The Oxygen Sensor Mechanism of PHD

2020/2/6

Literature Seminar

B4 Fujiyoshi

Contents

- Introduction
  - The Novel Prize in Physiology or Medicine 2019
  - Mechanism of hypoxia response
- Basic characteristics of PHD
- Structural analysis of PHD2
- Contribution of residue of PHD2 to O_2 dependent reactivity
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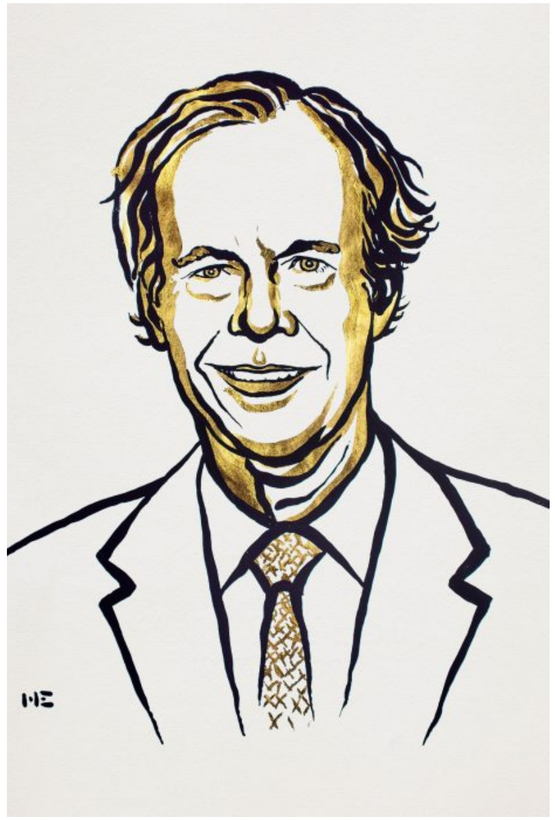
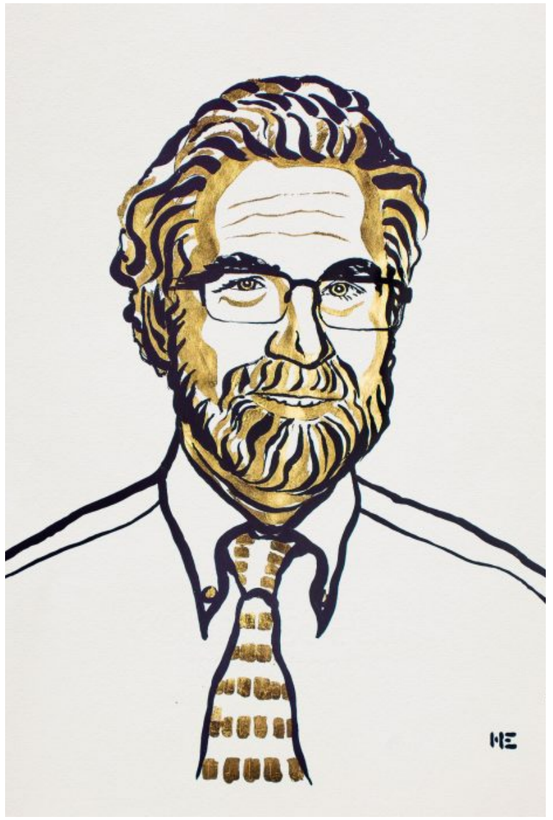
The Novel Prize in Physiology or Medicine 2019

Discovery: How cells sense and adapt to oxygen availability



William G. Kaelin Jr

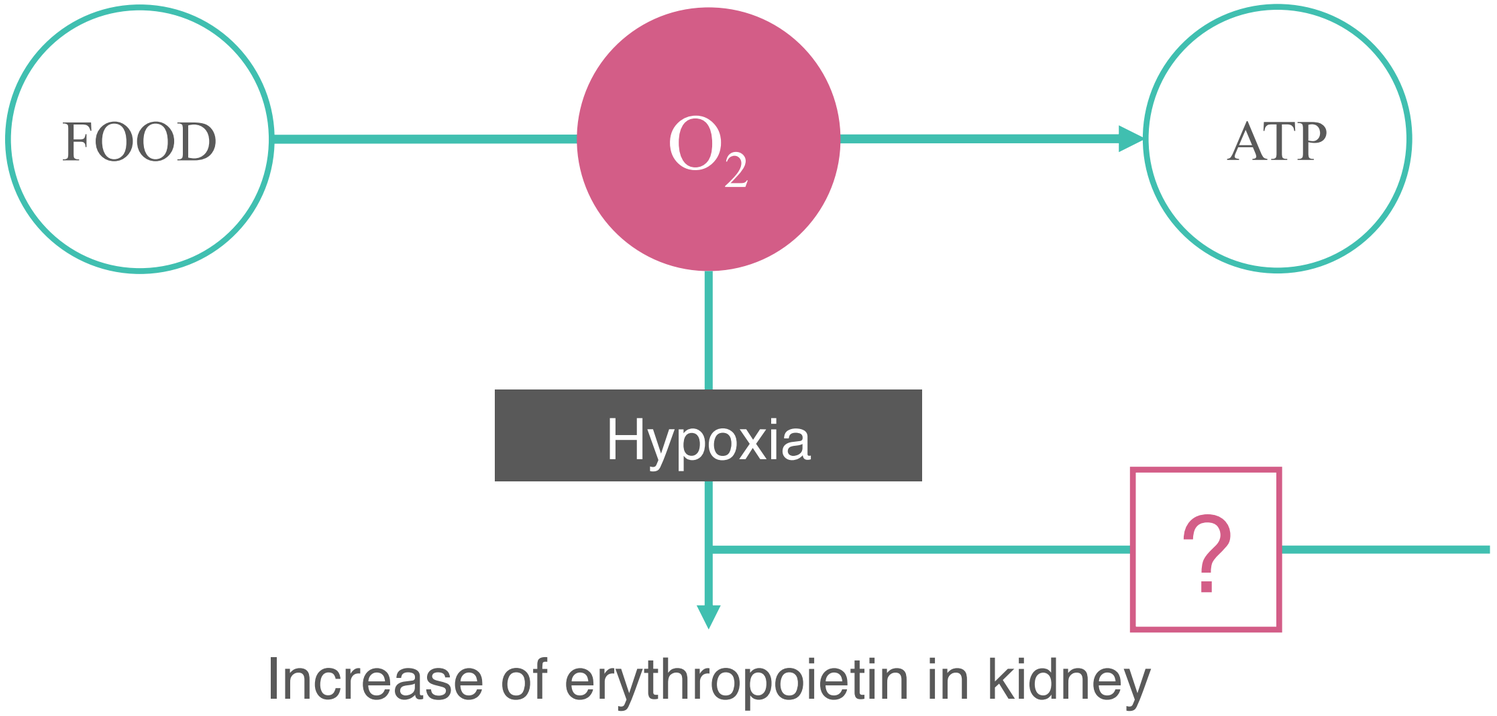
Sir Peter J. Ratcliffe



Gregg L. Semenza

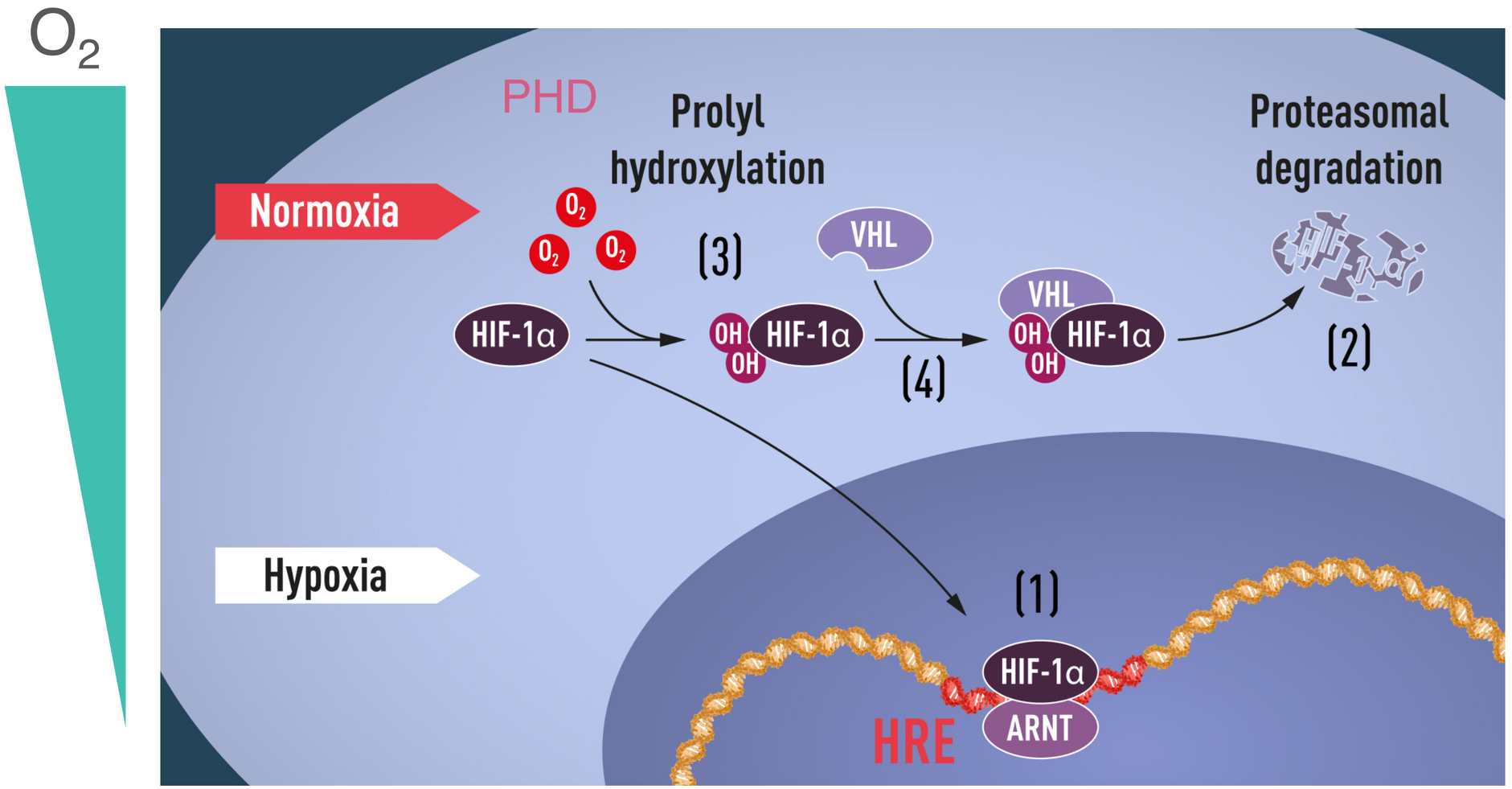
(The Nobel Prize in Physiology or Medicine 2019)

Question: How do cells sense and adapt to the oxygen availability?



