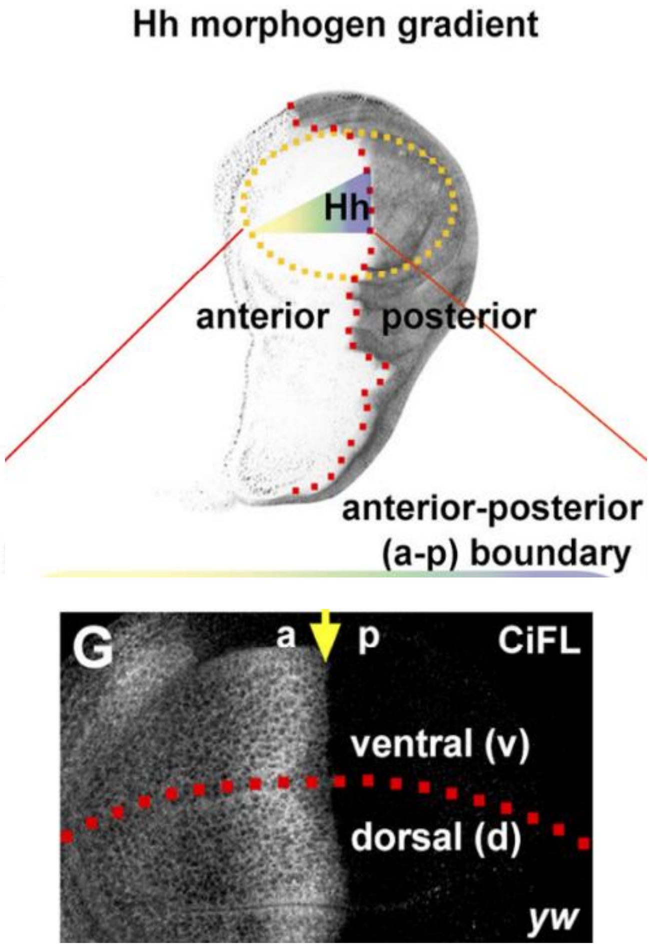
Gao et.al,2019



Nahmad et.al,2009



Zhu et.al,2004



Zhu et.al,2020

02
Background

2 Background

2.1 Sequential phosphorylation of Smo

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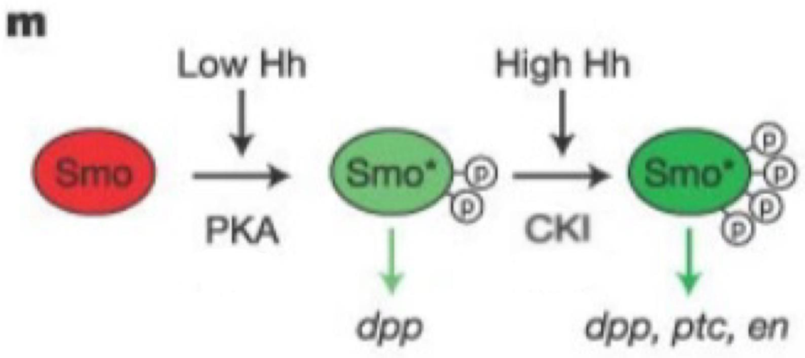
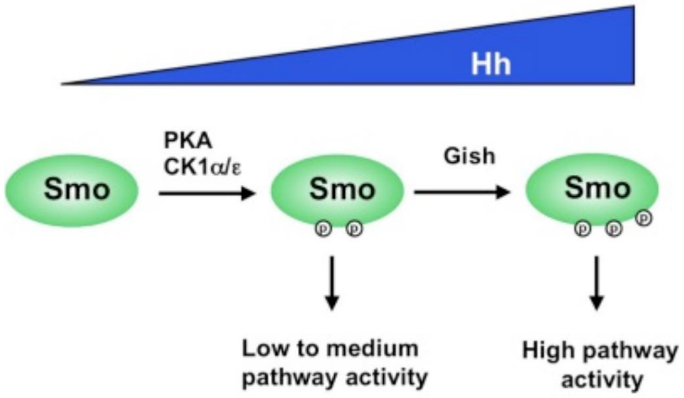
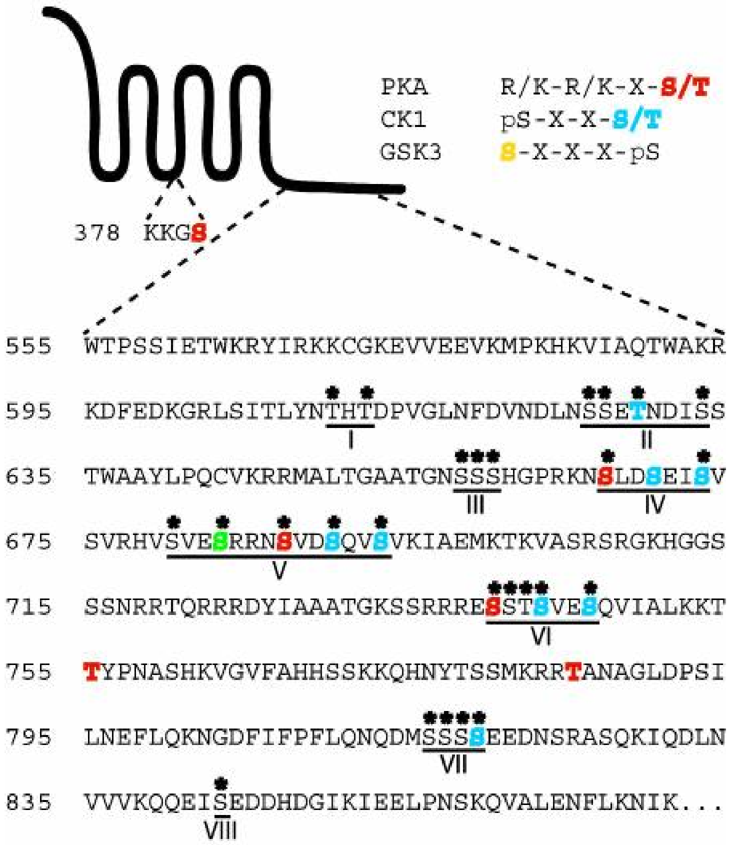
Smo is activated by sequential phosphorylation of PKA, CKI and GSK



Jia et.al,2004



Li et.al,2016



Zhang et.al,2004

2 Background

2.2 Previous research

Science Signaling

Current Issue   First release papers   Archive   About

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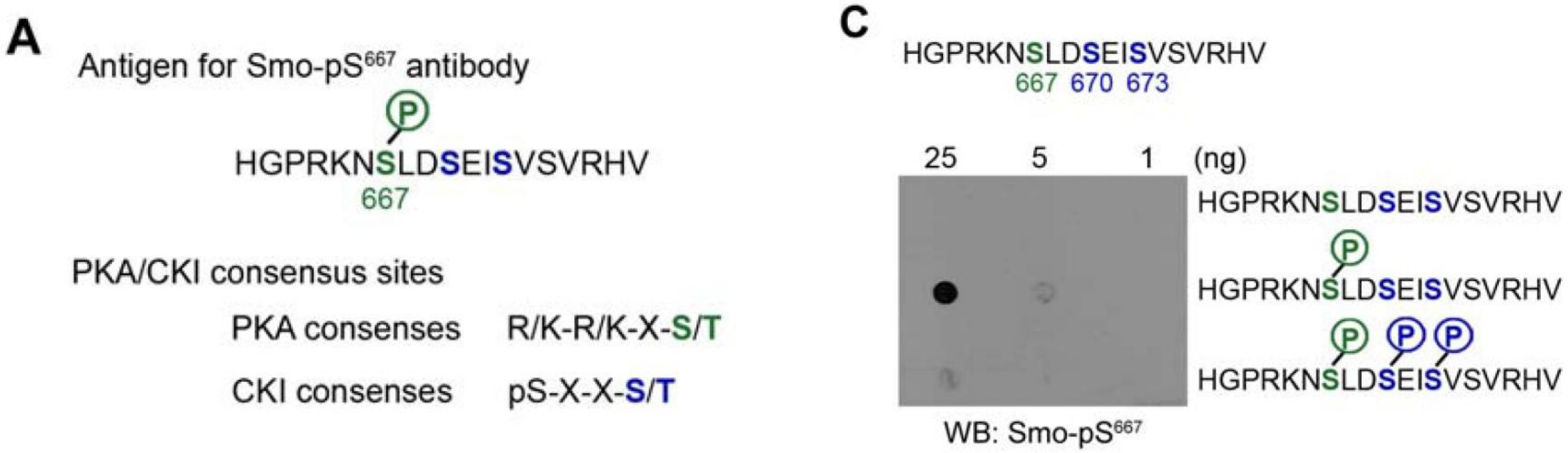
RESEARCH ARTICLE | DEVELOPMENT

Sequential Phosphorylation of Smoothened Transduces Graded Hedgehog Signaling

YING SU, JASON K. OSPINA, JUNZHENG ZHANG, ANDREW P. MICHELSON, ADAM M. SCHOEN, AND ALAN JIAN ZHU   Authors Info & Affiliations

- Since kinases phosphorylate Smo, which phosphatases regulate the dephosphorylation of Smo?
- How to distinguish Smo with different phosphorylation levels?

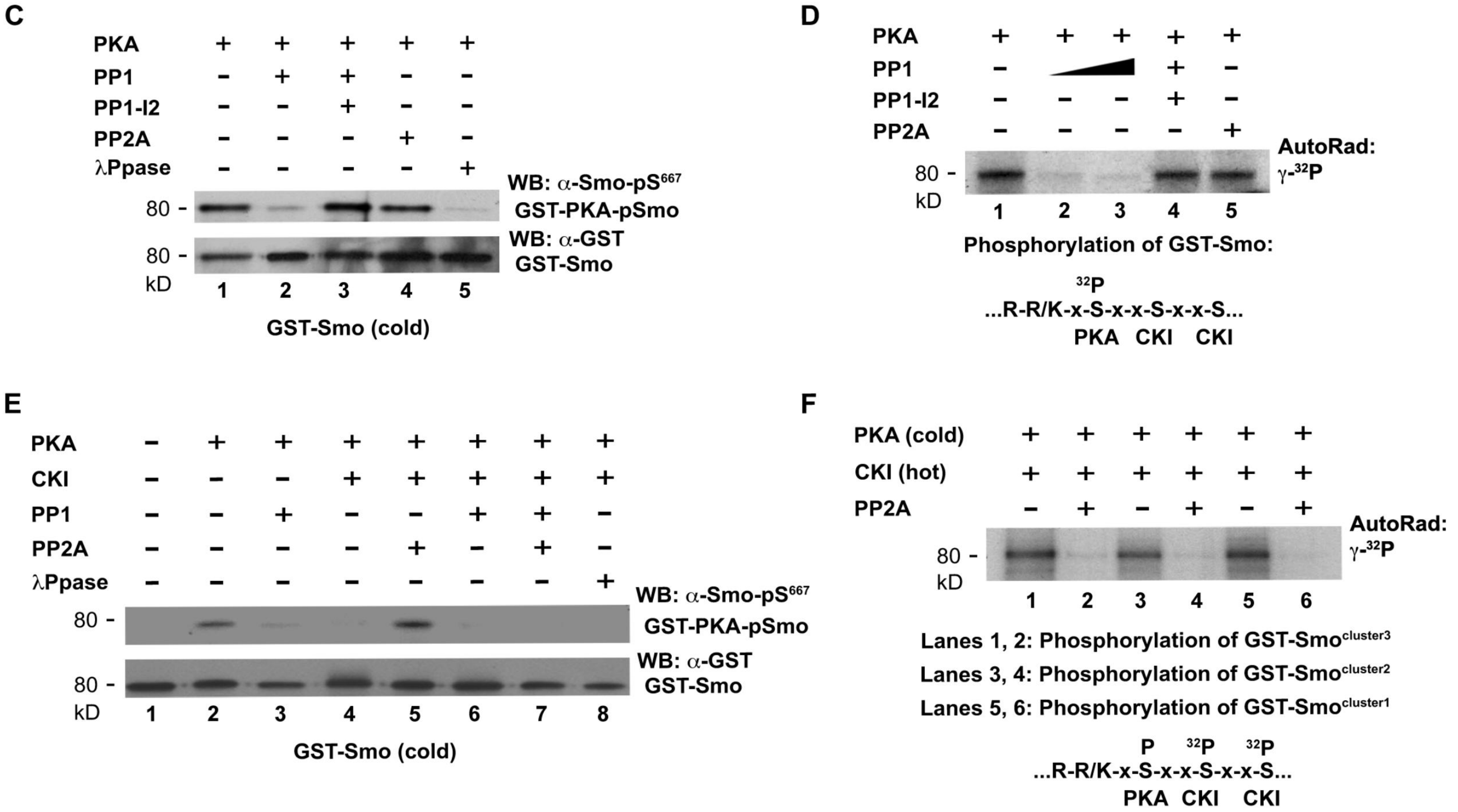


EILISPOT

Su et.al,2011

2 Background

2.2 Previous research

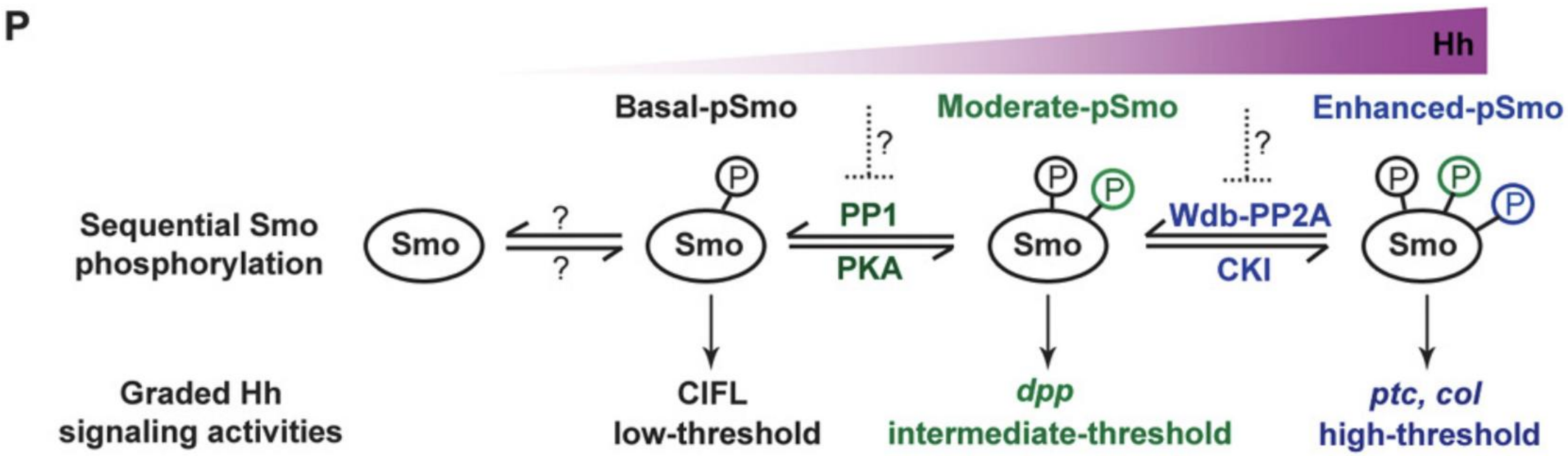


Su et.al,2011

2 Background

2.2 Previous research

PP1 and PP2A respectively antagonize PKA and CKI activities to maintain sequential phosphorylation of Smo

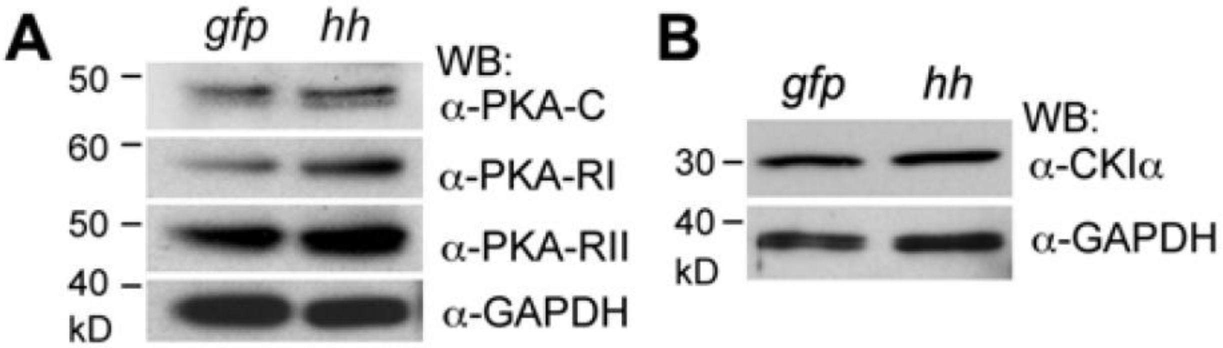


Su et.al,2011

2 Background

2.2 Previous research

- How such Smo-associated kinases and phosphatases sense Hh signaling gradient?
- How they in turn collectively coordinate graded Smo phosphorylation?



How such Smo-associated *phosphatases* sense and regulate Hh signaling gradient?