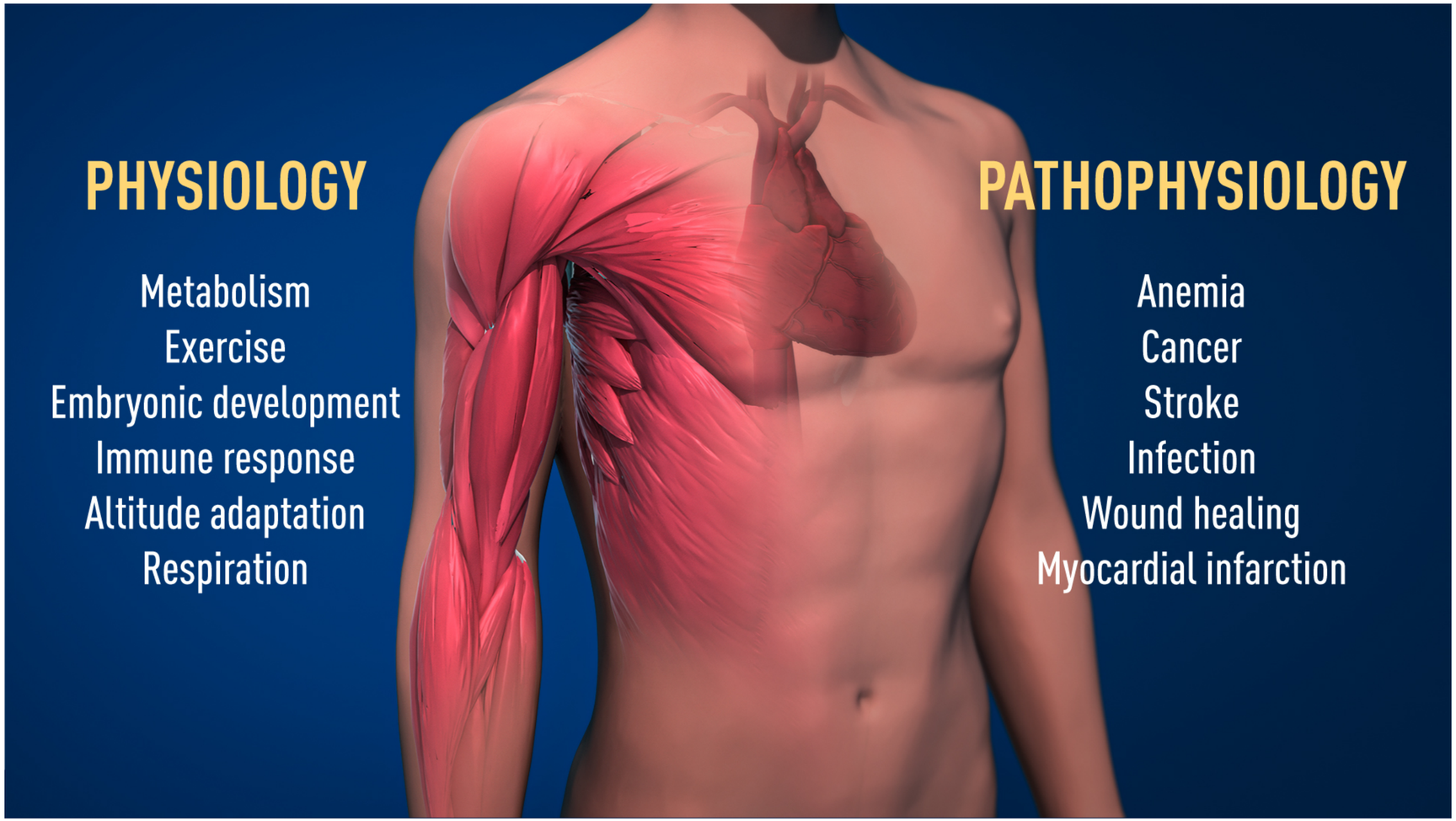
William G. Kaelin Jr., Sir Peter J. Ratcliffe and Gregg L. Semenza identified molecular machinery that regulates the activity of genes in response to varying levels of oxygen.

Overall mechanism of hypoxia response



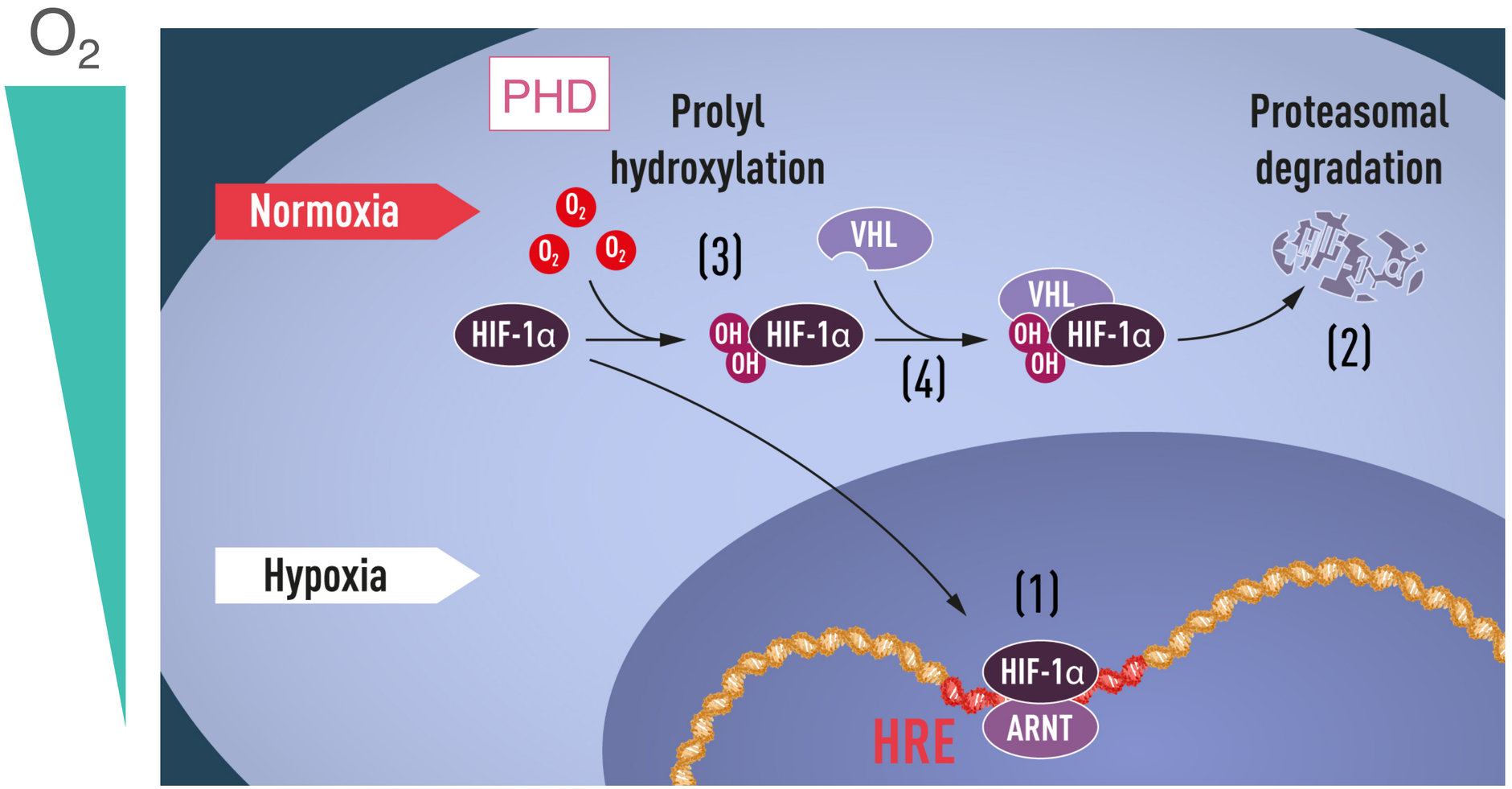
(The Nobel Prize in Physiology or Medicine 2019)

Physiology and pathology related to hypoxia response



(The Nobel Prize in Physiology or Medicine 2019)

Main topic: prolyl hydroxylase domain (PHD)



(The Nobel Prize in Physiology or Medicine 2019)