Hh signaling does not affect PKA or CKI abundance or activity

Let's Begin——

Su et.al,2011

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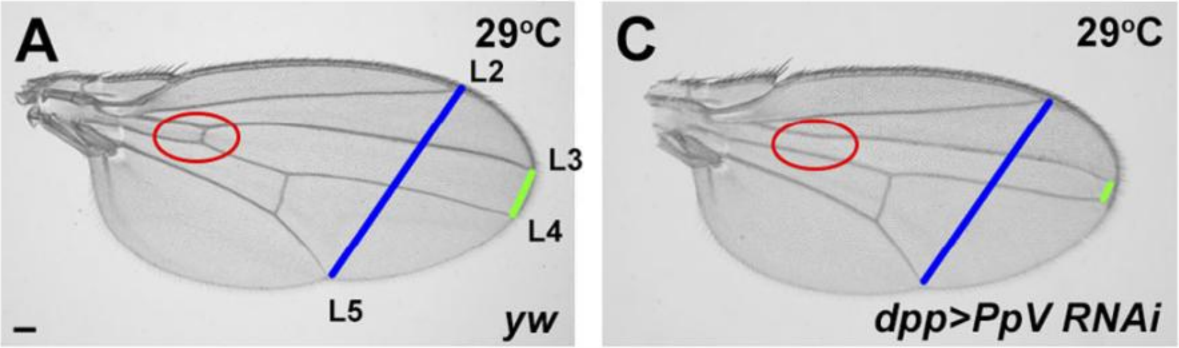
03

Reseach

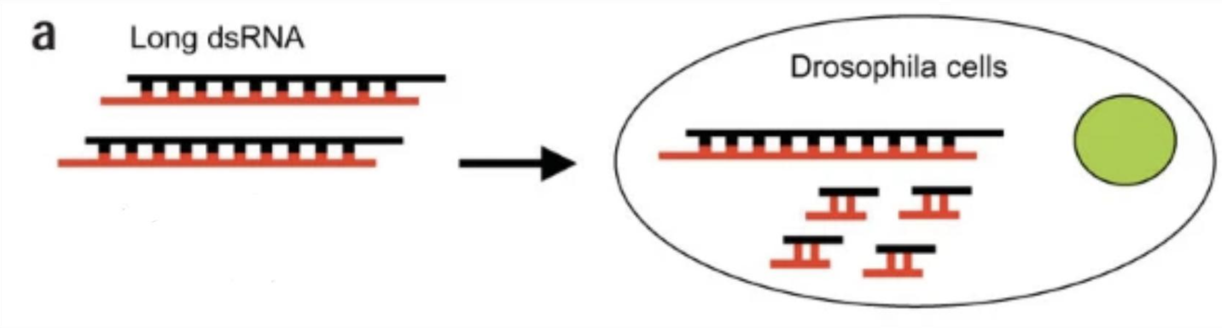
3 Research

3.1 Identification of the PpV



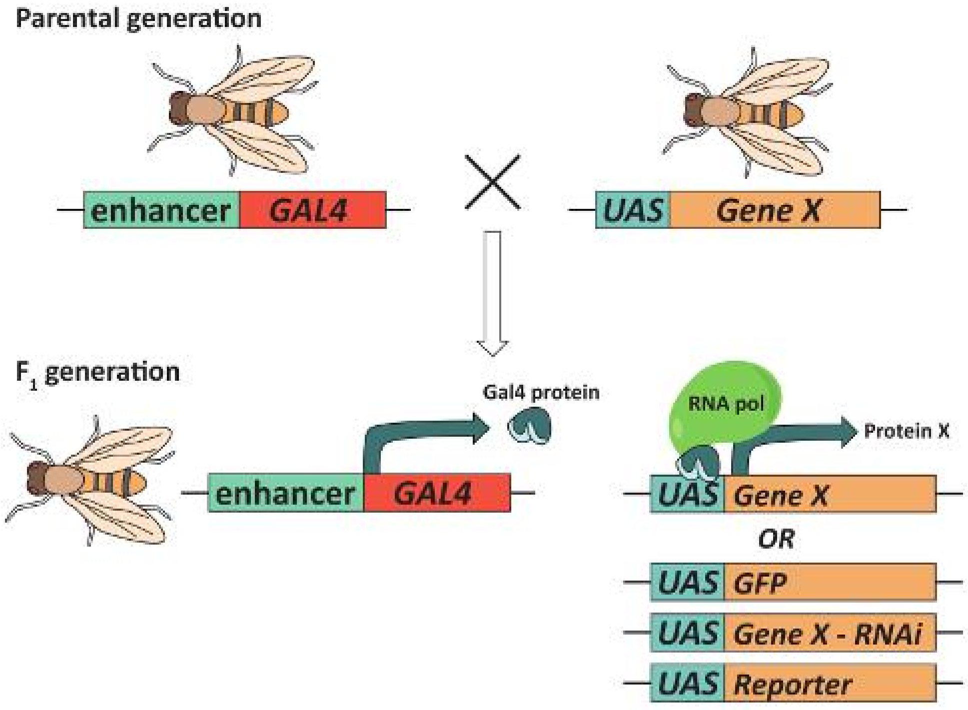
L3-L4 distance   anterior cross vein(ACV)

- RNAi Screening



Sharma et.al,2009

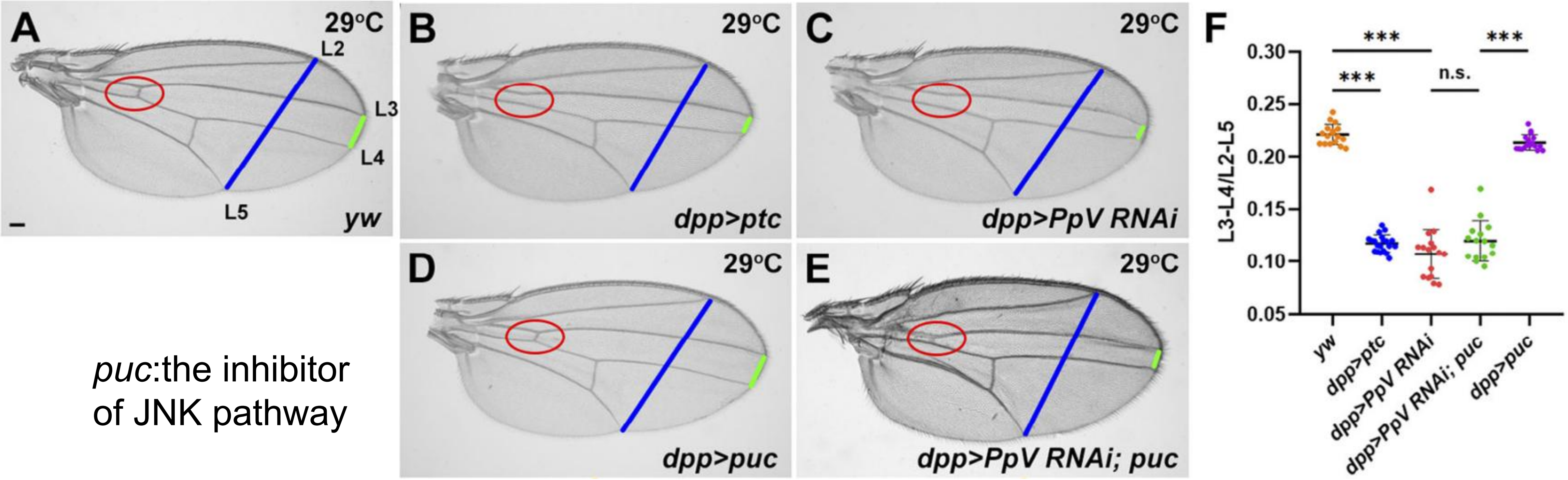
- Gal4-UAS system



Kelly et.al,2017

3.1 Identification of the PpV

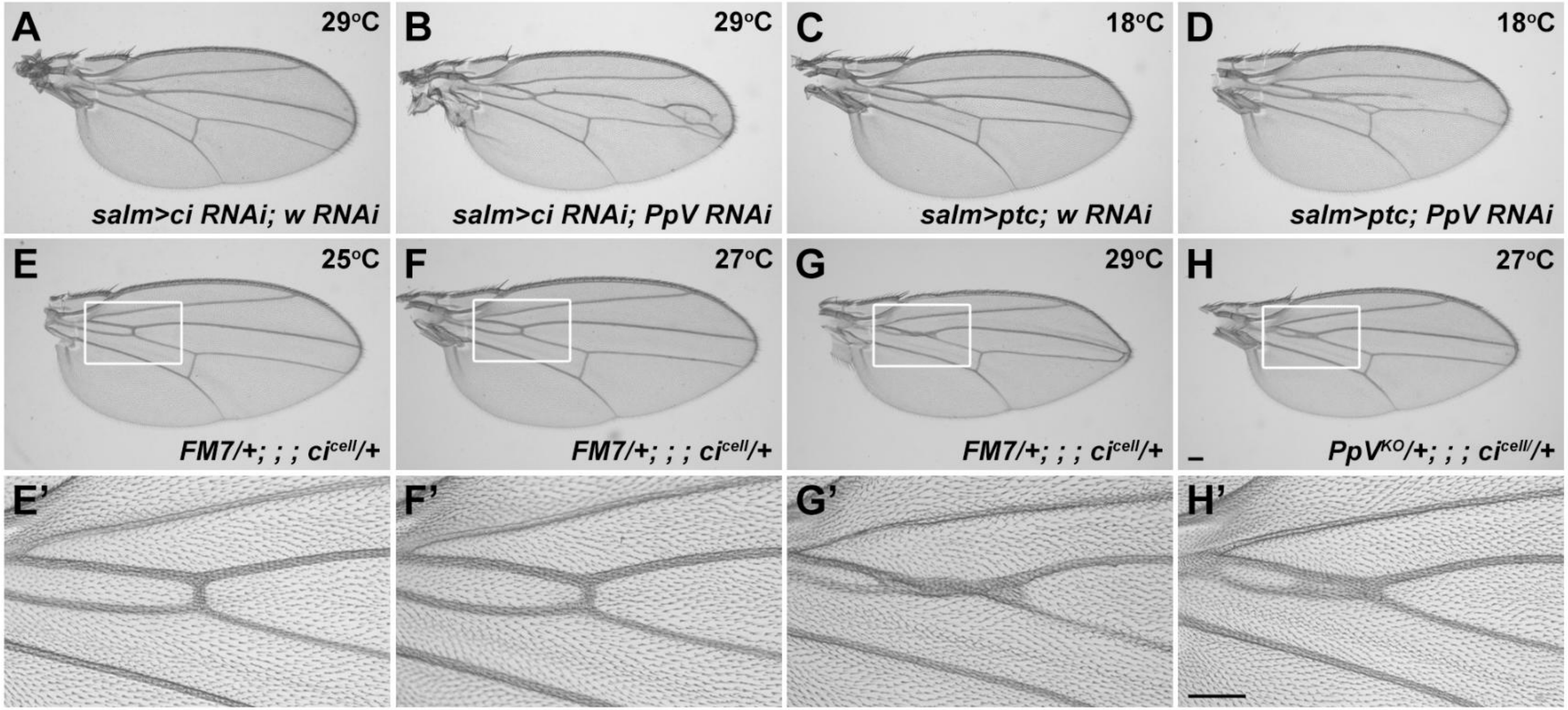
The decrease in the distance between L3 and L4 is a sign of the decrease in Hh signaling



puc:the inhibitor of JNK pathway

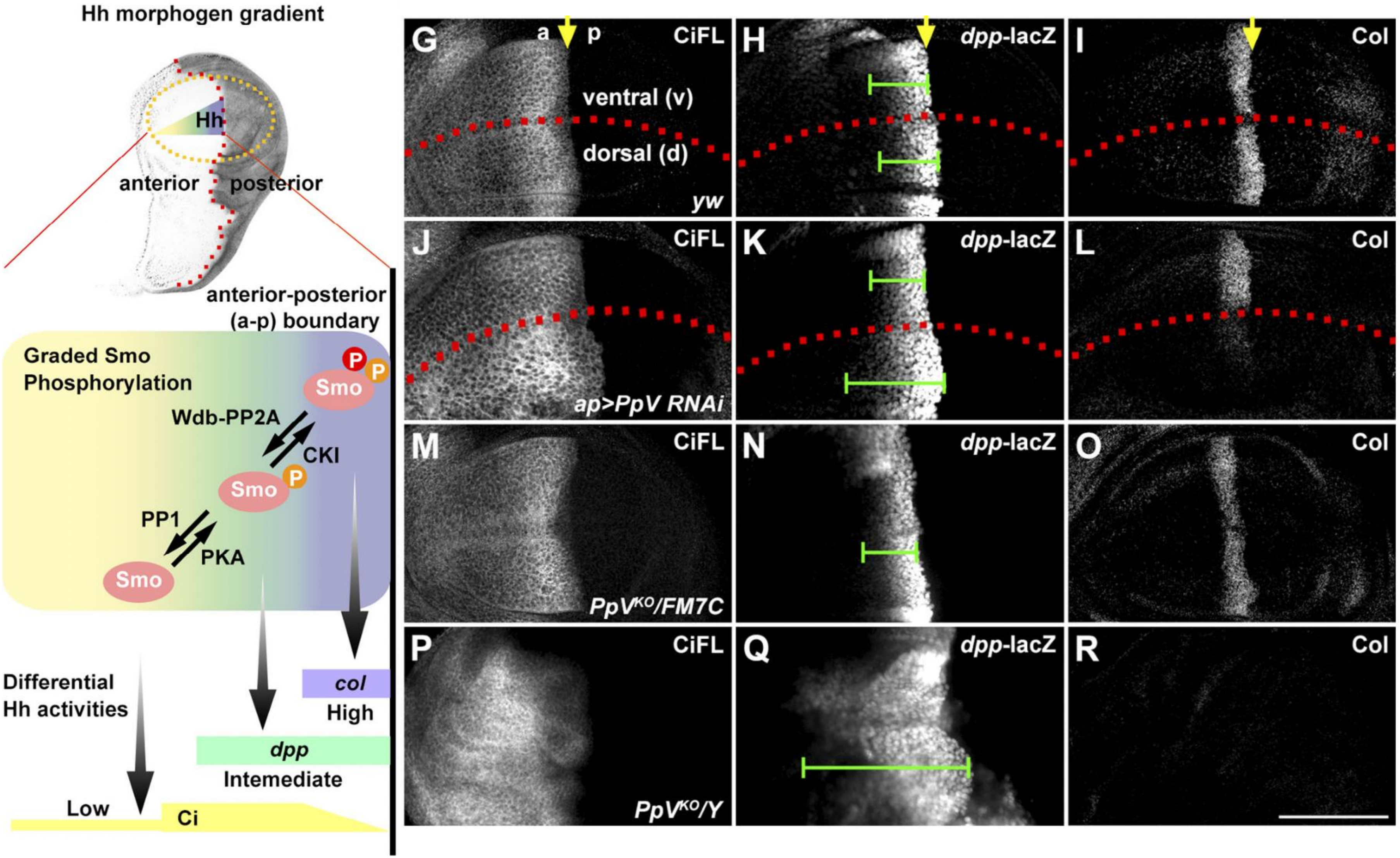
3.1 Identification of the PpV

There is a requirement of PpV activity in mediating Hh signaling in Drosophila wing development



3 Research

3.1 Identification of the PpV

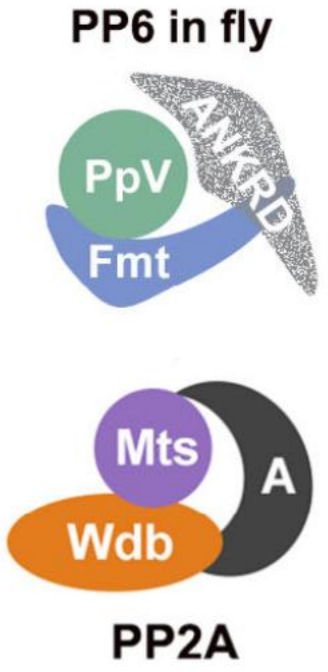


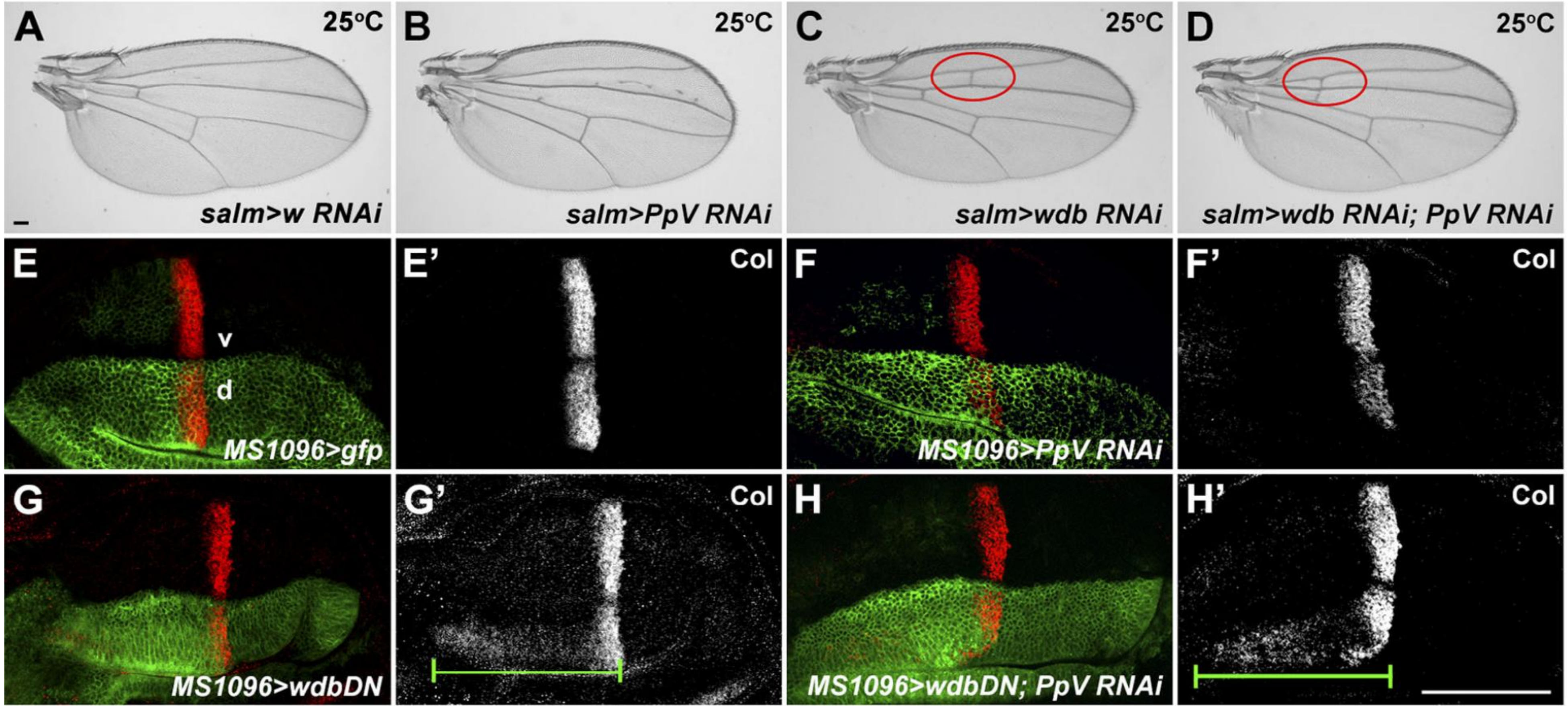
PpV inhibits phenotypes at low and intemediate Hh signal, but promotes phenotypes at high Hh signal