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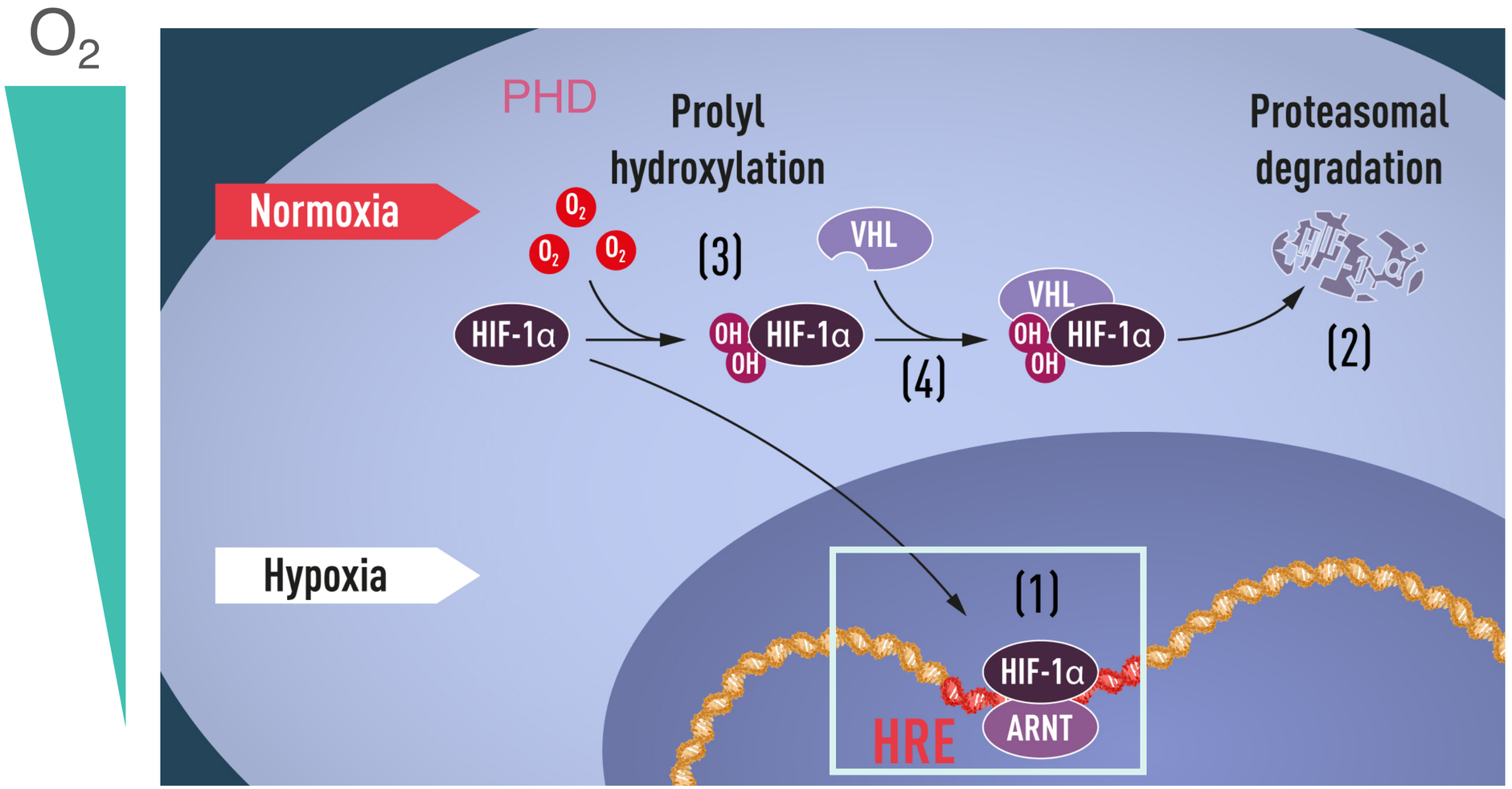
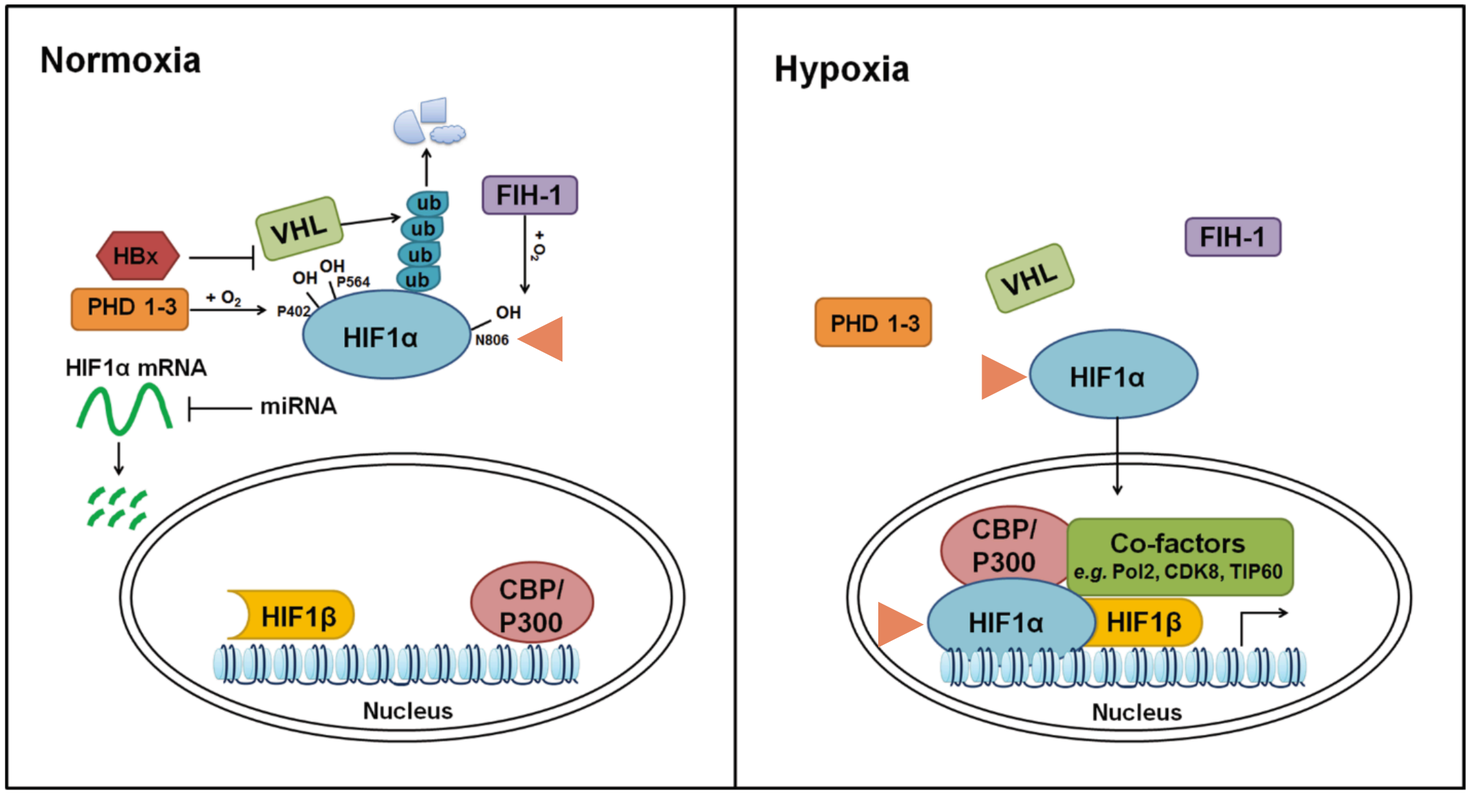
Transcriptional activation by HIF-α in hypoxia



(The Nobel Prize in Physiology or Medicine 2019)

Transcriptional activation by the interaction of HIF1α and p300/CBP

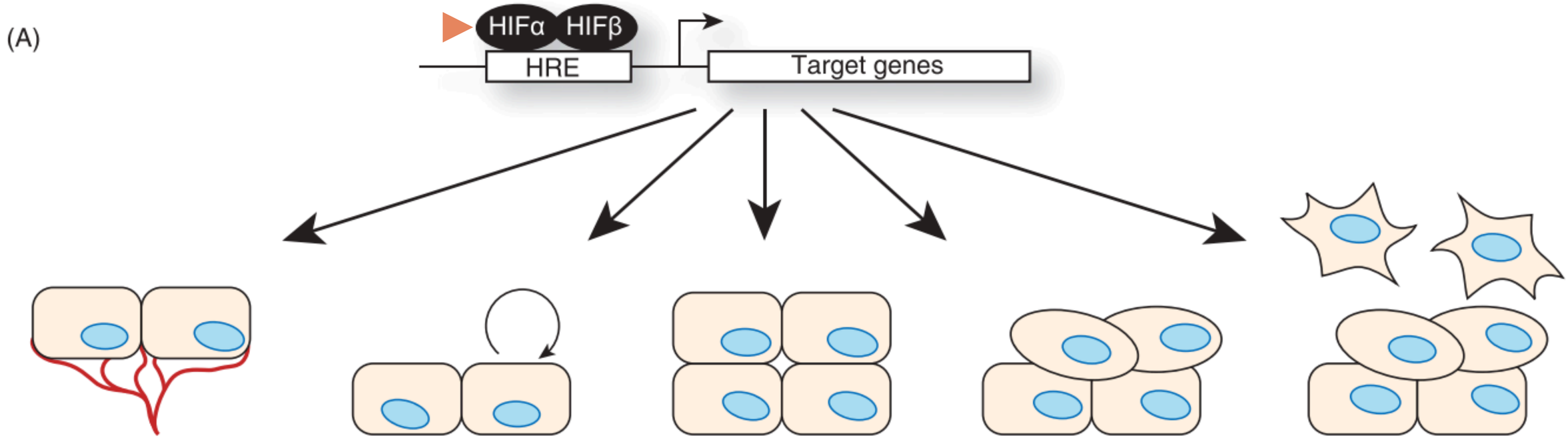


HIF1α in Hypoxia

1. No degradation
2. Nucleus translocation
3. Complex formation with HIFβ, CBP/p300 and other co-factors
4. Transcription activation

Chen, C., and Lou, T. (2017).
Oncotarget;Vol 8, No 28.