3.2 PpV acts upstream of wdb

Loss of both wdb and PpV expression phenocopied the defect associated with wdb RNAi alone

wdb is epistatic to PpV





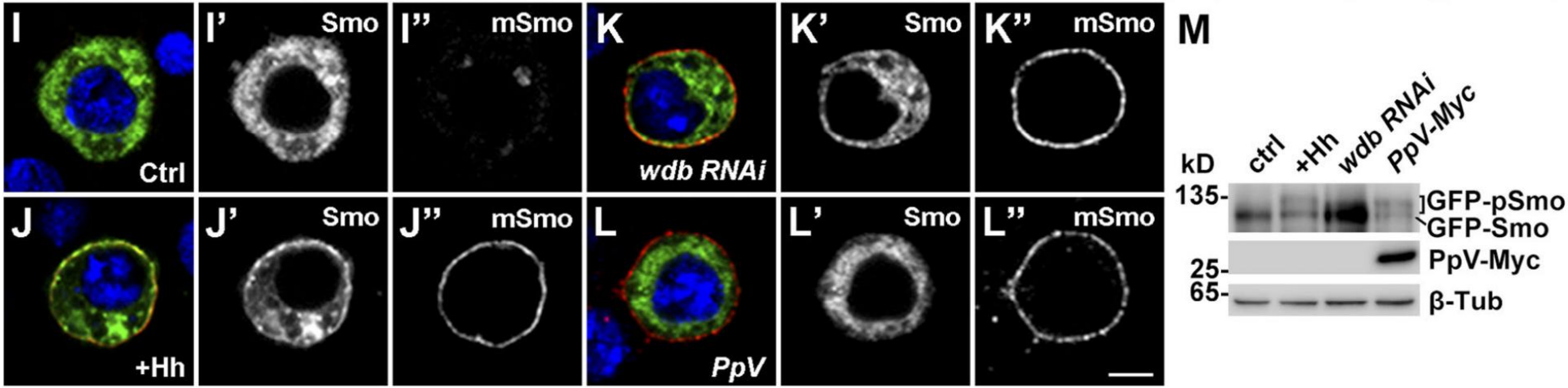
3 Research

3.2 PpV acts upstream of wdb

PpV acts upstream of wdb to modulate Smo phosphorylation in Hh signaling

Schneider2 (S2) cell

Long et.al,2019

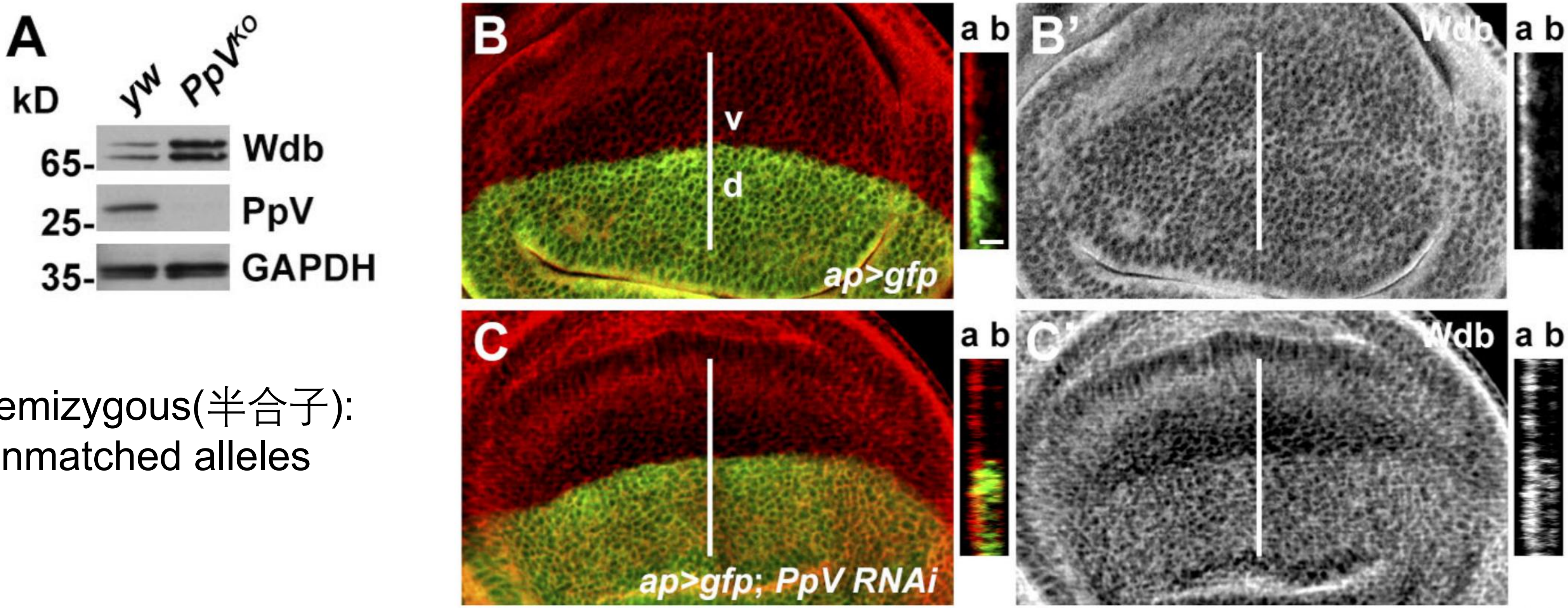


Smo:GFP Smo mSmo:membrane Smo

3 Research

3.3 PpV regulates Wdb protein abundance

The amount of **endogenous** Wdb was significantly increased in cell lysates extracted from hemizygous PpV^{KO} larvae

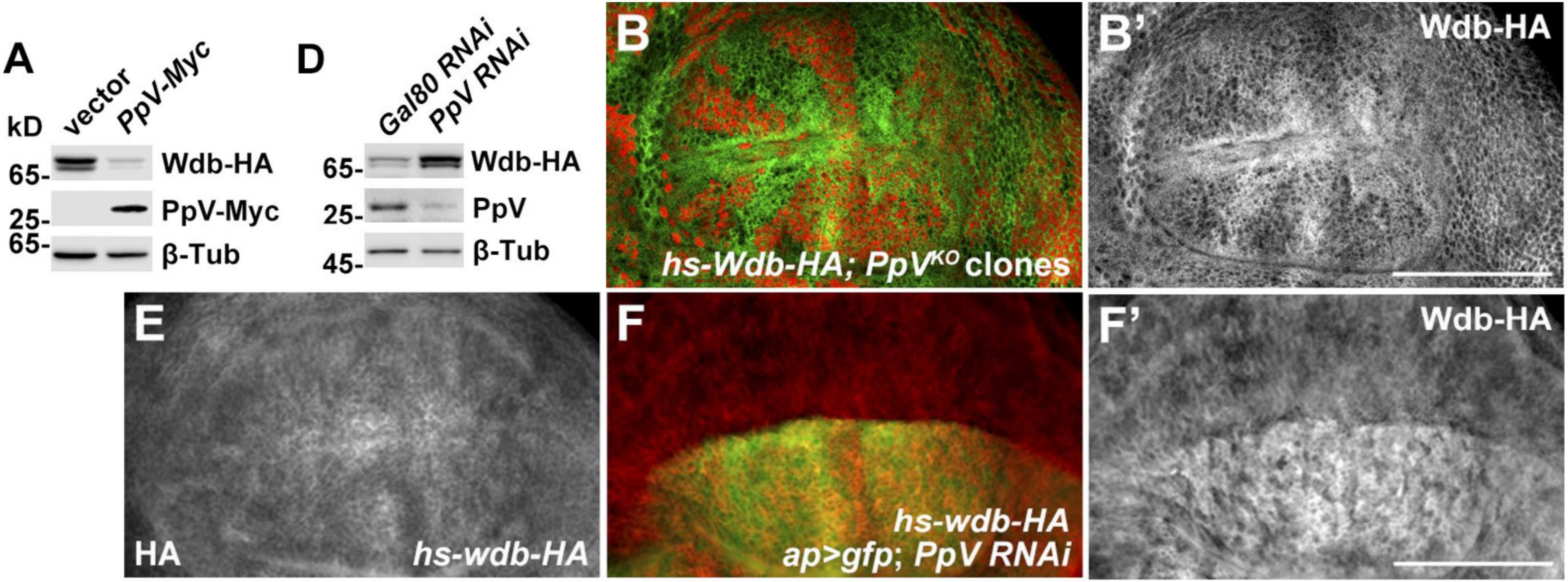


hemizygous(半合子):
Unmatched alleles

3 Research

3.3 PpV regulates Wdb protein abundance

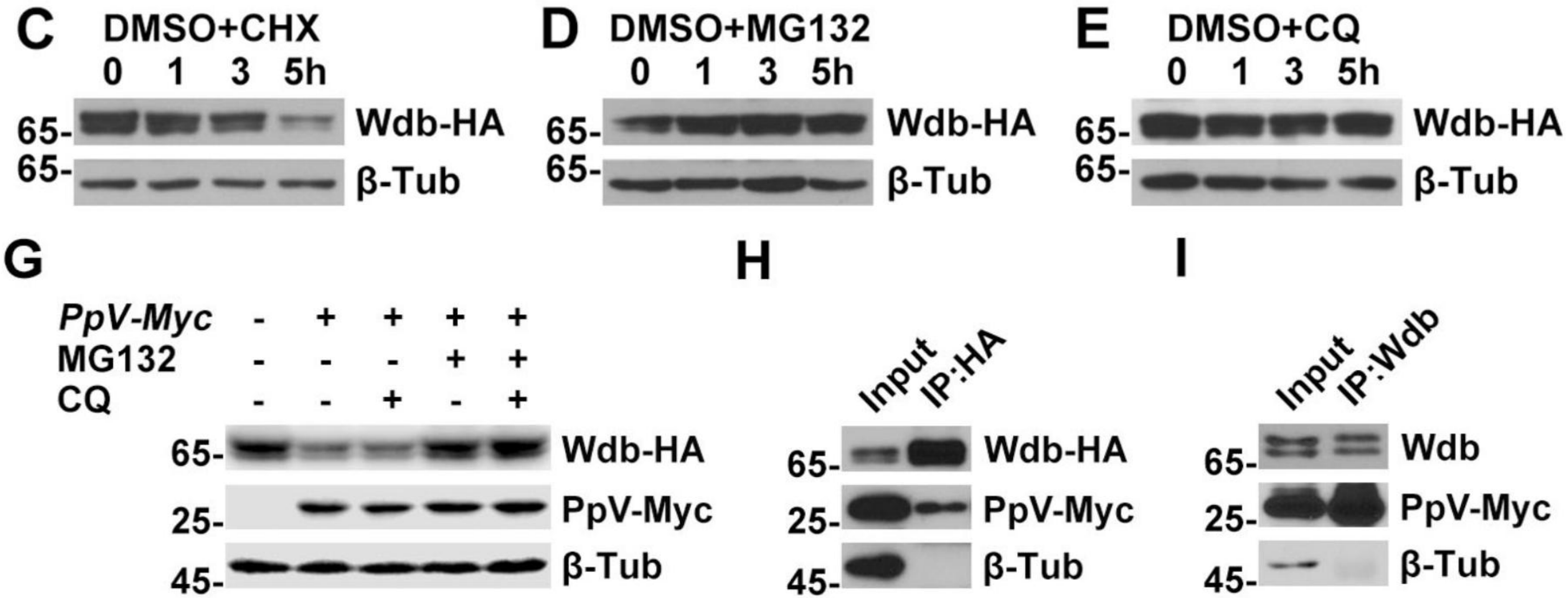
PpV knock-down results in the increase of **exogenously** expressed hs-Wdb-HA → **The regulation of wdb by PpV occurs after transcription**

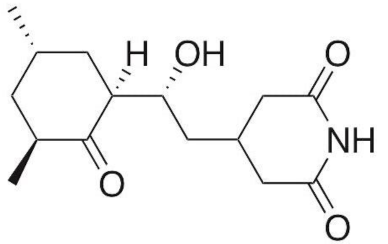


3 Research

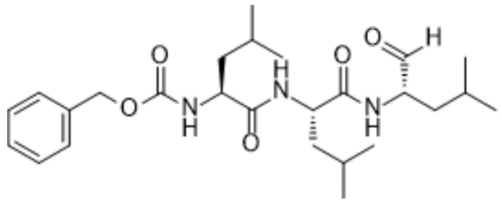
3.3 PpV regulates Wdb protein abundance

PpV may regulate the proteasome degradation of Wdb by direct interaction

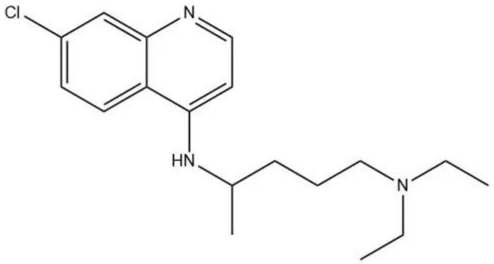




CHX



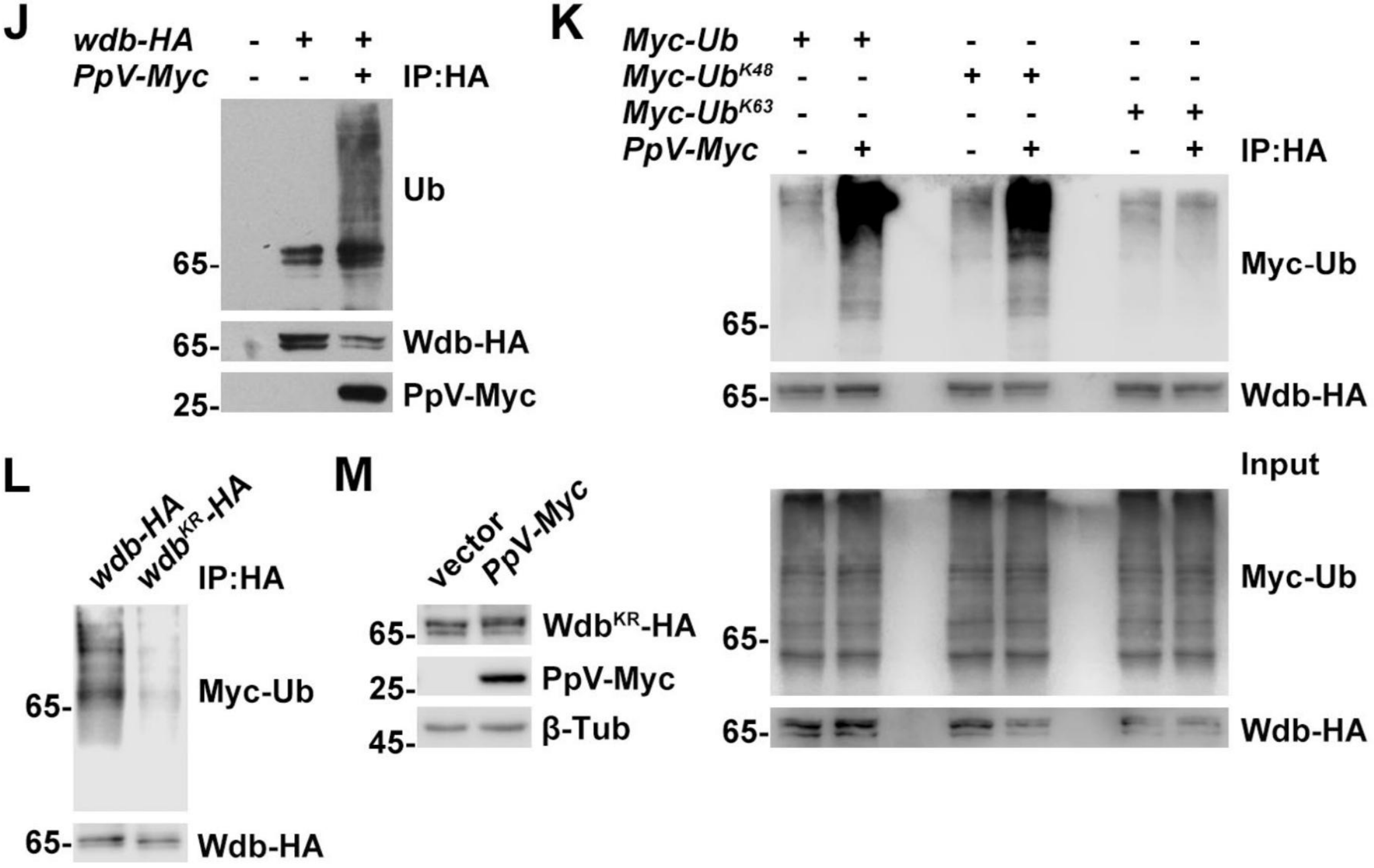
MG132



CQ

3 Research

3.3 PpV regulates Wdb protein abundance

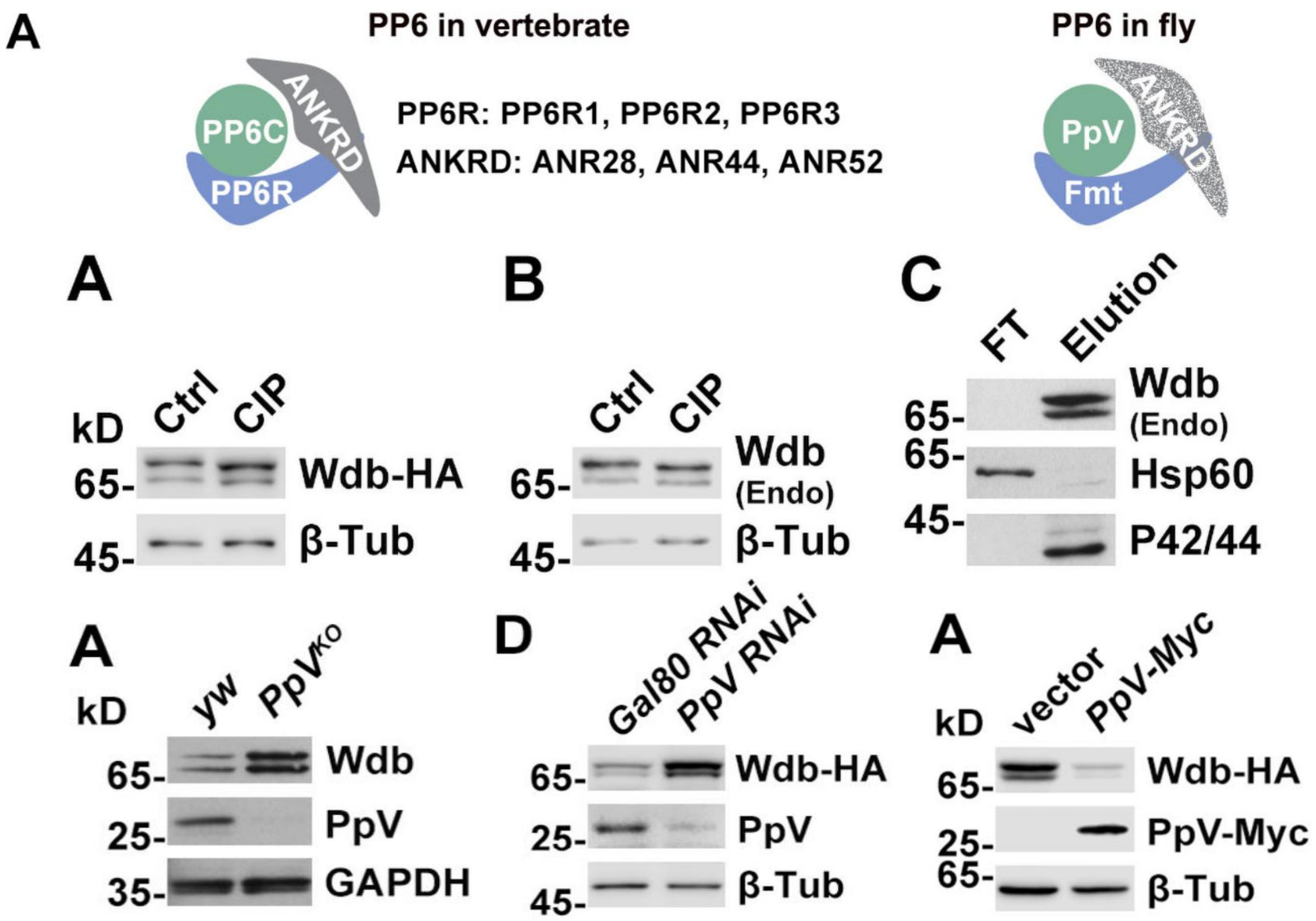


How does PpV facilitates Wdb degradation?

PpV promotes the K48 poly-Ub on modification of Wdb for proteasome degradation

3 Research

3.4 Effect of PpV phosphatase activity

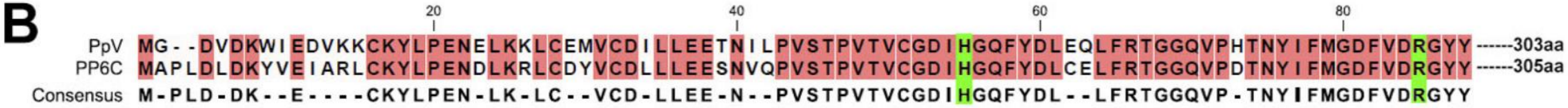


PpV does not seem to affect the electrophoretic mobility of wdb

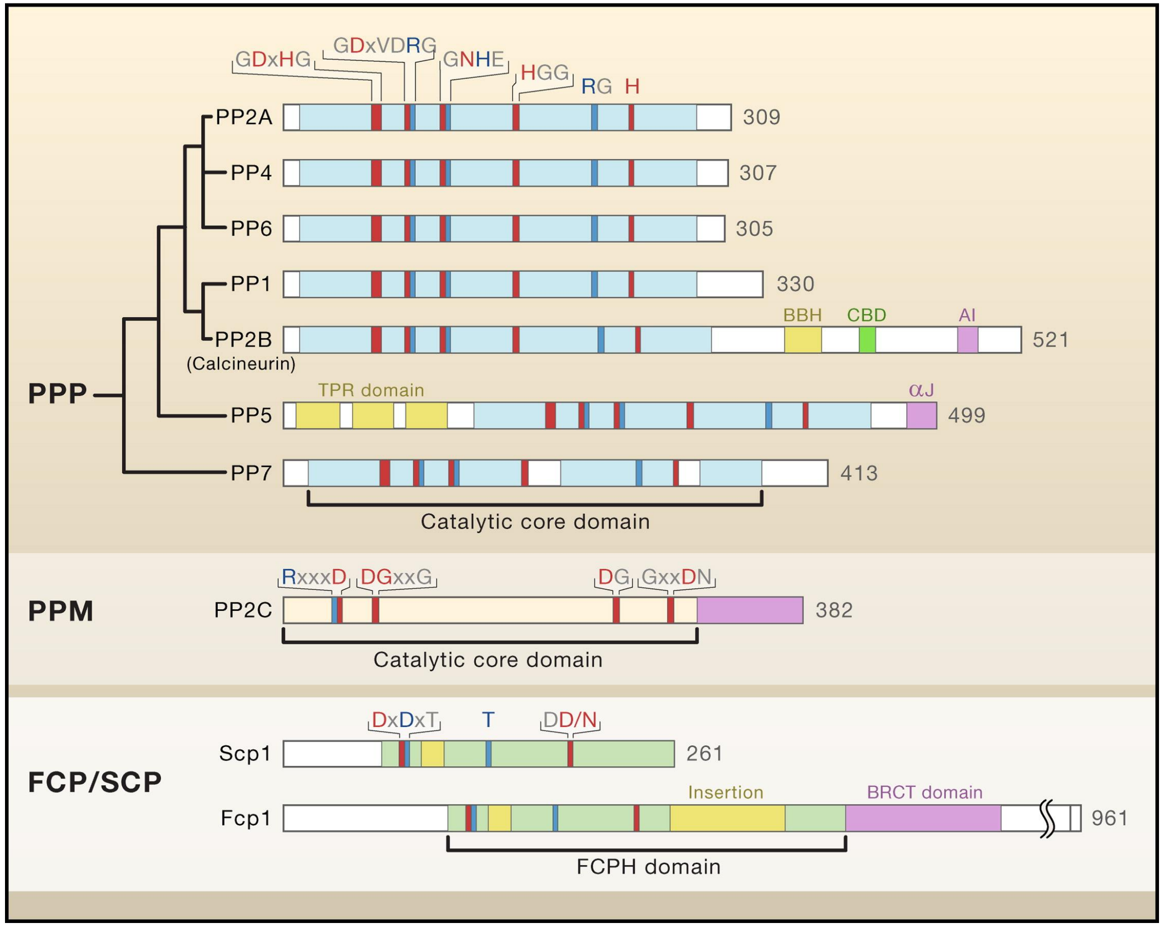
3 Research

3.4 Effect of PpV phosphatase activity

B

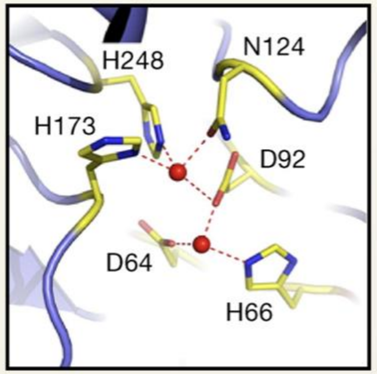


Catalytically inactive mutant PpV*: PP6C$^{H55Q/R85A}$/PpV$^{H53Q/R83A}$



How do H53Q and R83A affect the structure and function of PpV?

B



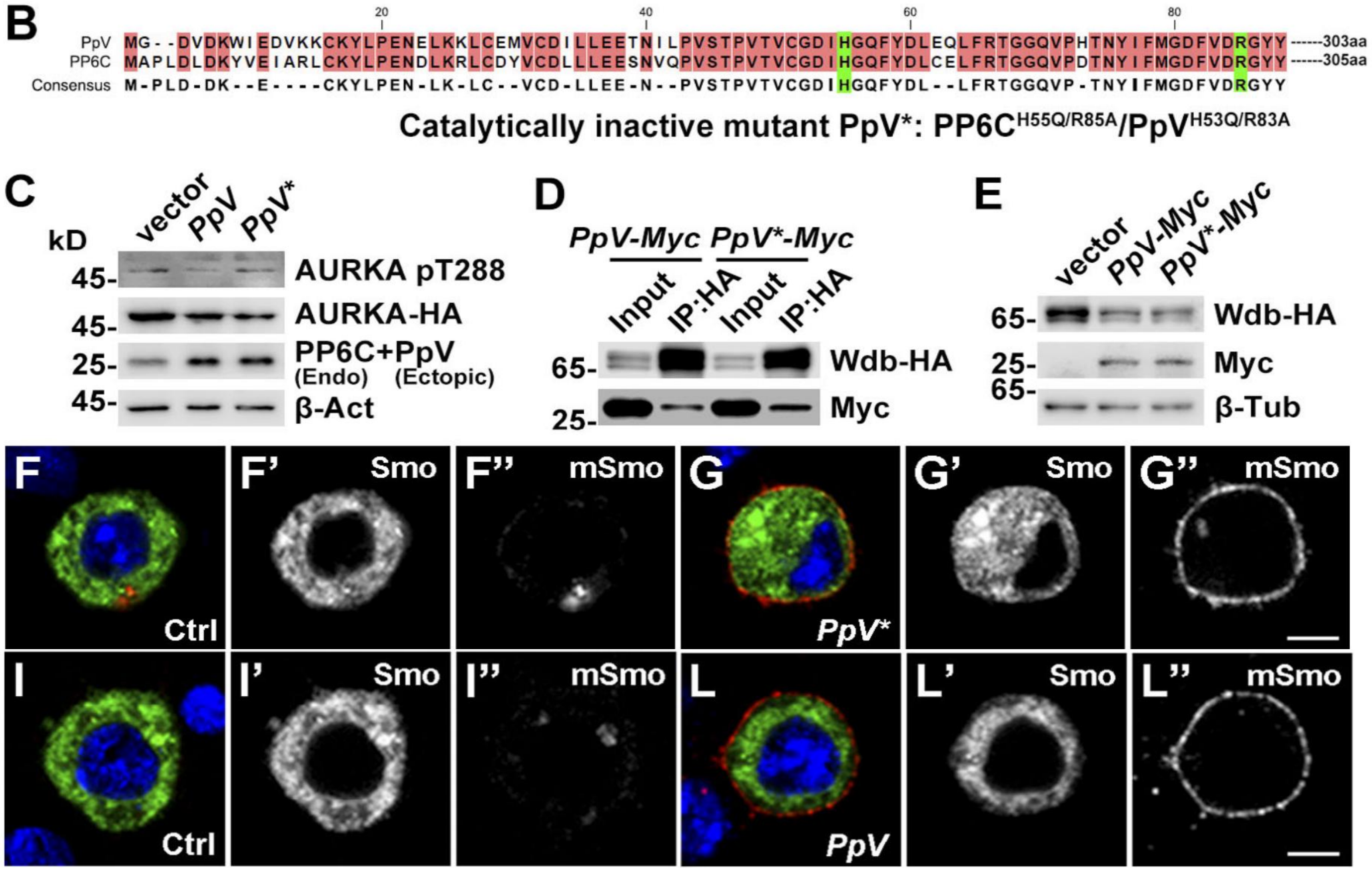
Conserved active site residues in PPP

PP1	D64	H66	D92	N124	H173	H248
PP2A	D57	H59	D85	N117	H167	H241
PP2B	D90	H92	D118	N150	H199	H281
PP4	D54	H56	D82	N114	H164	H238
PP5	D242	H244	D271	N303	H352	H427
PP6	D53	H55	D81	N113	H163	H237
PP7	D84	H86	D113	N149	H197	H303

Shi, *Cell*, 2009

3 Research

3.4 Effect of PpV phosphatase activity



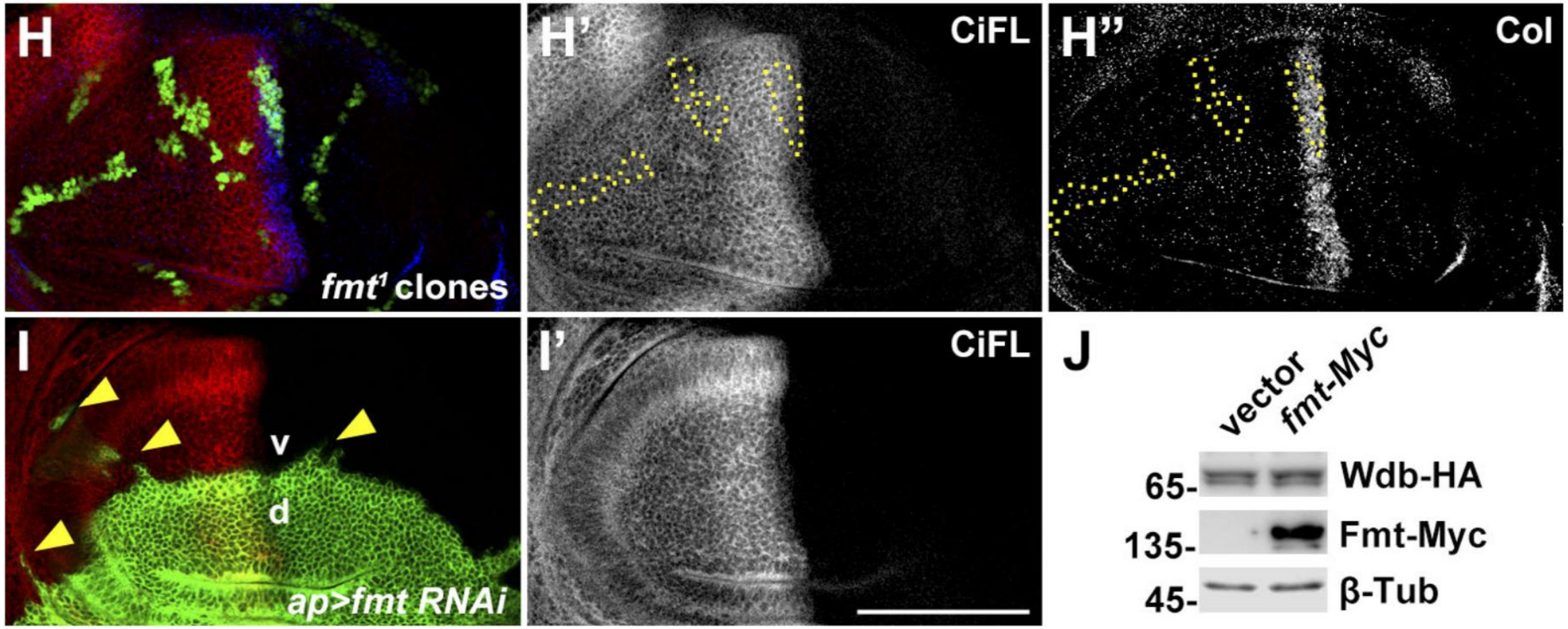
The phosphatase activity of PpV did not affect the regulation of Wdb

3 Research

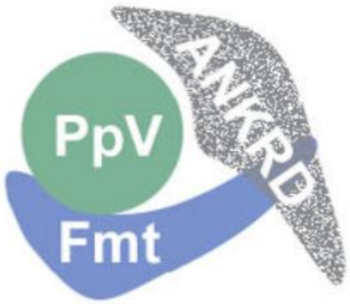
3.4 Effect of PpV phosphatase activity

The absence of fmt, the only regulatory subunit of ppv, did not affect its regulation of Hh phenotype

PpV facilitates Wdb protein degradation independent of its phosphatase activity



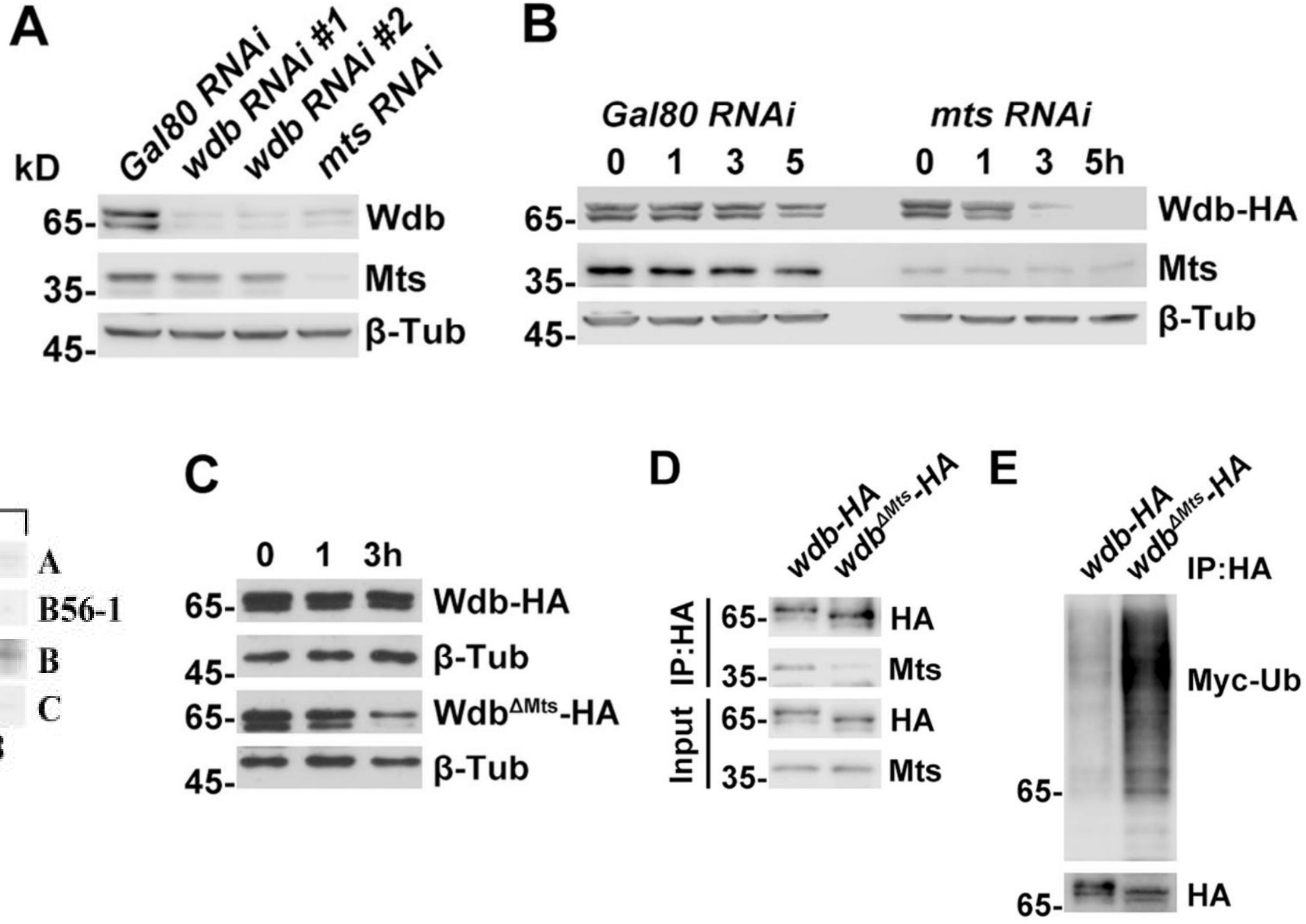
PP6 in fly

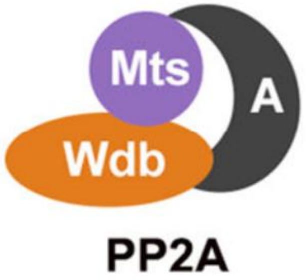


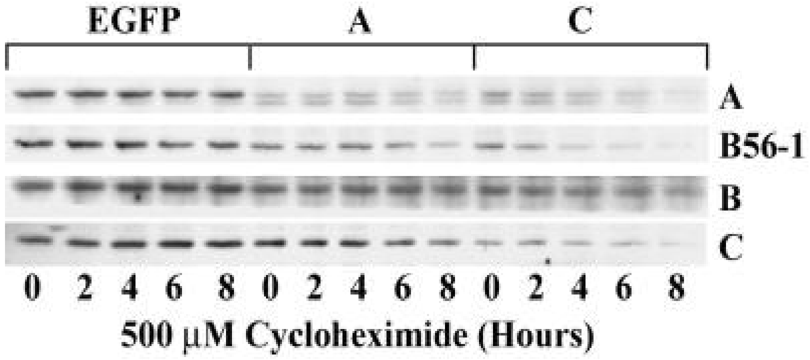
3 Research

3.5 Mechanism of PpV inhibiting Wdb activity

Removal of the catalytic subunit mts from the PP2A complex decreases Wdb stability