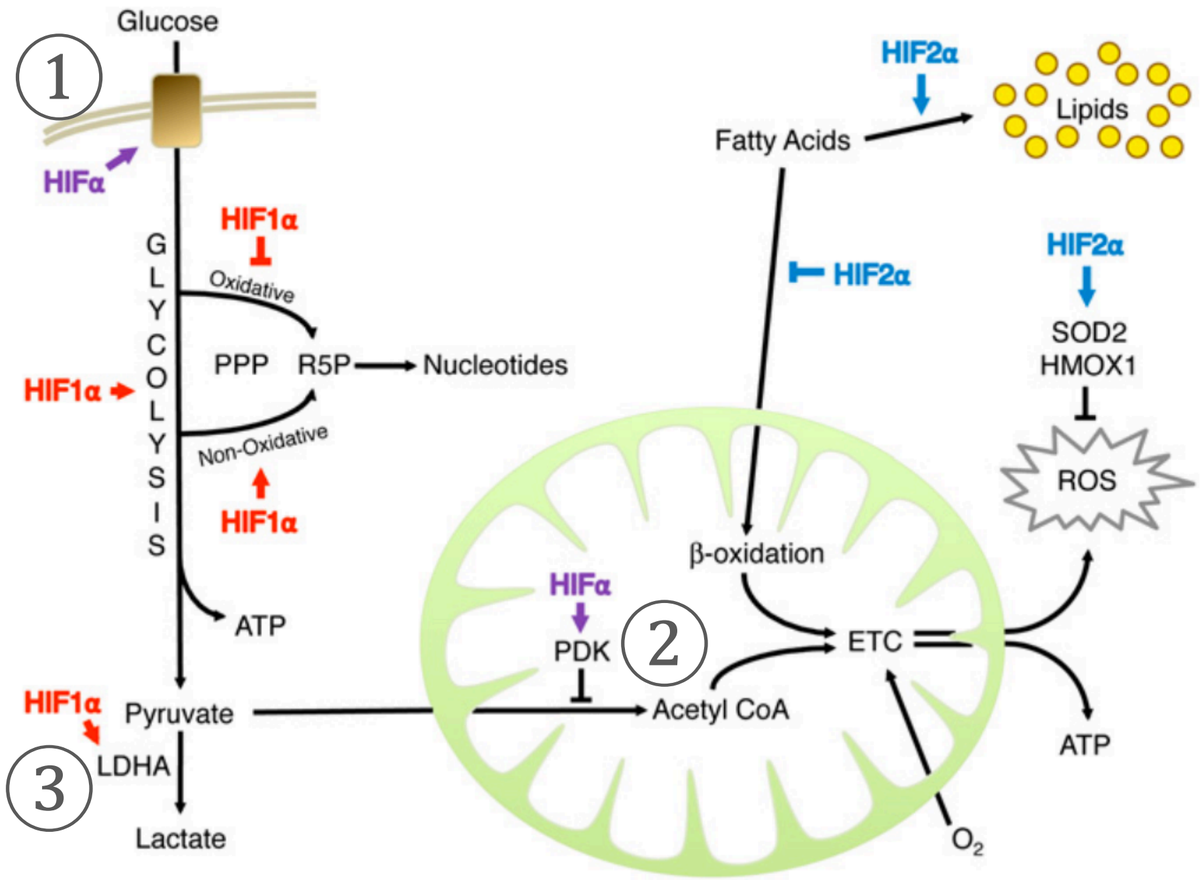
HIFα target genes for hypoxia response



Angiogenesis O_2 supply	Stemness Self renewal	Proliferation	EMT
Metabolism	Redox Homeostasis	Apoptosis	Metastasis Invasion

Dengler, V.L., Galbraith, M.D., and Espinosa, J.M. (2014). Crit. Rev. Biochem. Mol. Biol. 49, 1–15.

HIFα modulate cellur metabolism.



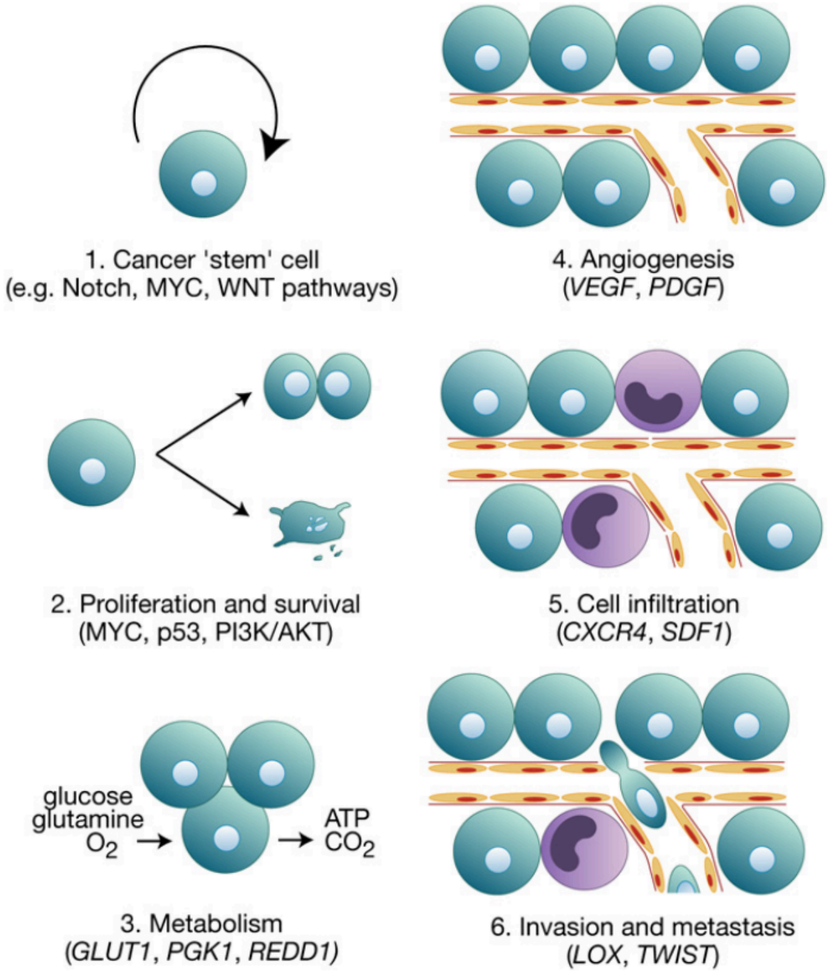
In hypoxia, the aerobic metabolism is switched to the anaerobic metabolism.

HIFα inducts

1. GLUT1
    1. To increase the glucose transportation (much glucose for anaerobic metabolism)
2. PDK
    1. To inhibit the conversion from pyruvate to acetylCoA (Stop TCA cycle)
3. LDHA
    1. To convert pyruvate to lactate (Oxidize NADH)

Majmundar, A.J., Wong, W.J., and Simon, M.C. (2010). Mol. Cell 40, 294–309.

Effect of HIFα on multiple steps of cansar development

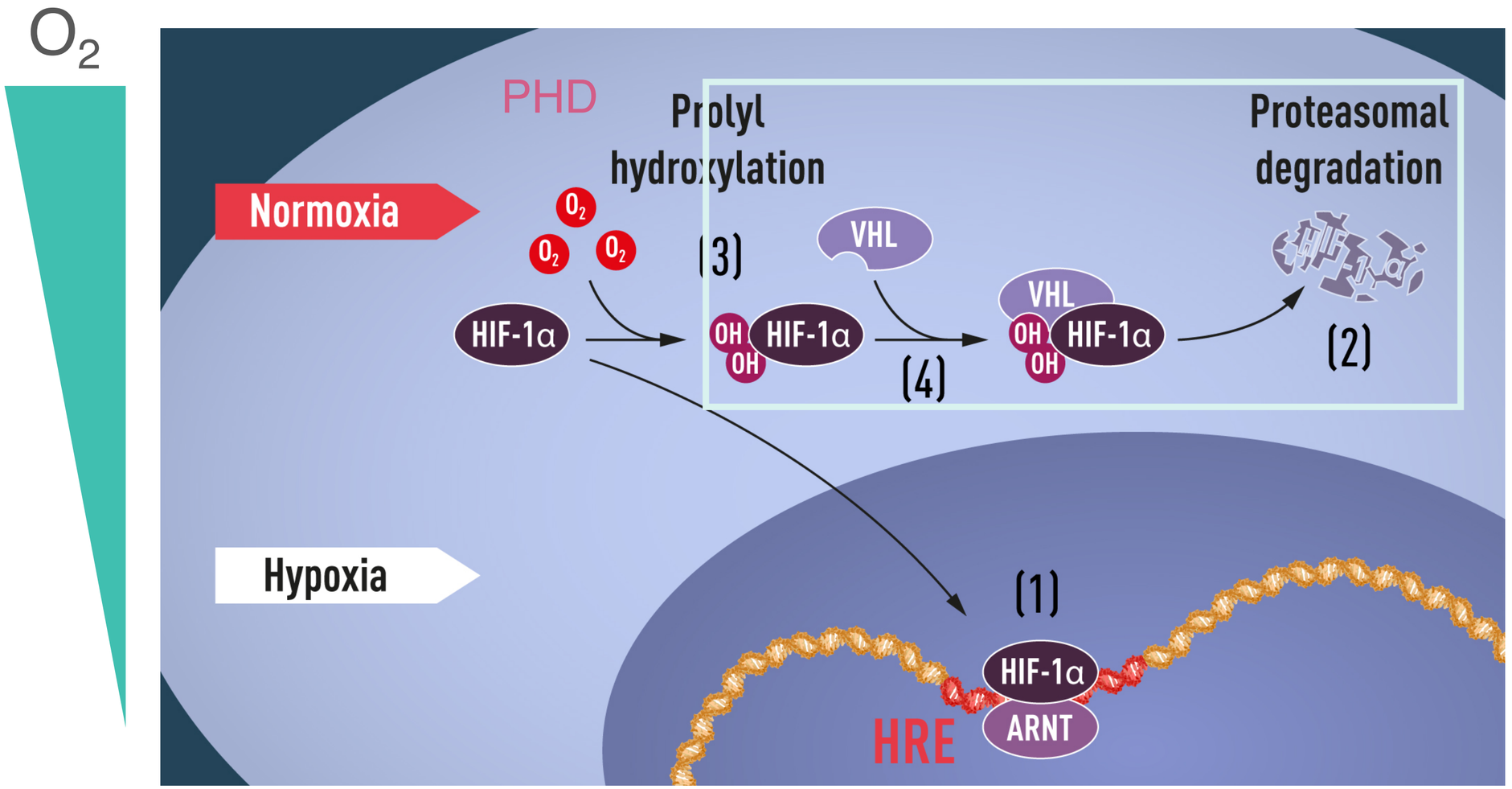


Rapidly proliferating cancer cells may outgrow their vascular network, limiting O_2 diffusion within the tumor.
→ Hypoxic stress
→ HIFα expression and the downstream activation of the hypoxic stress response are widespread in many cansers.