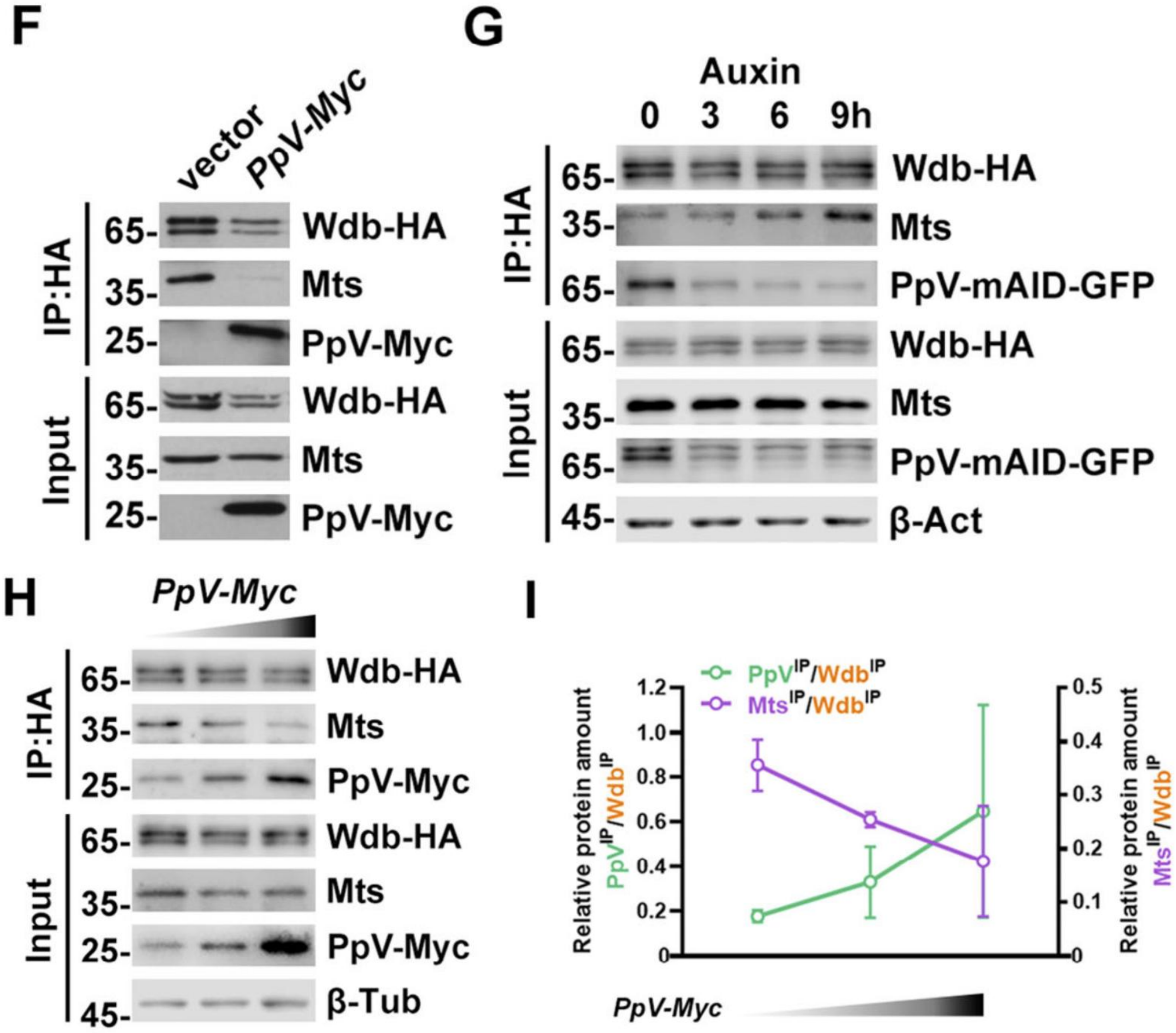




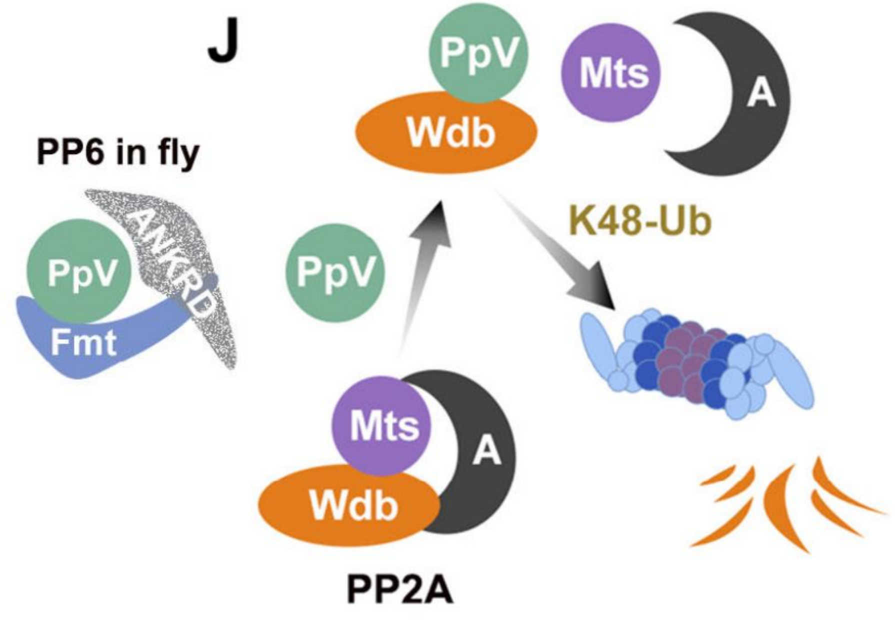
Silverstein,2002

3 Research

3.5 Mechanism of PpV inhibiting Wdb activity



PpV facilitates Wdb degradation by interference with the PP2A complex assembly



Is this the end? No!

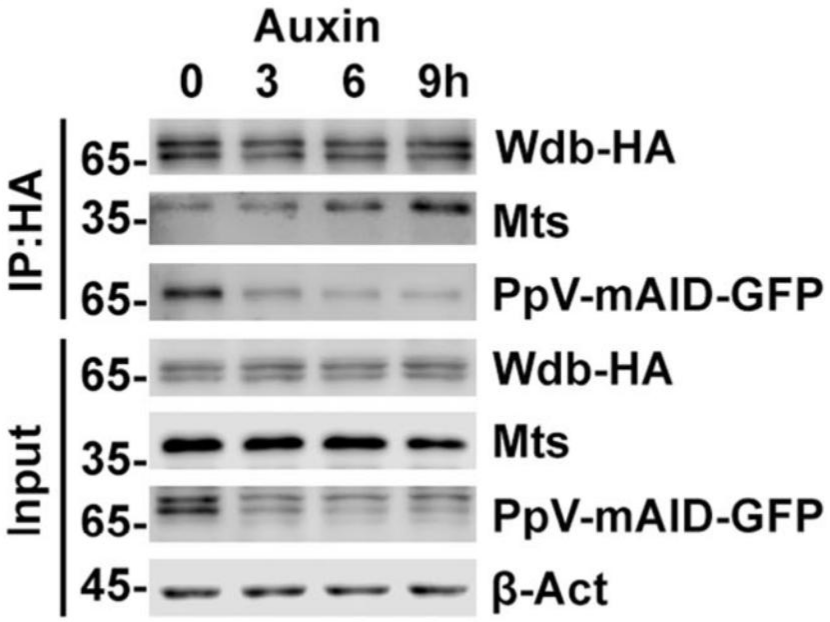
3 Research

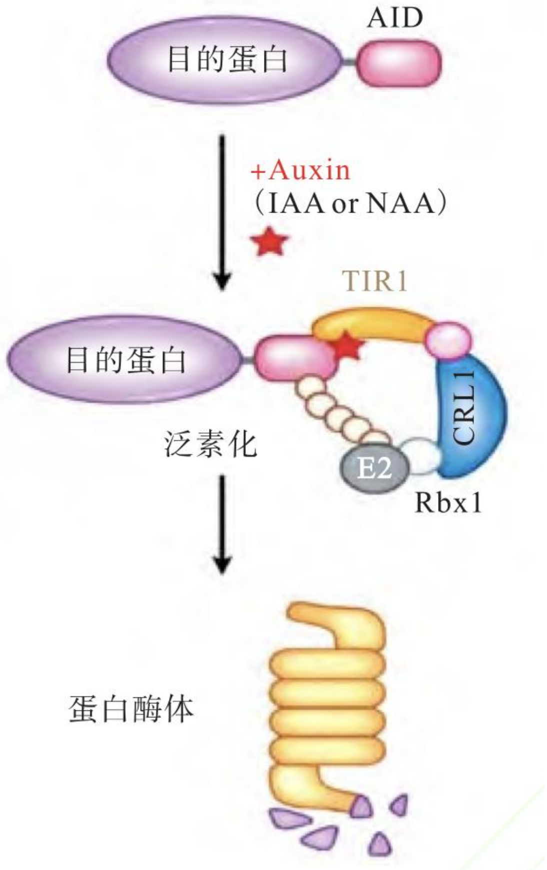
3.5 Mechanism of PpV inhibiting Wdb activity

- Auxin-inducible degron system

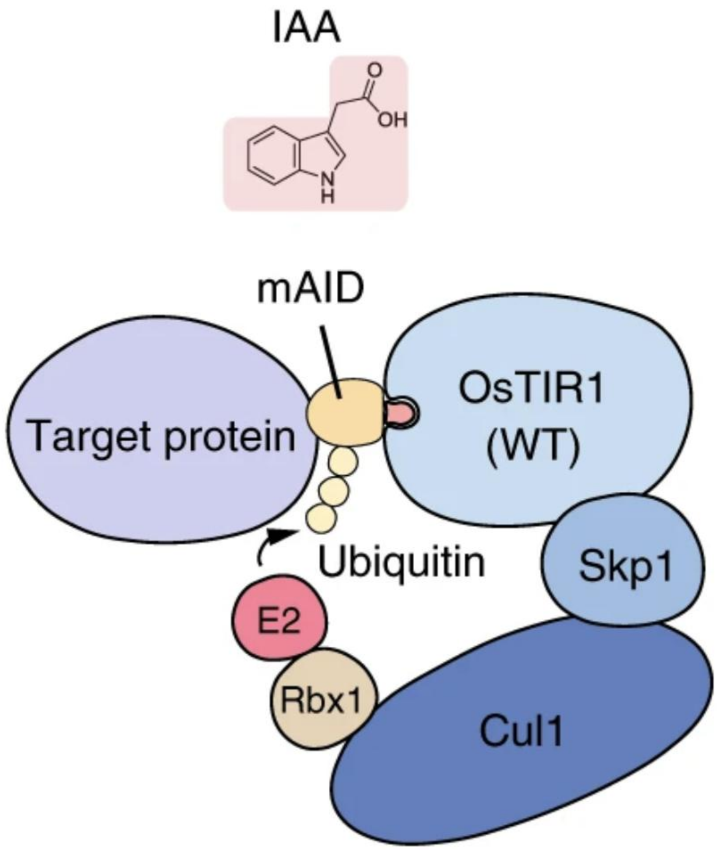
  Regulates protein degradation conditionally

G





Ma et.al,2018

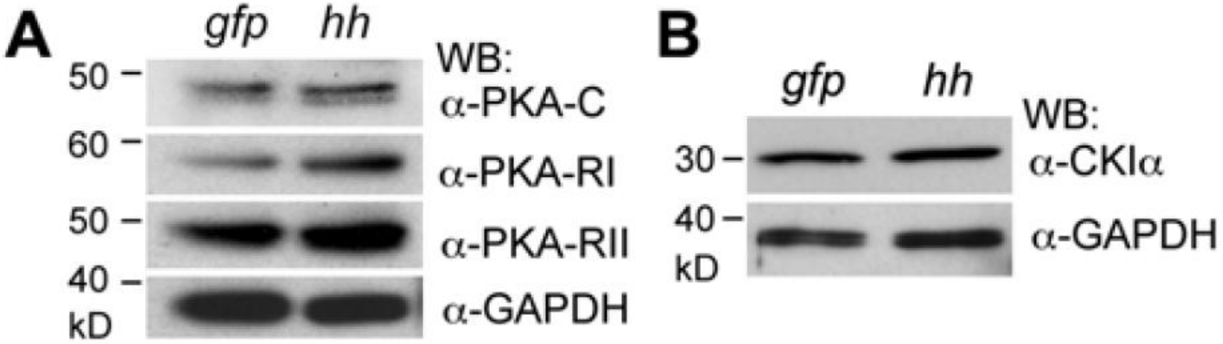


Aisha et.al,*Nat.Commun.*,2020

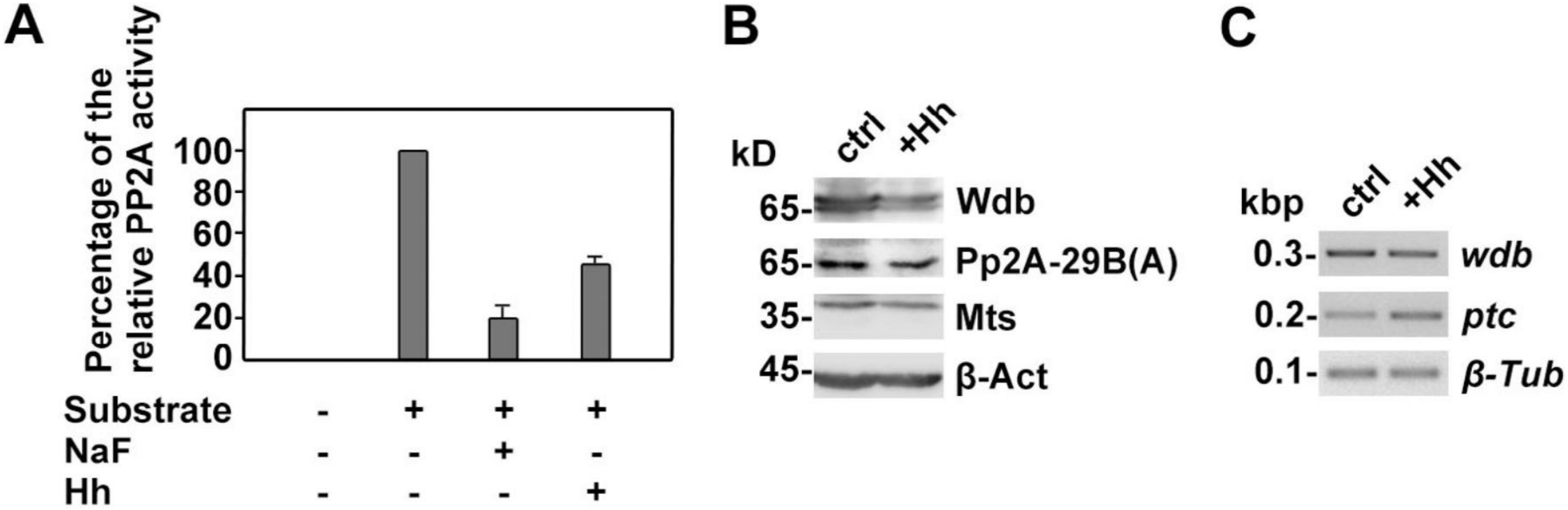
3 Research

3.6 PpV is regulated by the Hh signaling

The Hh signal reduces the abundance and activity of Wdb

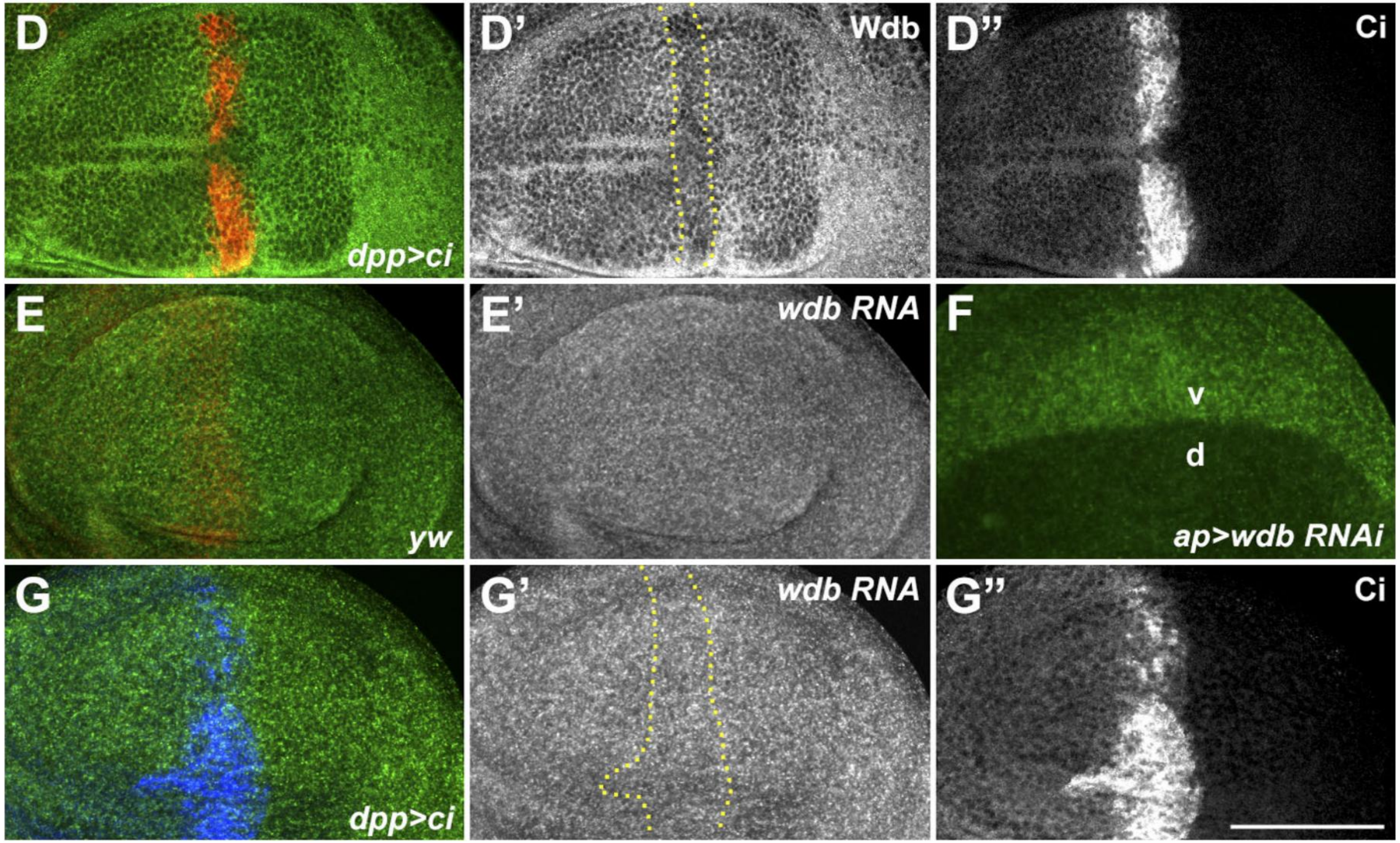


Zhu et.al,2011



3 Research

3.6 PpV is regulated by the Hh signaling

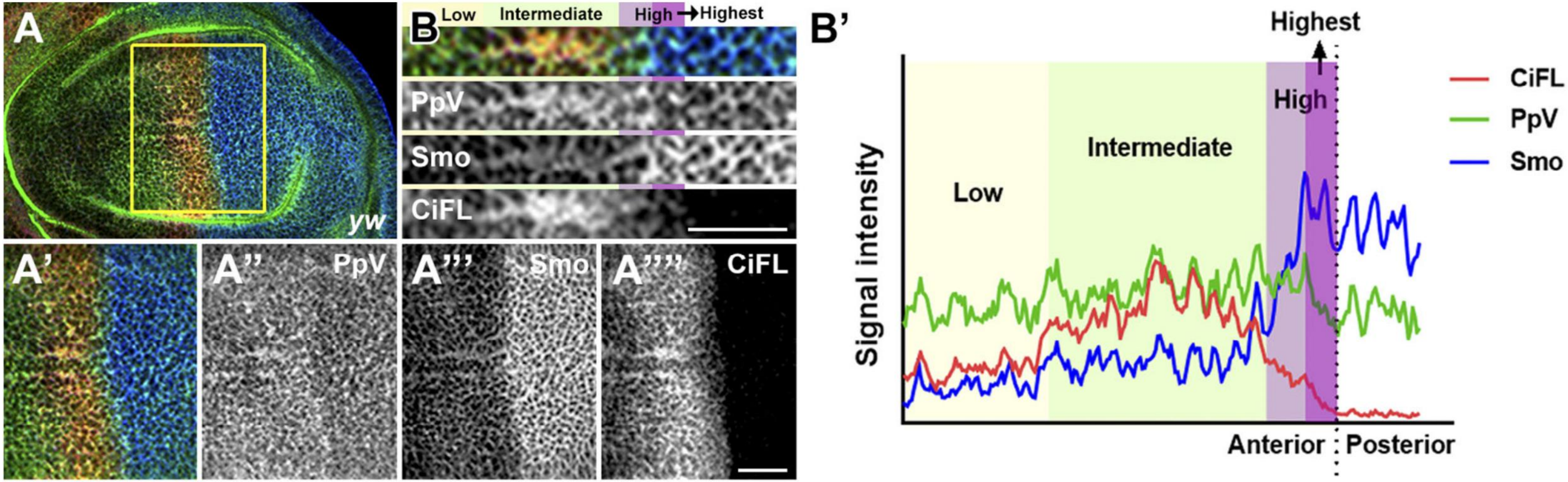


The inhibition of Wdb by Hh signal comes from post-transcriptional regulation

3 Research

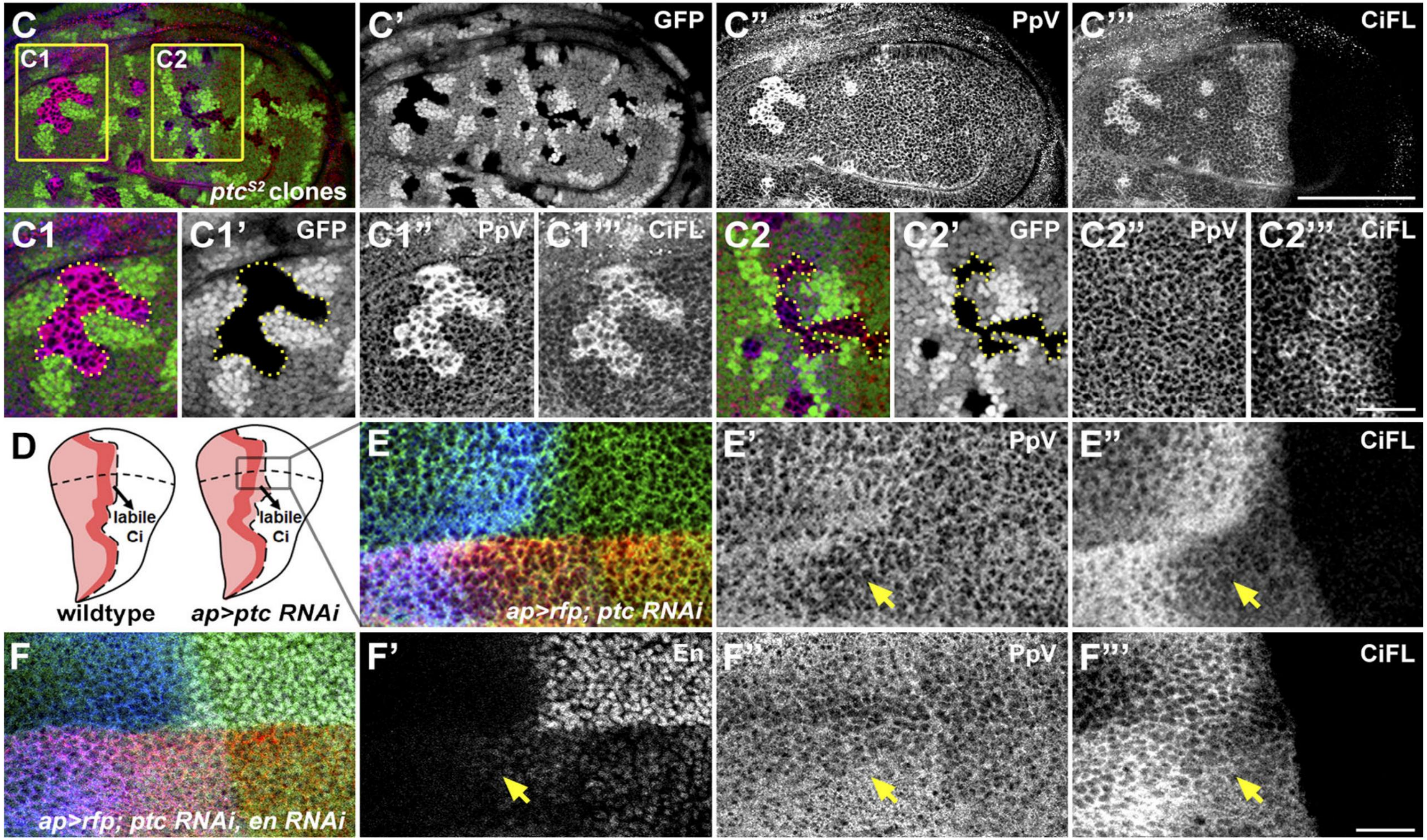
3.6 PpV is regulated by the Hh signaling

- **From ‘low’ to ‘high’, the signal intensity of PpV increases gradually**
- **But at the ‘highest’, PpV signal strength drops rapidly**



3 Research

3.6 PpV is regulated by the Hh signaling

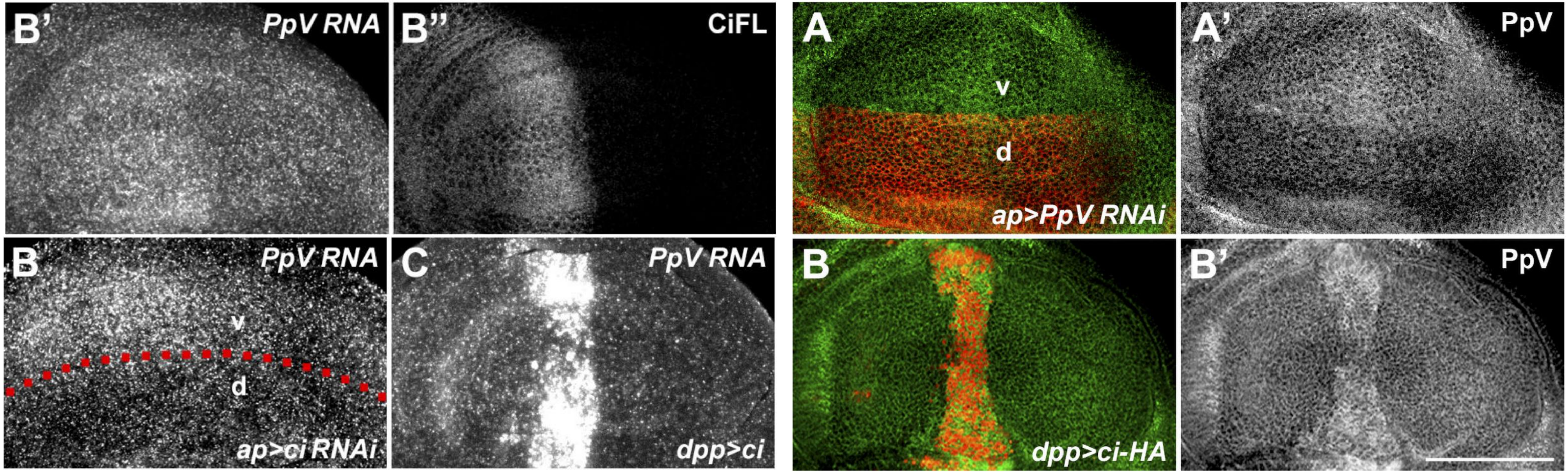


The expression of PpV correlates with graded Hh signaling

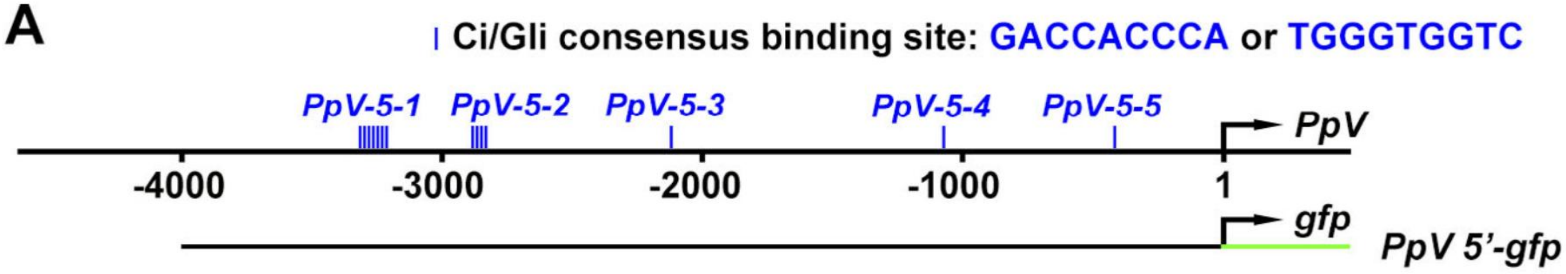
3 Research

3.7 PpV is a genuine Hh transcriptional target

Hh signaling may regulate PpV at the transcriptional level

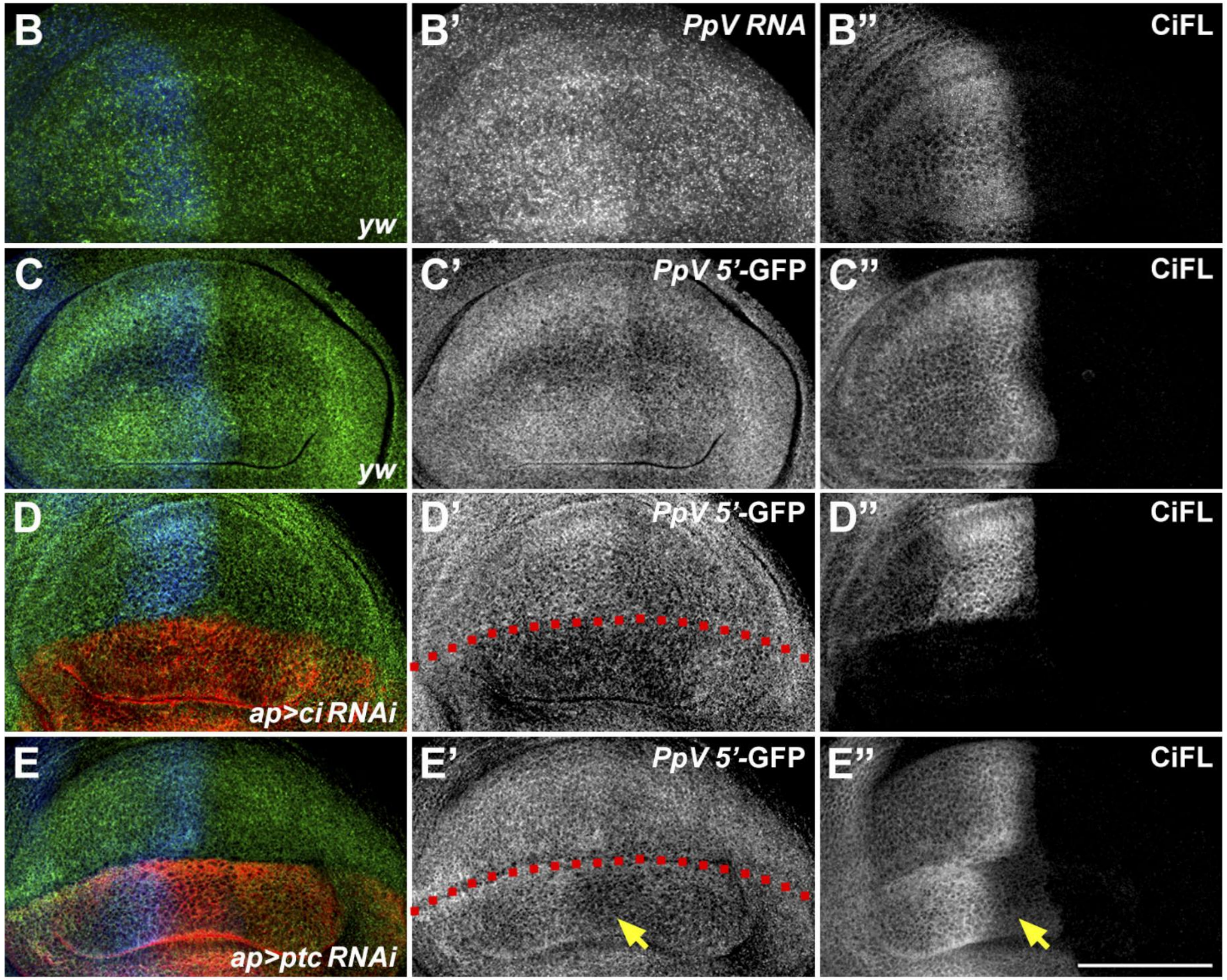


Genomatix MatInspector



3 Research

3.7 PpV is a genuine Hh transcriptional target

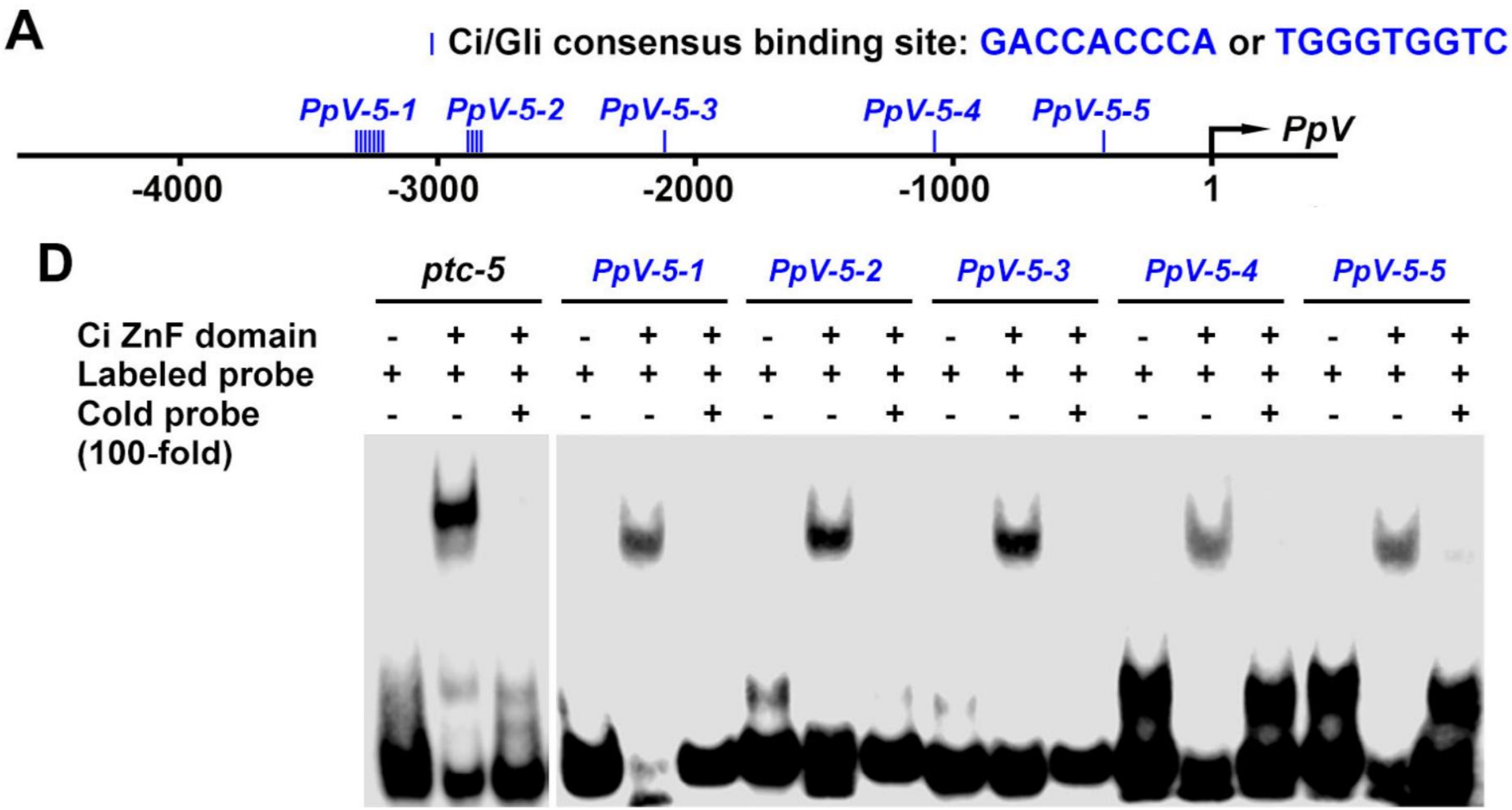


Ppv 5 '-GFP cloning of the PpV promoter reproduces the expression pattern of Hh signal for PpV regulation

3 Research

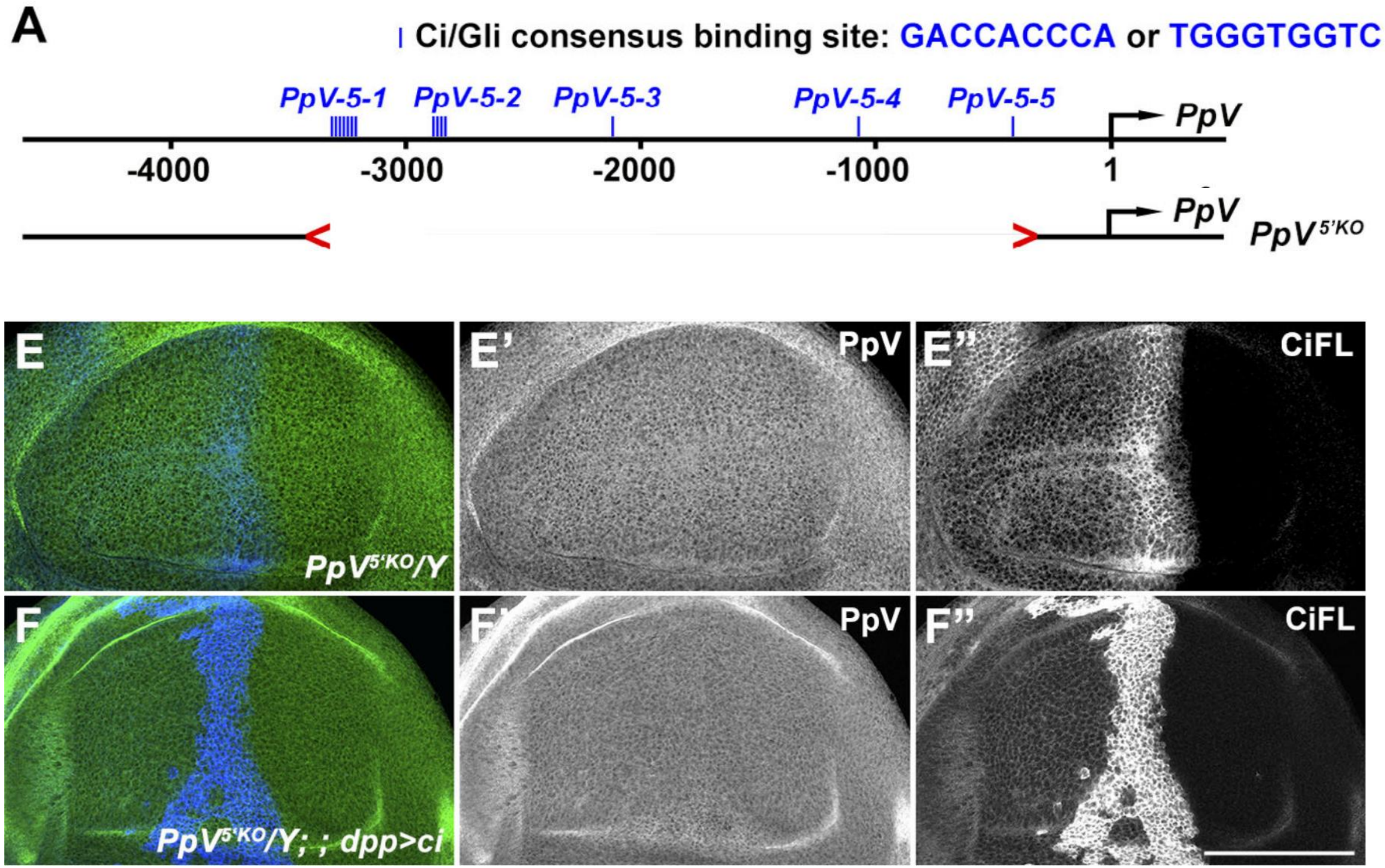
3.7 PpV is a genuine Hh transcriptional target

The EMSA experiment verified the specificity of Ci and Ci consensus binding site



3 Research

3.7 PpV is a genuine Hh transcriptional target



PpV^{KO} verification

PpV is a bona fide Hh signaling target whose transcription is maintained by graded Hh signaling!