1. Canser stem cell
2. Proliferation and suvival
3. Metabolism
4. Angiogenesis
5. Cell infiltration
6. Invasion and metastasis

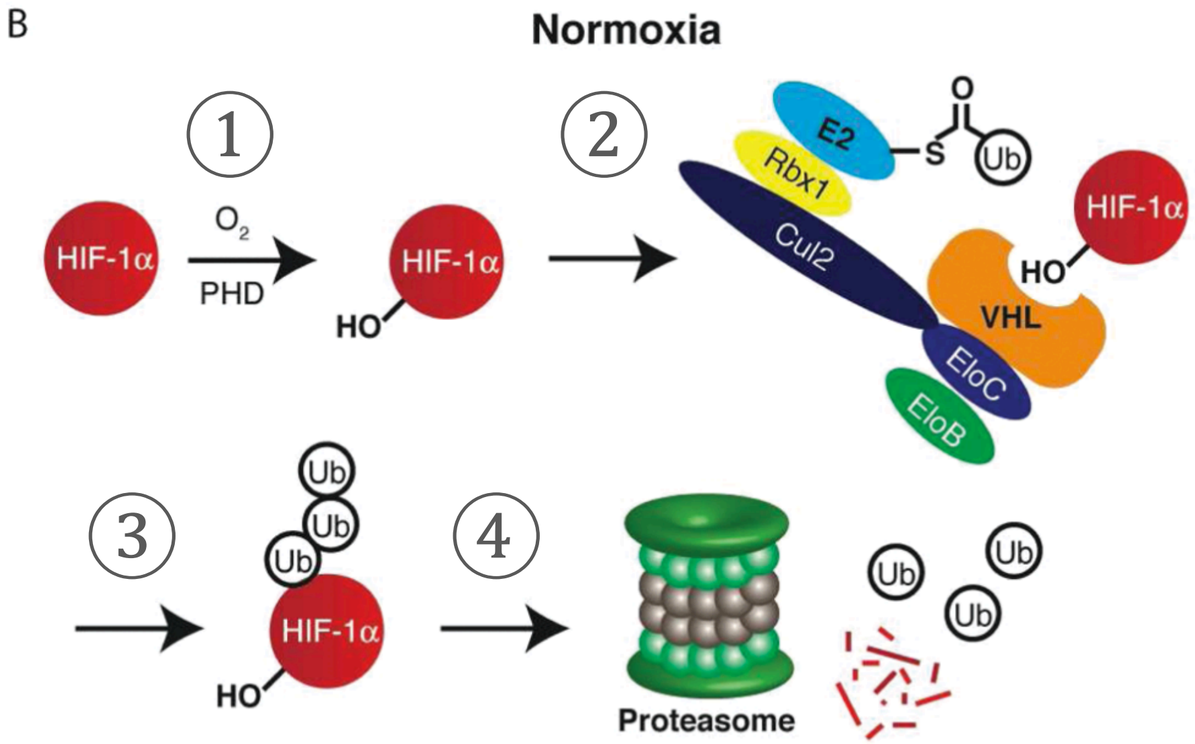
Majmundar, A.J, *et al.*,(2010)

VHL recognition of HIF-α and proteosomal degradation



(The Nobel Prize in Physiology or Medicine 2019)

HIFα is ubiquitination by E3 ligase and degraded by proteasome.

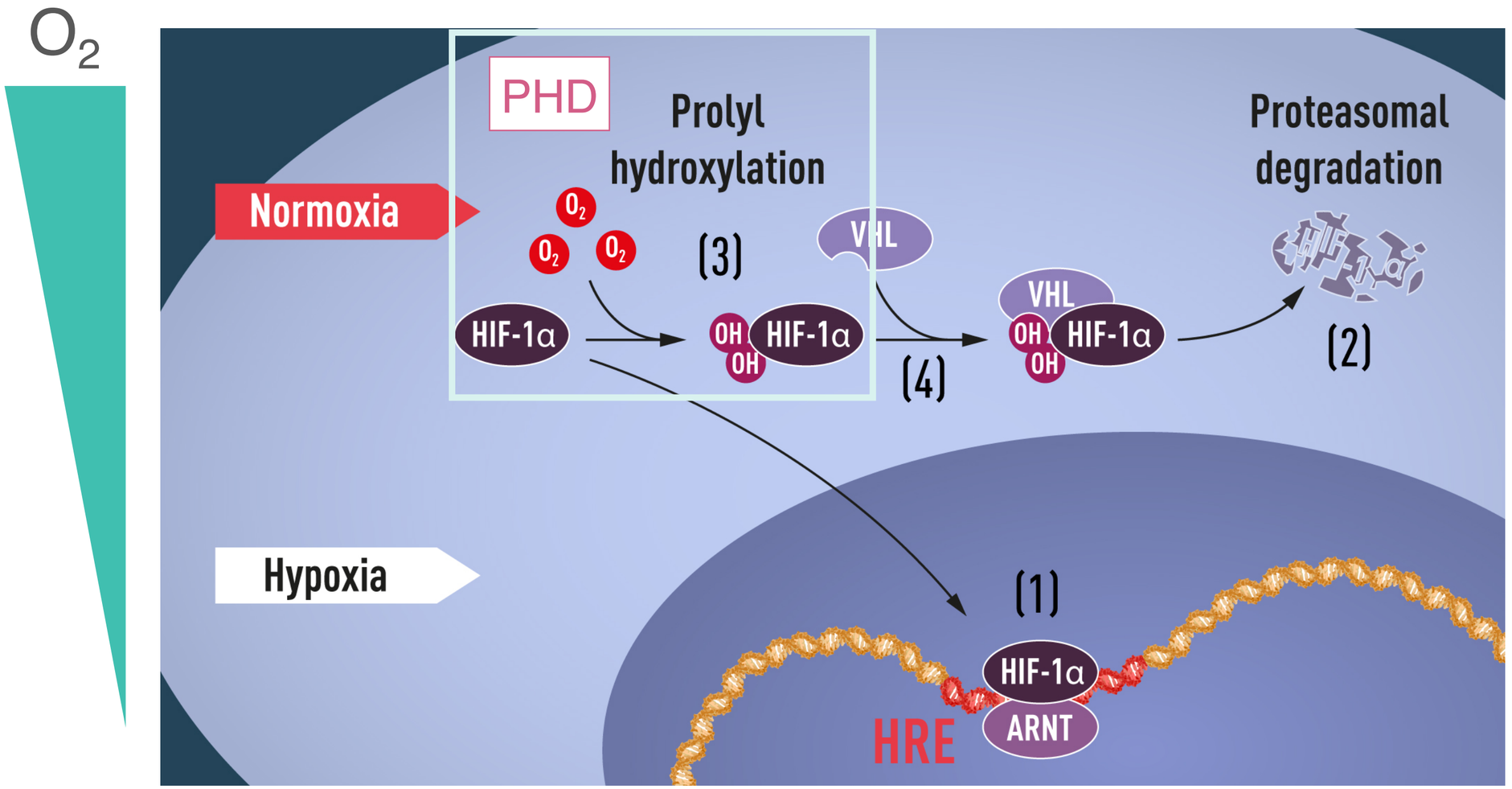


HIFα in normoxia

1. Hydroxylation of proline
2. Recognition of hydroxyproline by VHL
3. Ubiquitibnation by E2
4. Degradation by proteasome

Buckley, D.L., Van Molle, I., Gareiss, P.C., Tae, H.S., Michel, J., Noblin, D.J., Jorgensen, W.L., Ciulli, A., and Crews, C.M. (2012). J. Am. Chem. Soc. 134, 4465–4468.

PHD is an oxygen sensor in hypoxia response



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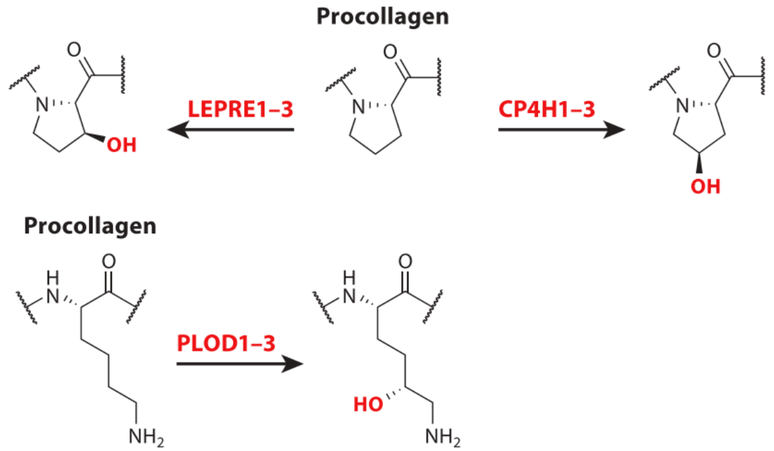
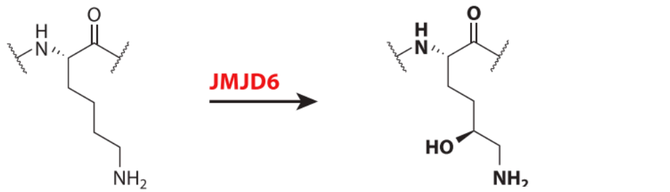
Fe(II)/2OG dependent oxygenase