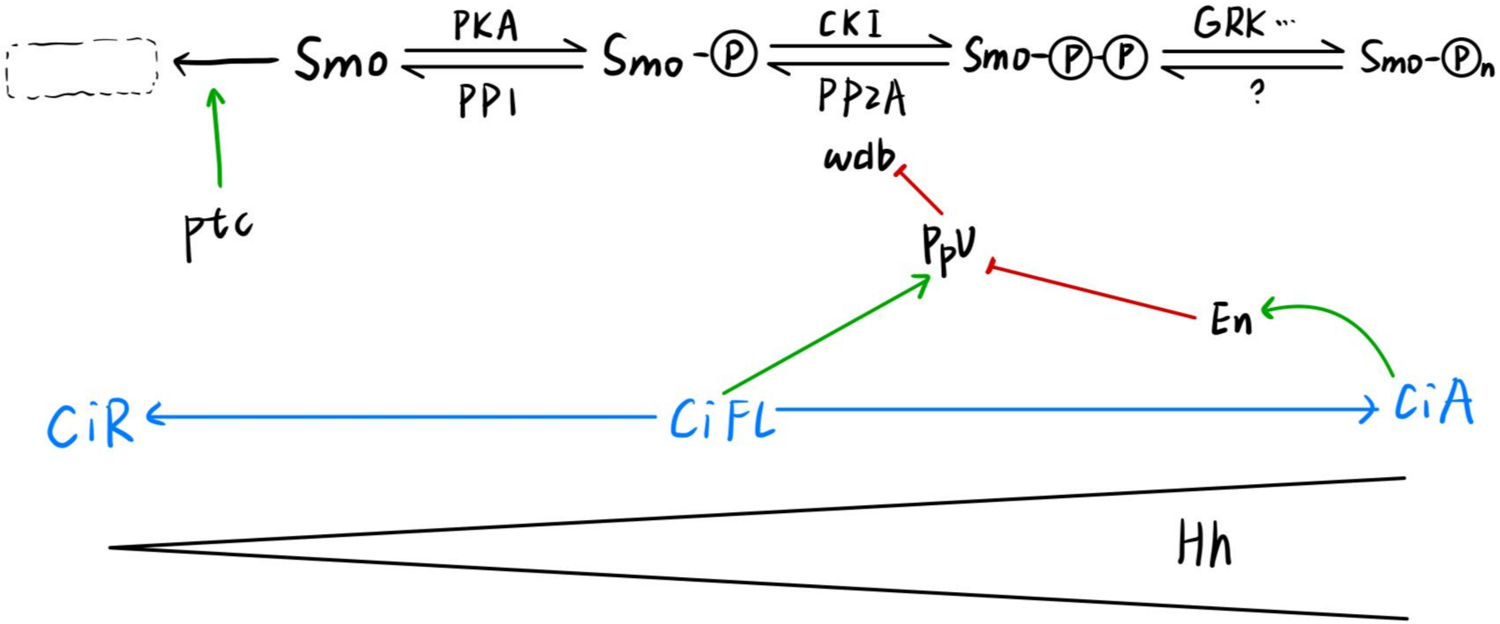
04

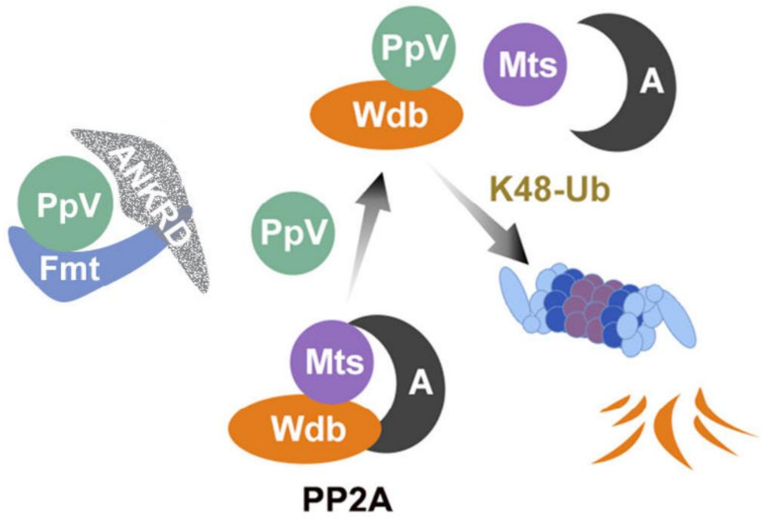
Discussion

4 Discussion

- **4.1 PpV acts as a graded Hh signaling sensor in the feedback control of Hh signaling homeostasis**
  - act both as a sensor and target of Hh signaling
  - participate in feedback regulatory loops
  - contribute to the robustness and reliability of Hh signaling

How to regulate?



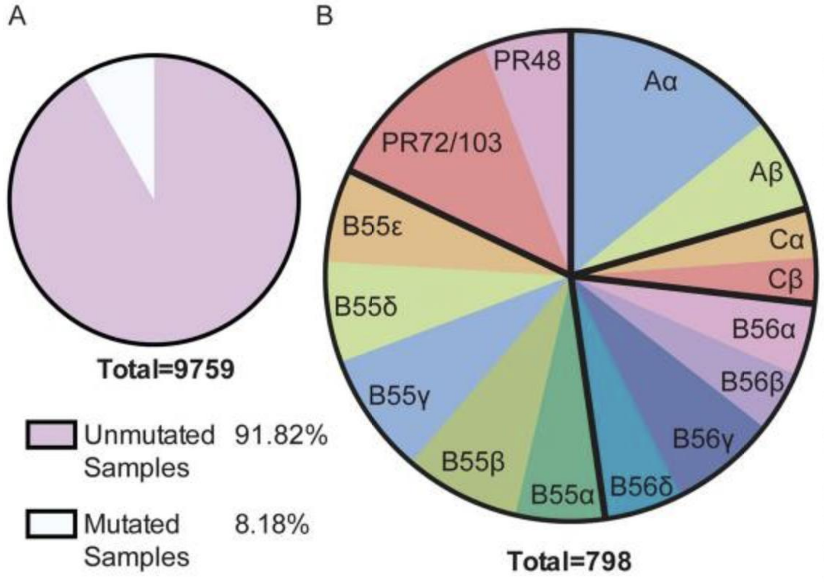


4 Discussion

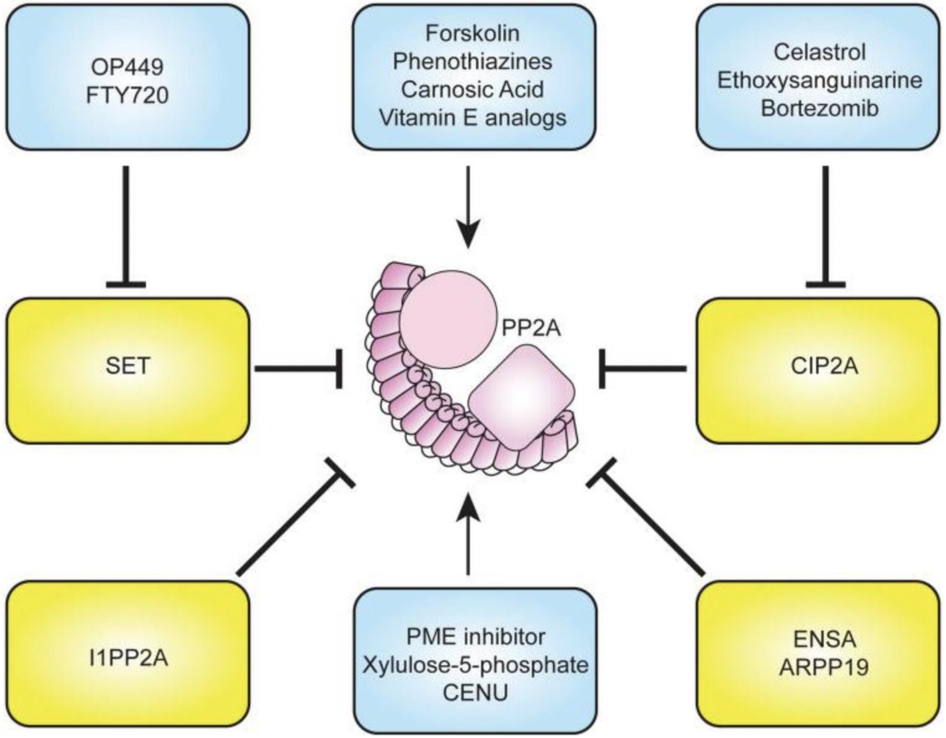
- **4.2 What are the applications of this discovery?**

PP2A:cancer suppressor gene

Screening of disease agents targeting PP2A disorders, especially cancer agents



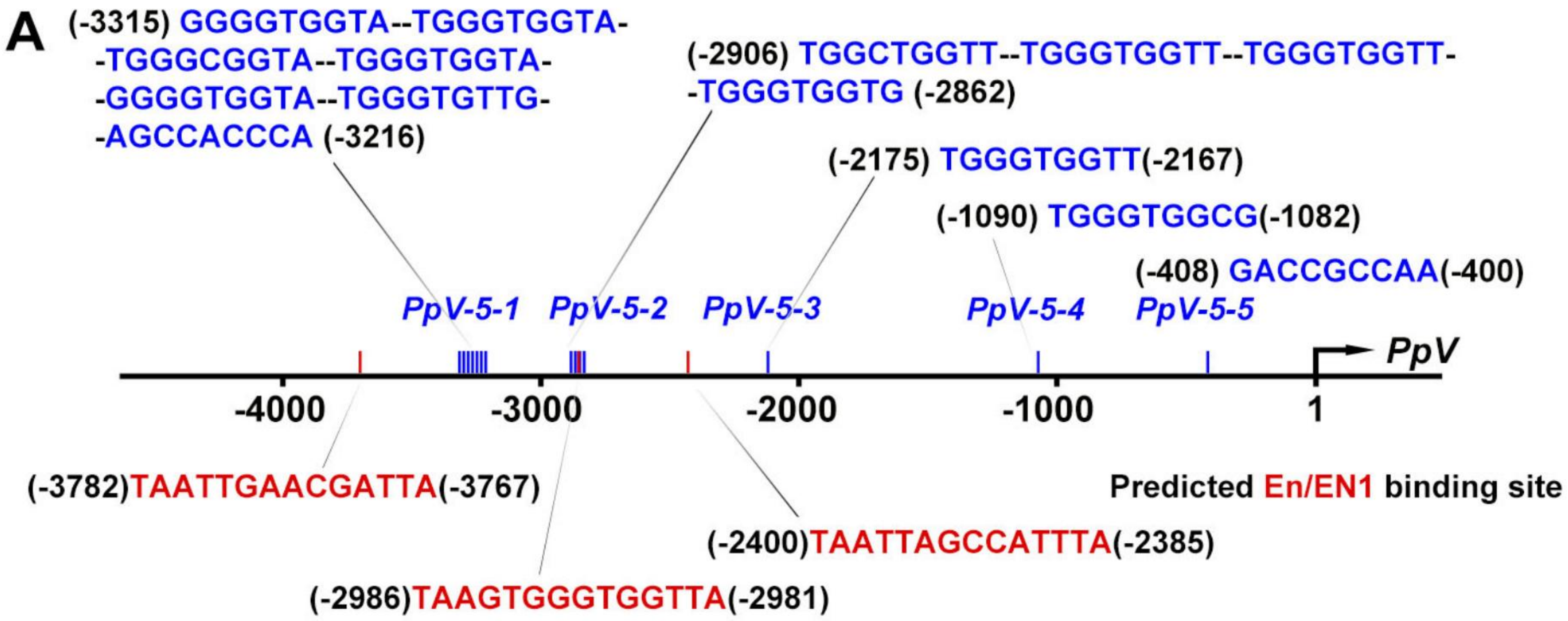
Name	Gene	Percentage
Aα	PPP2R1A	14.29%
Aβ	PPP2R1B	6.27%
Cα	PPP2CA	3.26%
Cβ	PPP2CB	3.01%
B56α	PPP2R2A	4.76%
B56β	PPP2R2B	4.01%
B56γ	PPP2R2C	7.02%
B56δ	PPP2R2D	5.14%
B55α	PPP2R5A	5.89%
B55β	PPP2R5B	7.64%
B55γ	PPP2R5C	8.02%
B55δ	PPP2R5D	6.64%
B55ε	PPP2R5E	6.14%
PR72/103	PPP2R3A	12.16%
PR48	PPP2R3B	5.76%



Sangodkar,2016

4 Discussion

- **4.3 Possible future research directions**
  - How does En regulate PpV at the transcriptional level
  - How does PpV sense Hi signal concentration
  - Formation principle of Hh concentration gradient
  - ......



4 Discussion

- **4.4 Difference between Modern and traditional research methods**

What's the difference between the traditional biochemical paradigm shown in the main paper and the modern molecular and genetical paradigm shown in the supplementary paper?

- Traditional
  - More macroscopic
  - Various chemical reagents
  - Organic chemistry experiment
  - Filtration, extraction...
  - Chromatography
  - Electrophoresis
  - Radioactivity detection
  - Condition-based exploration of chemical molecules
- Morden
  - microcosmic
  - Gene cloning and transfection
  - Gene editing technology
  - Gene knockout and RNAi
  - In situ hybridization
  - Fluorescence imaging technique
  - Antibodies and immunological techniques

4 Discussion

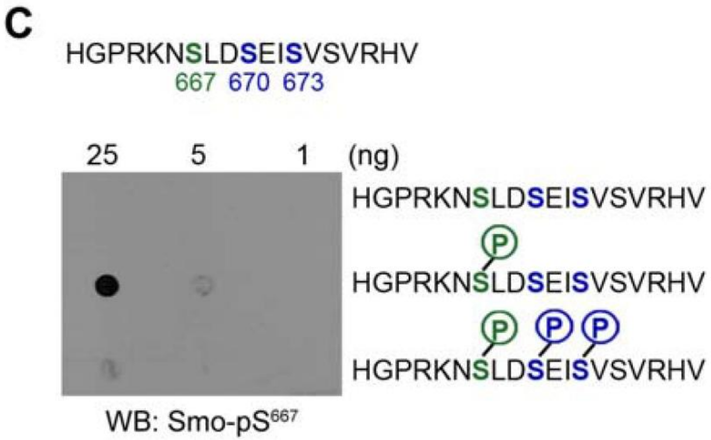
- **4.5 Modern research methods of protein phosphorylation**
  - How to prove that phosphorylase is phosphorylated when phosphorylase b is converted to phosphorylase a?
  - How do we study protein phosphorylation today in a modern way?
  - How do we study different phosphorylases?

Name	Class	Reaction
PhosPhorylase	Transferase (EC 2.4,2.7.7)	A−B + H−OP ⇌ A−OP + H−B
Phosphatase	Hydrolase (EC 3.1.3)	P−B + H−OH ⇌ P−OH + H−B
Kinase	Transferase (EC 2.7.1-2.7.4)	P−B + H−A ⇌ P−A + H−B

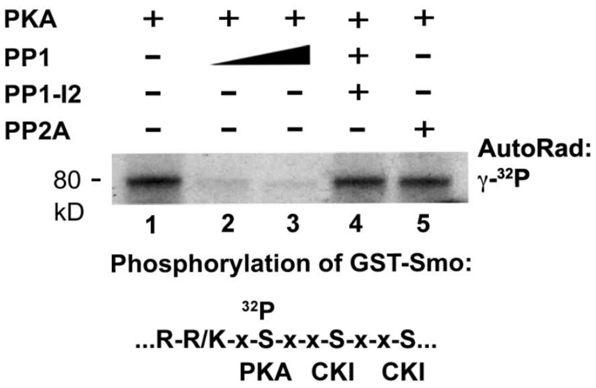
From wikipedia

4 Discussion

- Specific antibody detection

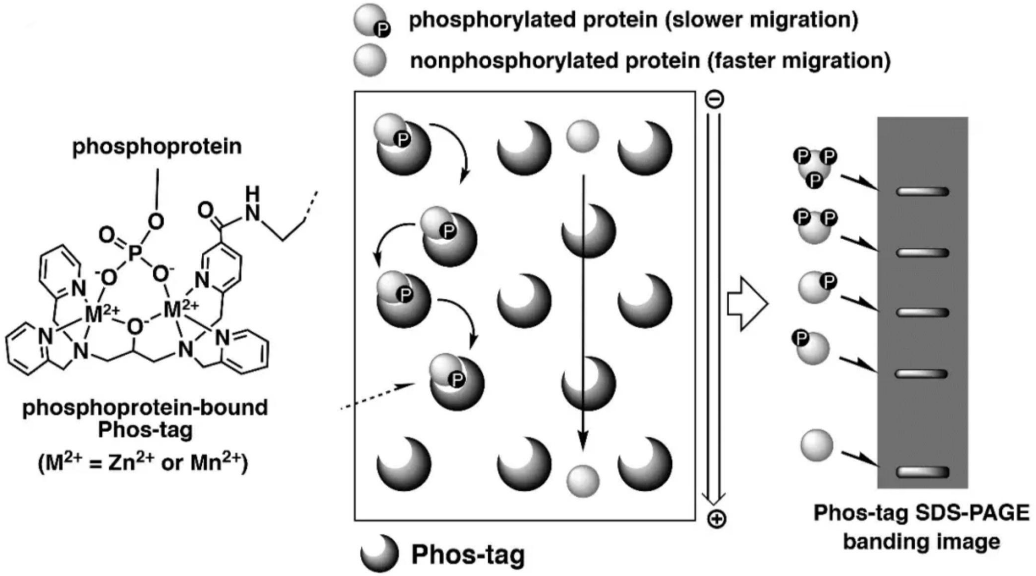


- Isotope detection



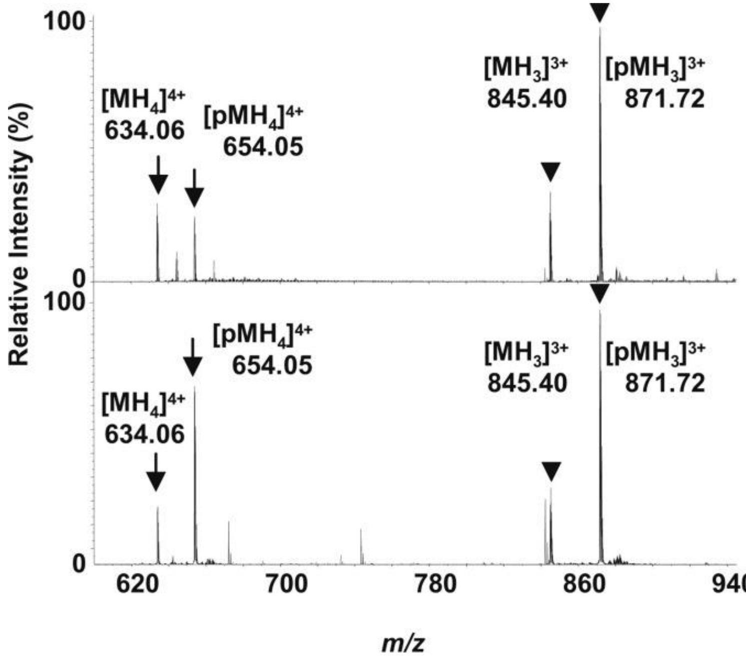
Liu,2011

- Phos-tag SDS-PAGE



Ke,2022

- MS



Steen,2006

4 Discussion

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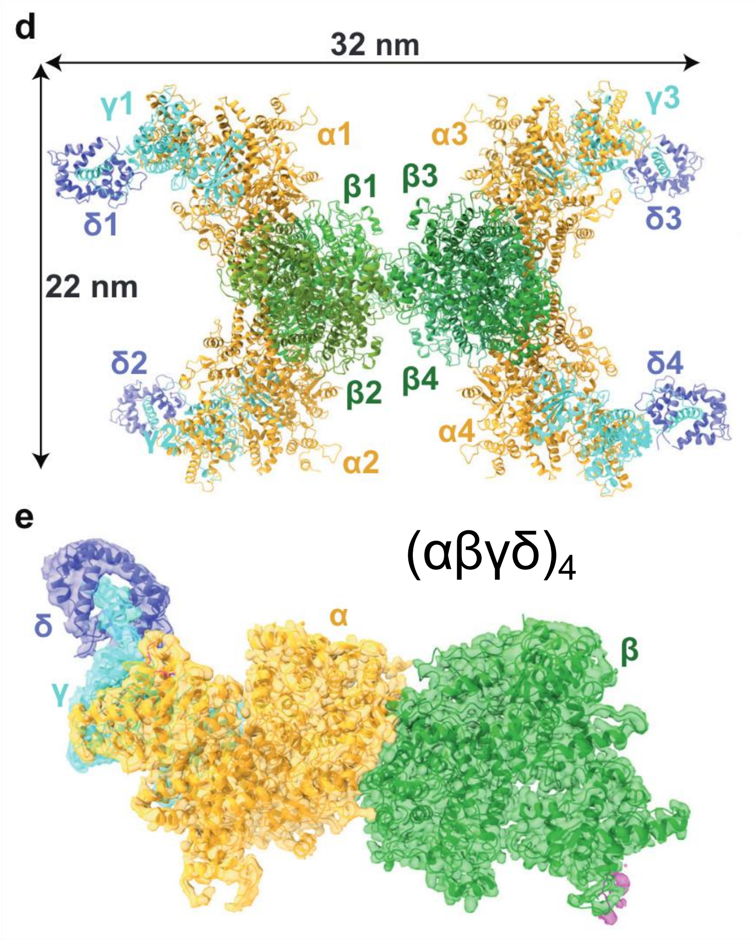
- 4.6 What activates the proteins(PhK) required to catalyze the conversion of phosphorylases b and a?