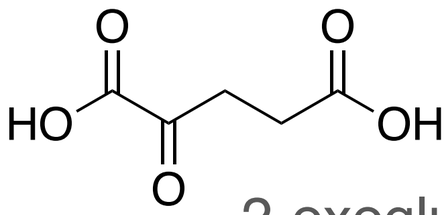
Hydroxylation of Pro in procollagen



Hydroxylation of Lys in histone

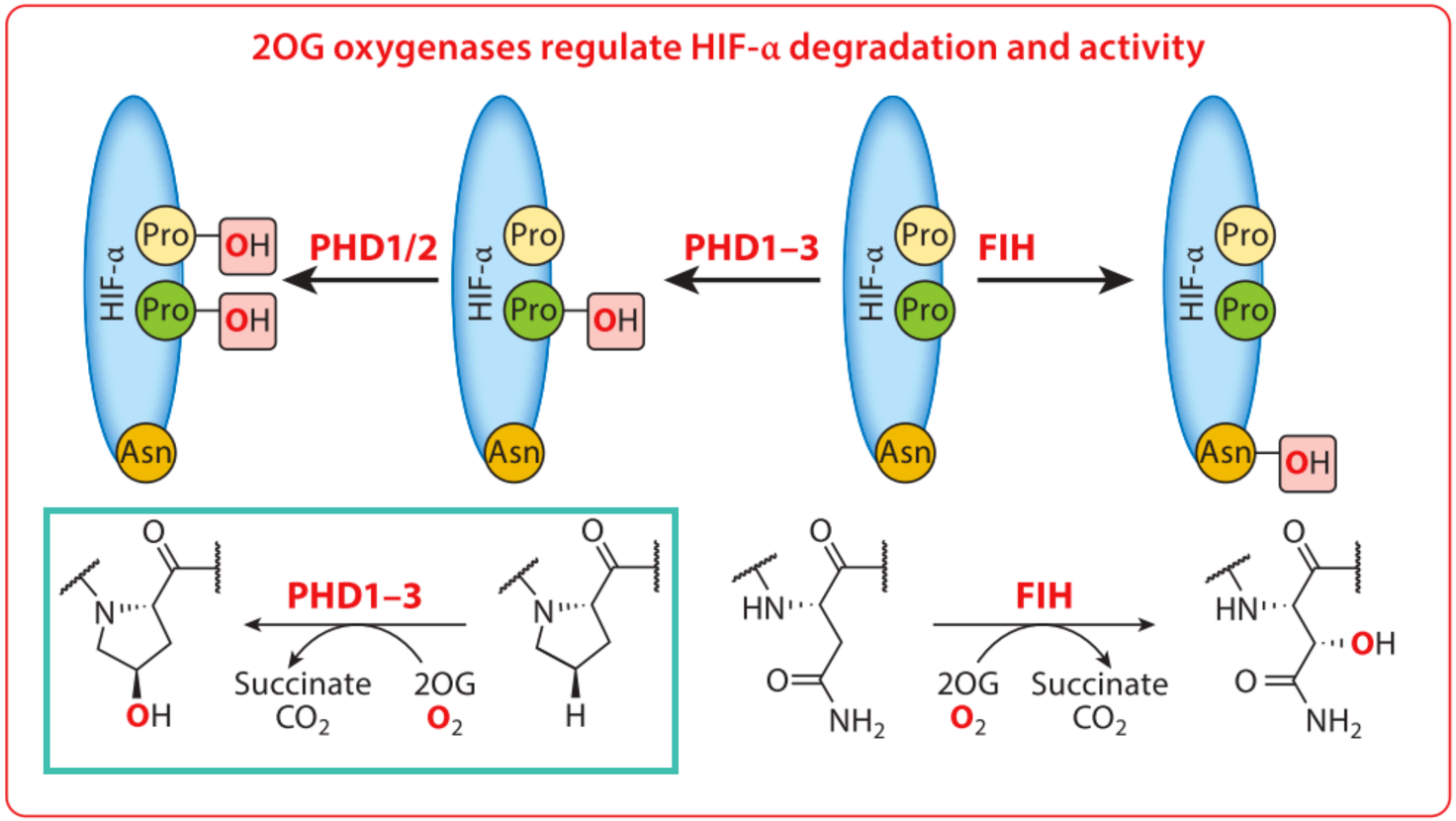


SR proteins
Histones
p53
ERα
HSP70



2-oxoglutarate (2OG)

Hydroxylation of Pro and Gln in HIFα



Islam, M.S., Leissing, T.M., Chowdhury, R., Hopkinson, R.J., and Schofield, C.J. (2018). Annu. Rev. Biochem. *87*, 585–620.

General catalytic cycle for Fe(II)/2OG-dependent oxygenase

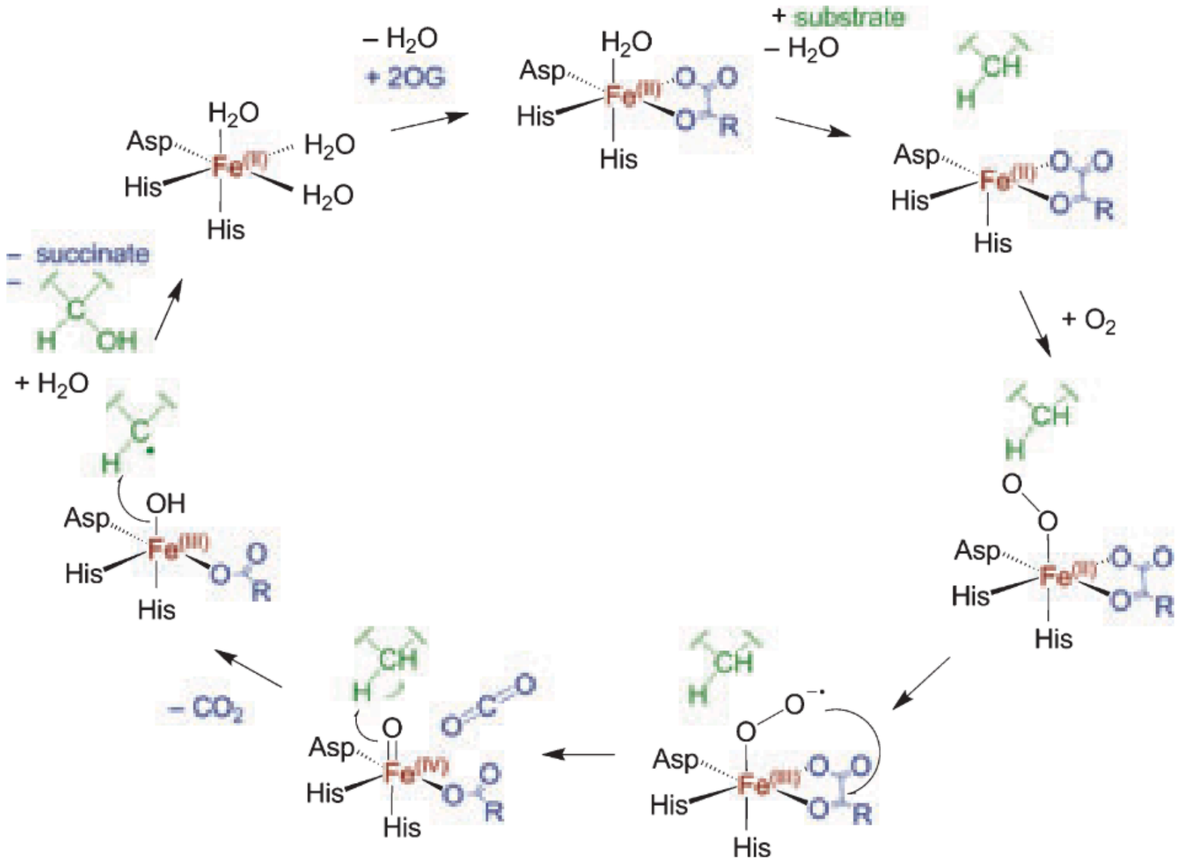
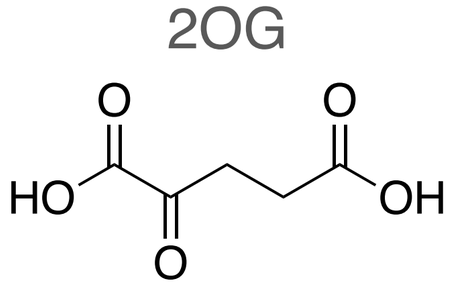


Fig. 1. Proposed general catalytic mechanism for the Fe(II)/2OG oxygenases.



2-oxoglutarate (2OG): decarboxylation to succinate

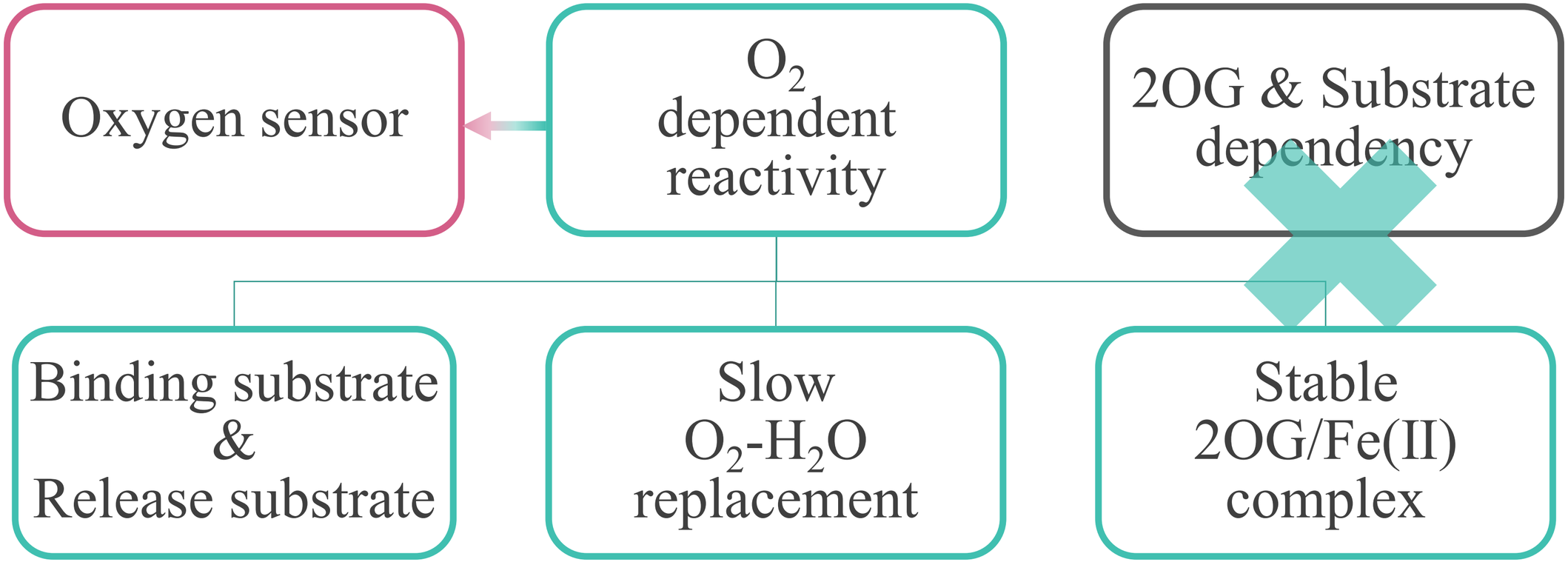
Fe(II): Fe(IV)=O a reactive intermediate

Two His and one Asp or Glu: Fe(II) ligand

Water: Fe(II) ligand replaced to O_2

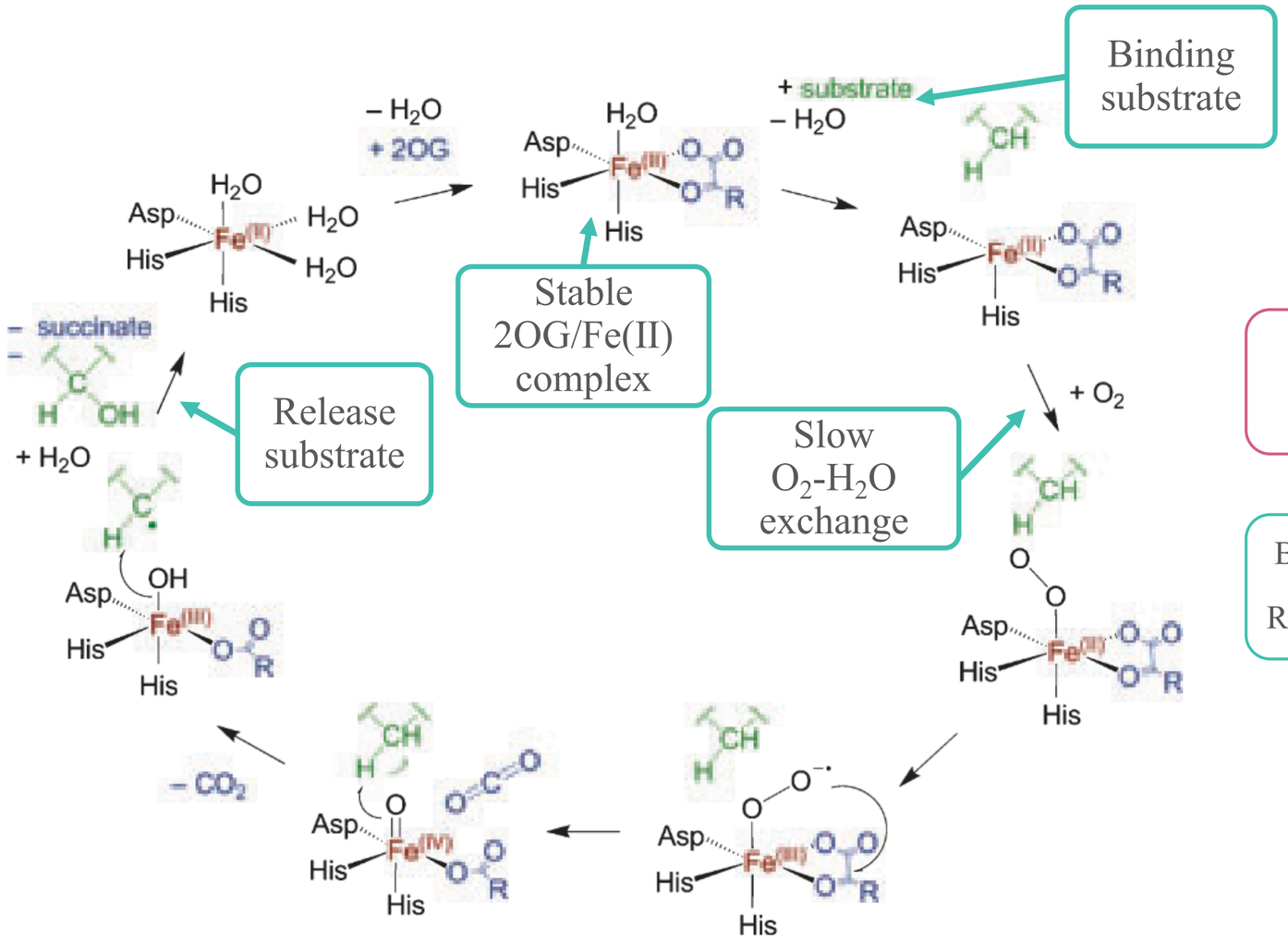
Flashman, E., Hoffart, L.M., Hamed, R.B., Bollinger Jr, J.M., Krebs, C., and Schofield, C.J. (2010). FEBS J. 277, 4089–4099.

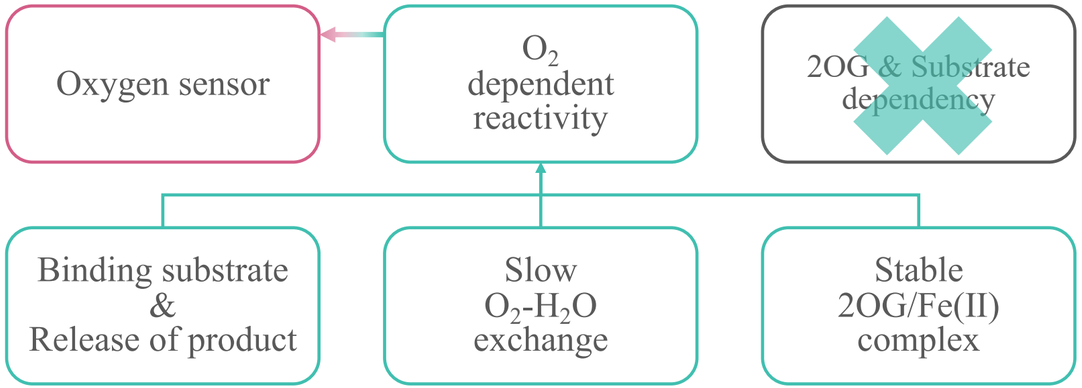
Essentials for O_2 dependent reactivity in PHD



$$v\left([S],[2OG],[O_2]\right)=\frac{k_{cat}\cdot[E]_T\cdot[O_2]}{K_m+[O_2]}\simeq\frac{k_{cat}\cdot[E]_T\cdot[O_2]}{K_m}\left(K_m\gg[O_2]\right)$$

The rate-determining steps for O_2 dependent reactivity

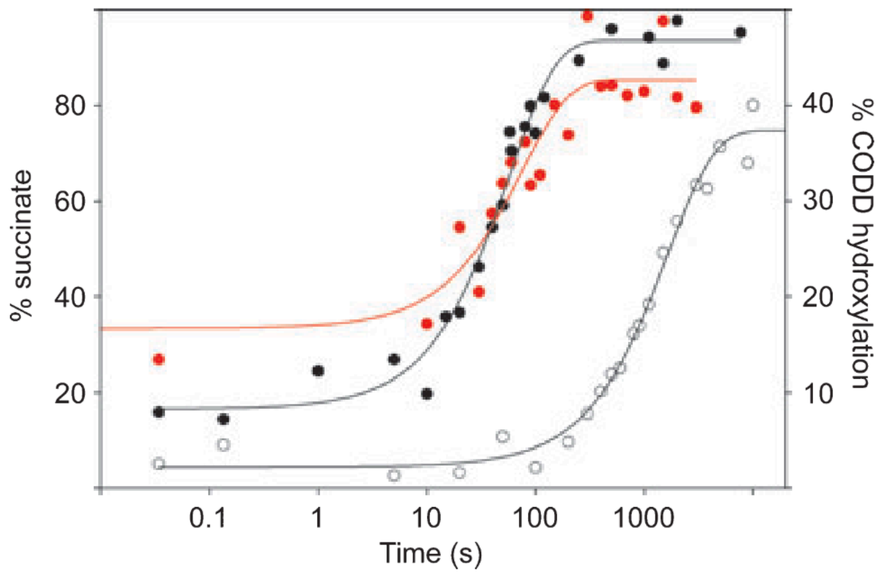




Flashman, E., *et al.*, (2010).

Substrate binding

Stable binding of substrate and easy release of product



PHD:Fe(II):2OG

Binding of substrate to enzyme generally stimulates a reaction in other hydroxylase. (**TauD**; w/ : w/o = 1000 : 1)

● 2OG to succinate with CODD

k_{cat} = 0.018 s^{-1}

○ 2OG to succinate without CODD

k_{cat} = 0.0006 s^{-1} → 30-fold slower

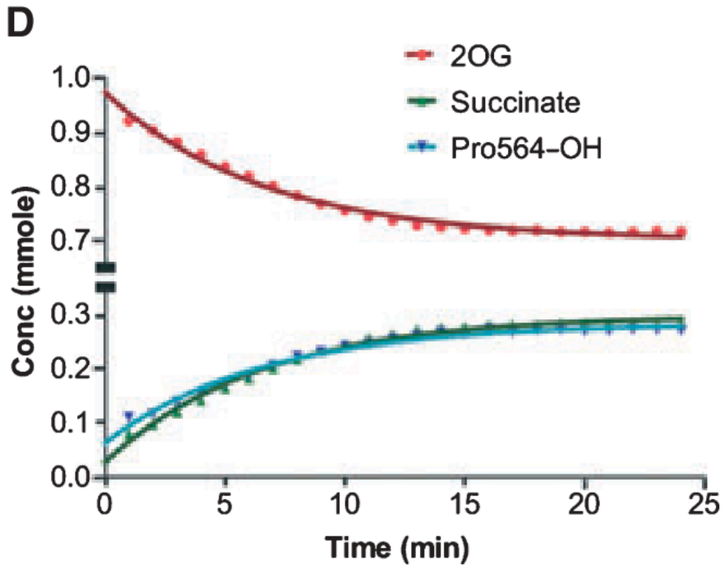
(● CODD hydroxylation: k_{cat} = 0.013 s^{-1})

(CODD:
C-terminal oxygen-dependent degradation domain)

→ In PHD2, substrate binding may **not be a rate-determiniting step**.