liver: AD→α_1R→G_q→PLCβ→IP_3→ER→Ca^{2+}
muscle: AP→DHPR→RyR1→SR→Ca^{2+}

Group	Type	Example
AGC	Ser/Thr	PKAI/II,PKCc/n/a,PKGI/II,GRK
CAMK	Ser/Thr	CaMK,PhK,eEF2K,MLCK,(AMPK)
CK1	Ser/Thr	CK1,TTBK,VRK
CMGC	Ser/Thr	CDK,CDKL,MAPK,GSK3,CLK,SRPK,DYRK,RCK,CK2
STE	Ser/Thr	STE20,STE11,STE7
TK	Tyr	RTK:EGFR,VEGFR,EphR,INSR CTK:Src,JAK
TKL	Ser/Thr/Tyr	TGFBR,Raf
Atypical	Ser/Thr	PDHK,ATM,ATR,mTOR
Other	Ser/Thr	Ikk,Wee

Manning et.al,*Science*,2002

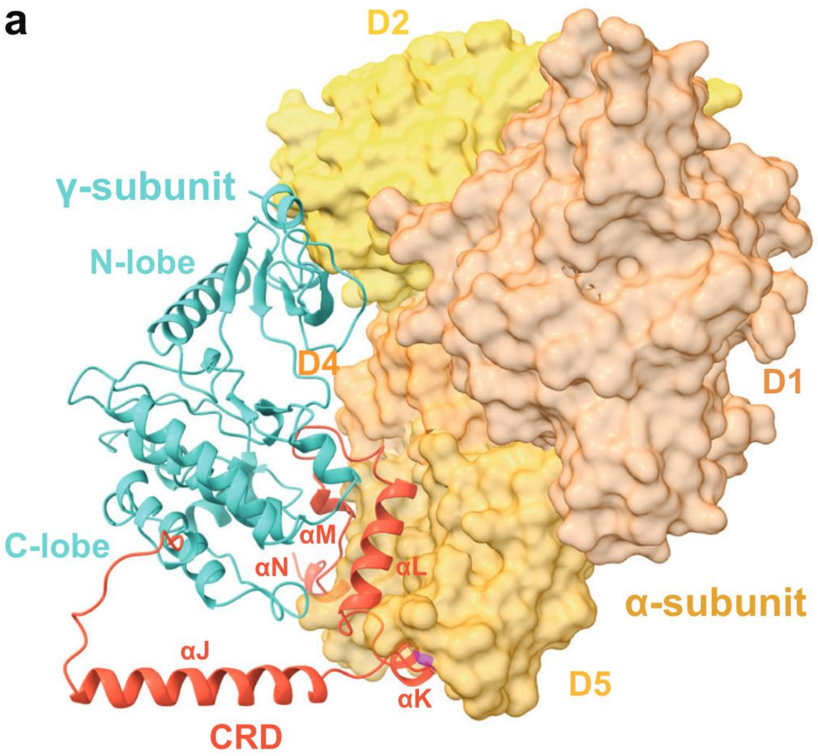
PKA **Ca^{2+}**

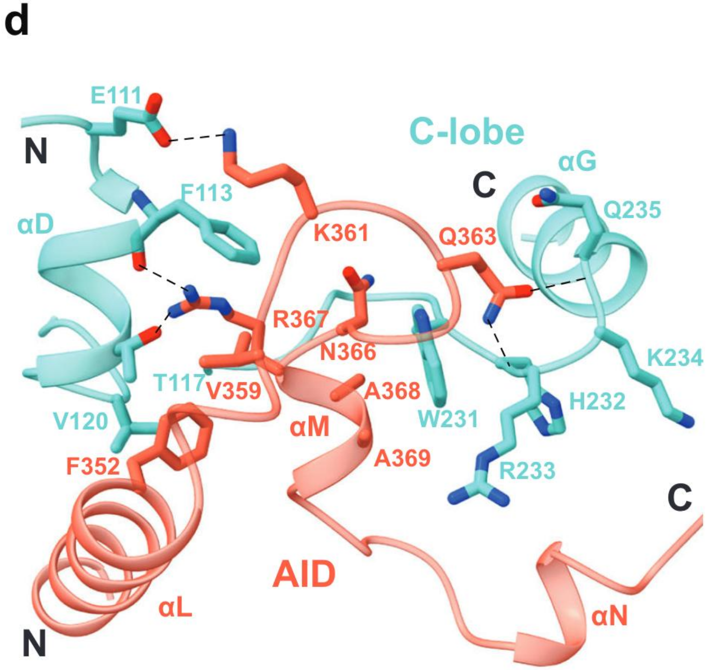


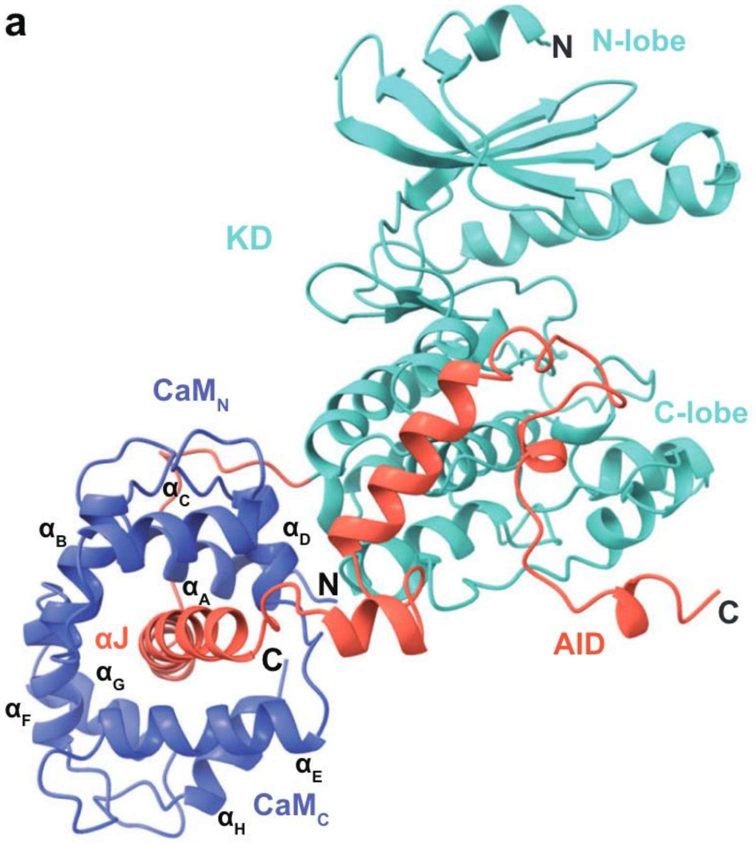
Xiao et.al,2024

4 Discussion

The KD domain of the γ subunit binds to the α subunit, the αJ hydrophobic helix of the CRD domain binds to the δ subunit, and the AID domain is inserted between the C-lobe and D5

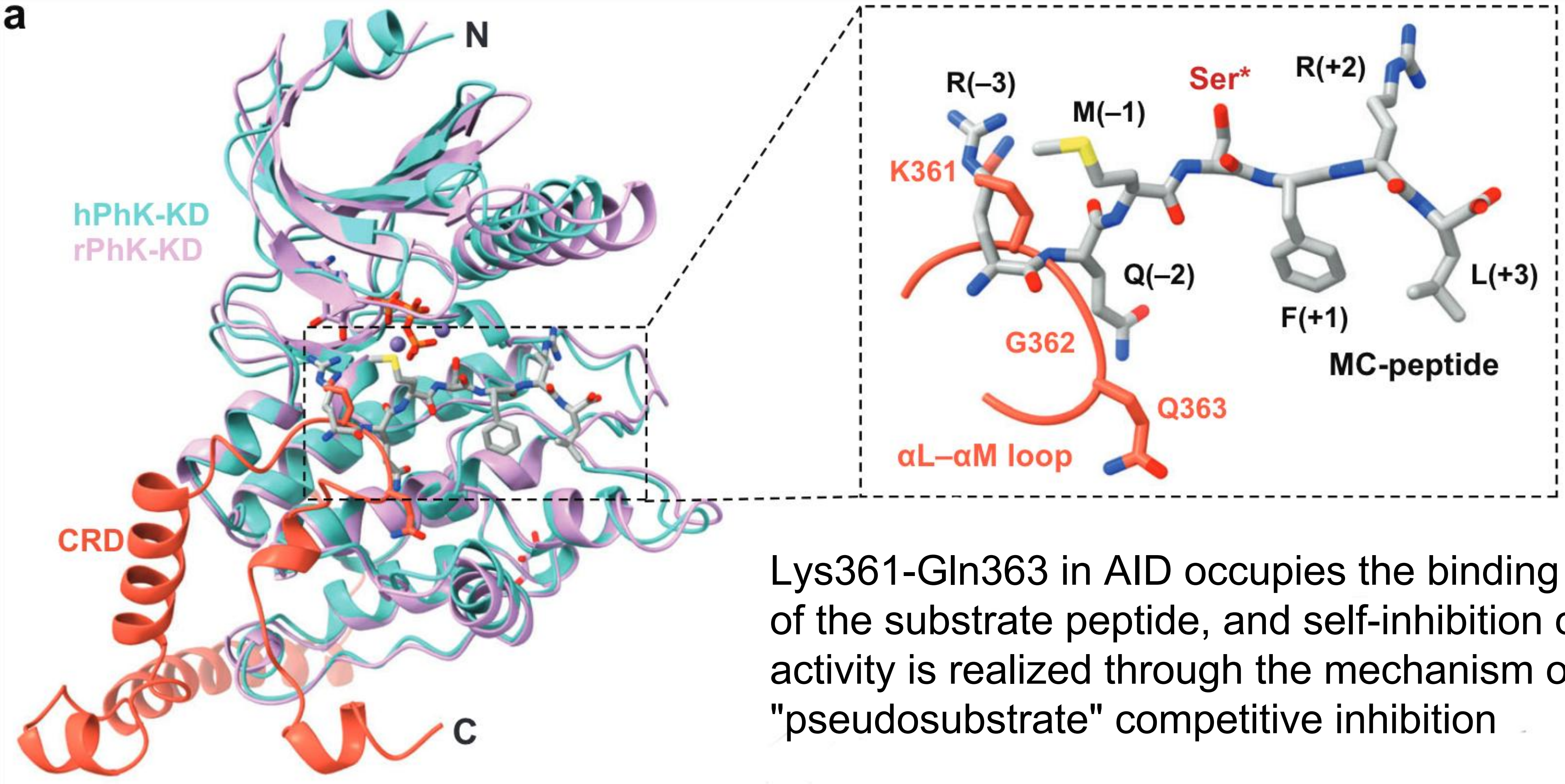






Xiao et.al,2024

4 Discussion

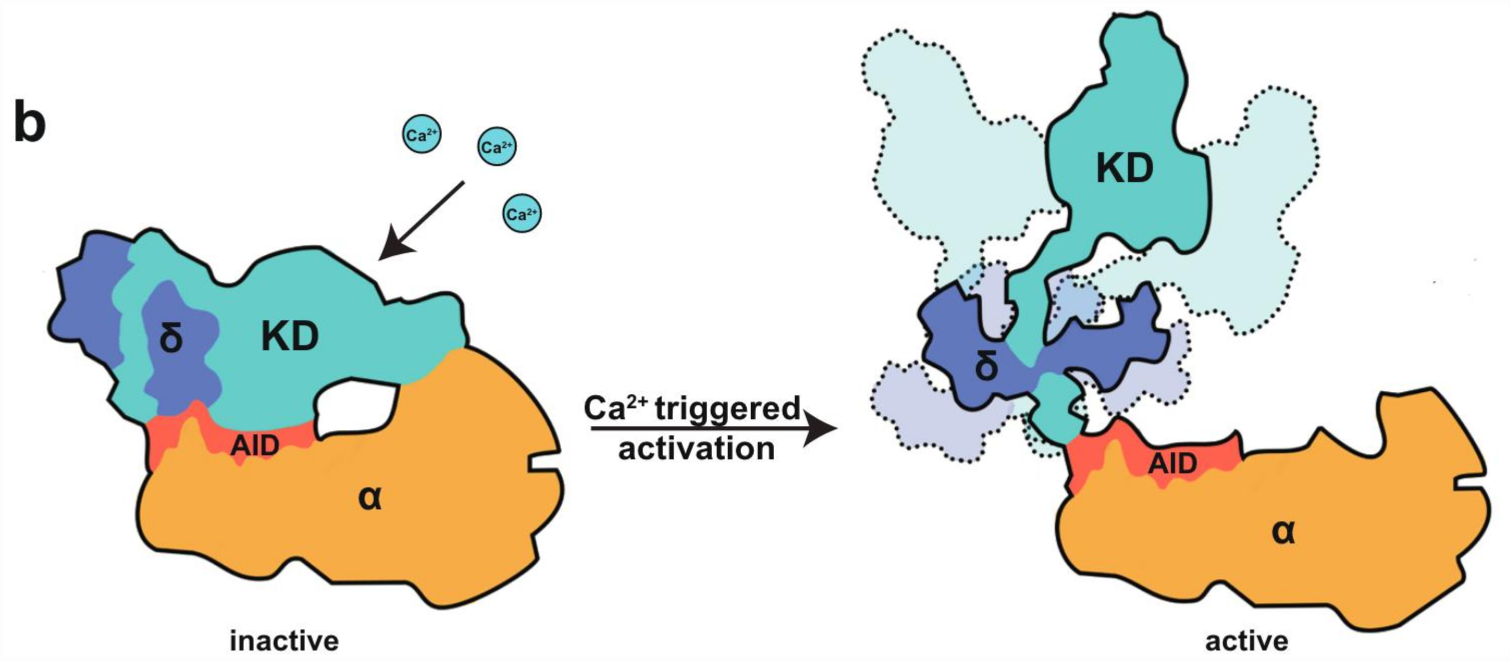


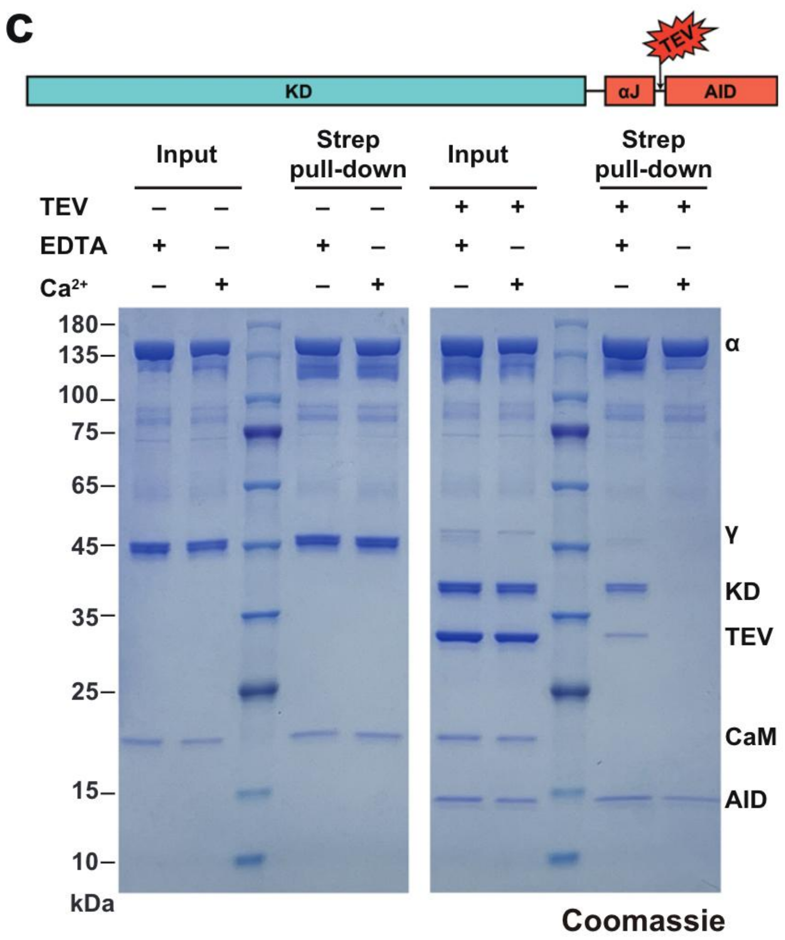
Lys361-Gln363 in AID occupies the binding position of the substrate peptide, and self-inhibition of kinase activity is realized through the mechanism of "pseudosubstrate" competitive inhibition

Xiao et.al,2024

4 Discussion

- **spring-loaded mechanism**





Xiao et.al,2024

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Thank You For your Listening！