Flashman, E., *et al.*, (2010).

Stable 2OG/Fe(II) complex



Previous EPR analysis showed that unstable 2OG chelation caused uncouple 2OG decarboxylaiton and produced reactive Fe ion, which was quenched by reductant such as ascorbate.

2OG decarboxylation is coupled to production of succinate and CODD hydroxylation.
→ 2OG/Fe(II) complex is quite stable.

2OG binding may **not be a rate-determining step** in PHD2

Flashman, E., *et al.*, (2010).

O_2 dependent reactivity

Enzyme	K_m		
	2-Oxoglutarate	Ascorbate	O_2
		μM	
HIF-P4H-1 = PHD1	60	170	230
HIF-P4H-2 = PHD2	60	180	250
HIF-P4H-3 = PHD3	55	140	230
C-P4H-I	20[a]	300[a]	40

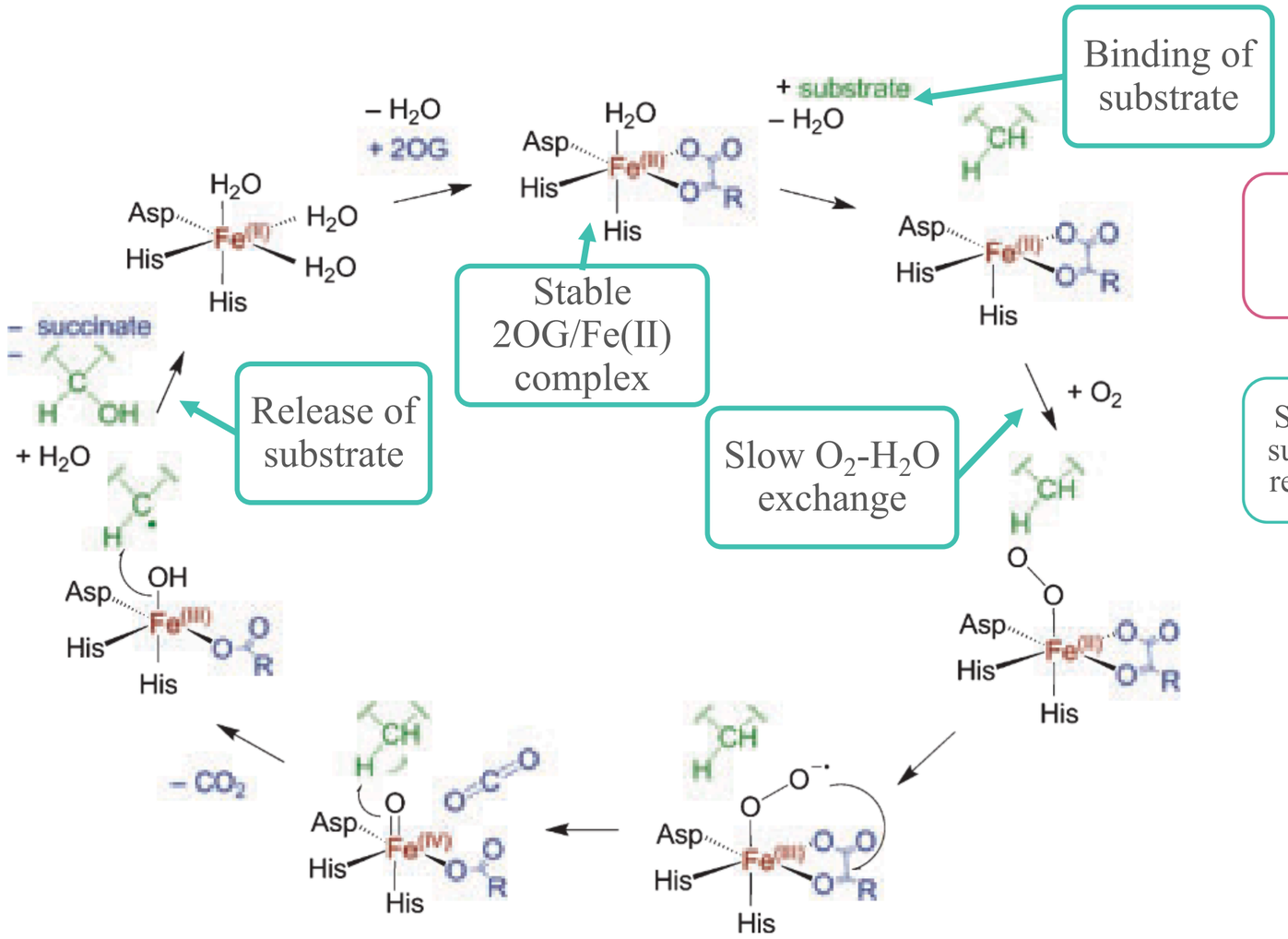
K_m for O_2 PHD2: 250 μM Other oxygenase: 40 μM

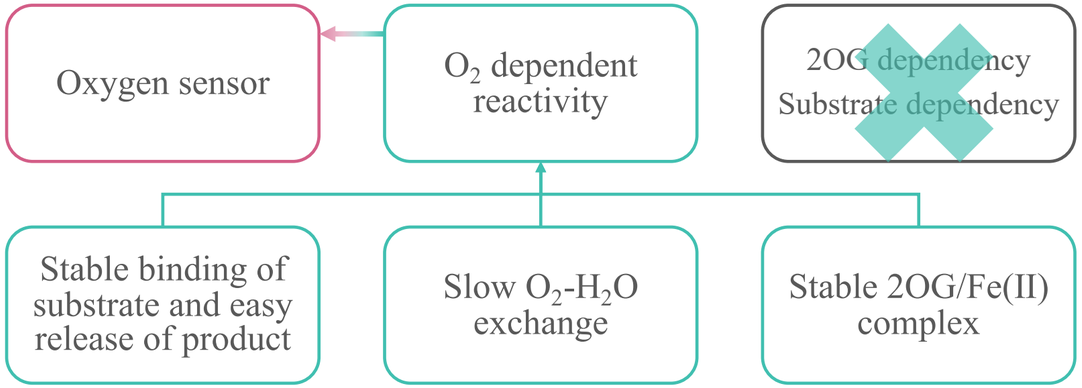
PHD2 has a higher K_m for O_2 than other oxygenases.

→ O2 binding may be **a rate-determining step** in PHD2.

Flashman, E., *et al.*, (2010).

The rate-determining steps for O_2 dependent reactivity





What structural properties do contribute to the O_2 dependent reactivity?

Flashman, E., *et al.*, (2010).

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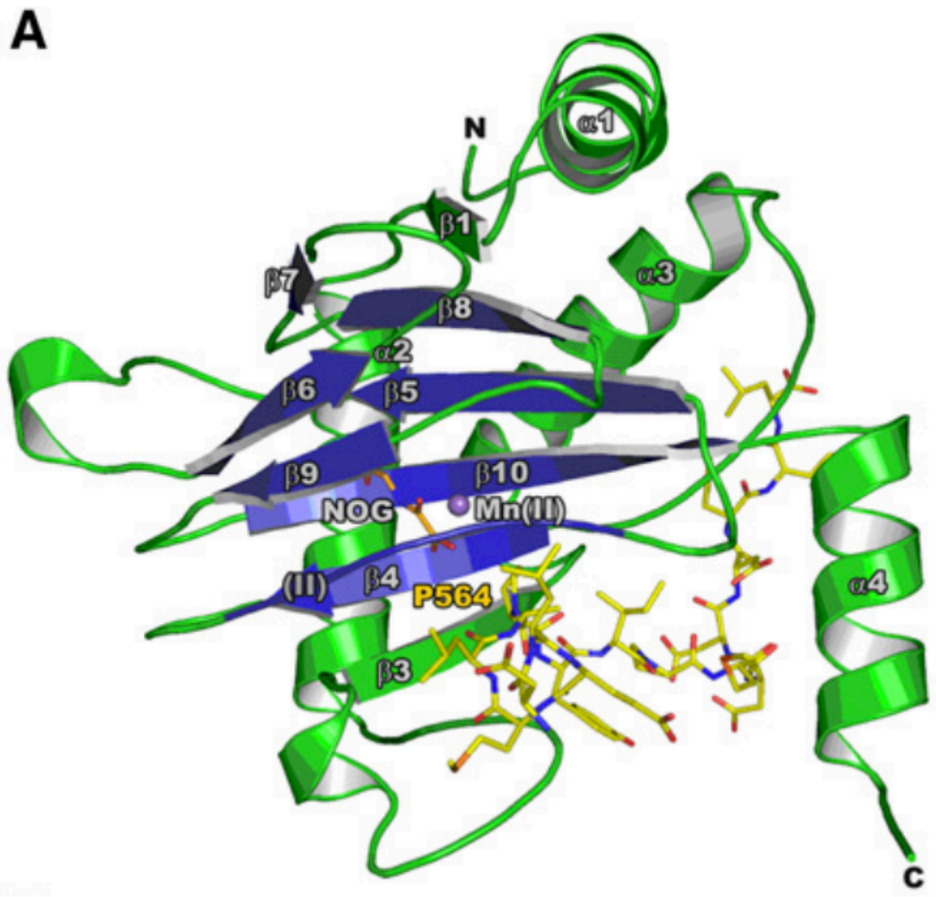
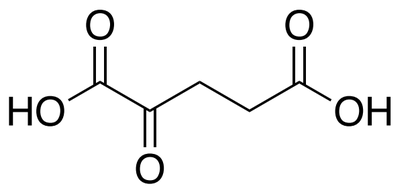
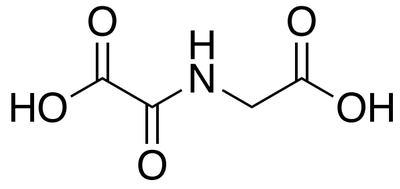
The structure of PHD2.NOG.Mn(II).CODD



Chowdhury, R., McDonough, M.A., Mecinović, J., Loenarz, C., Flashman, E., Hewitson, K.S., Domene, C., and Schofield, C.J. (2009). Structure 17, 981–989.



2OG



NOG

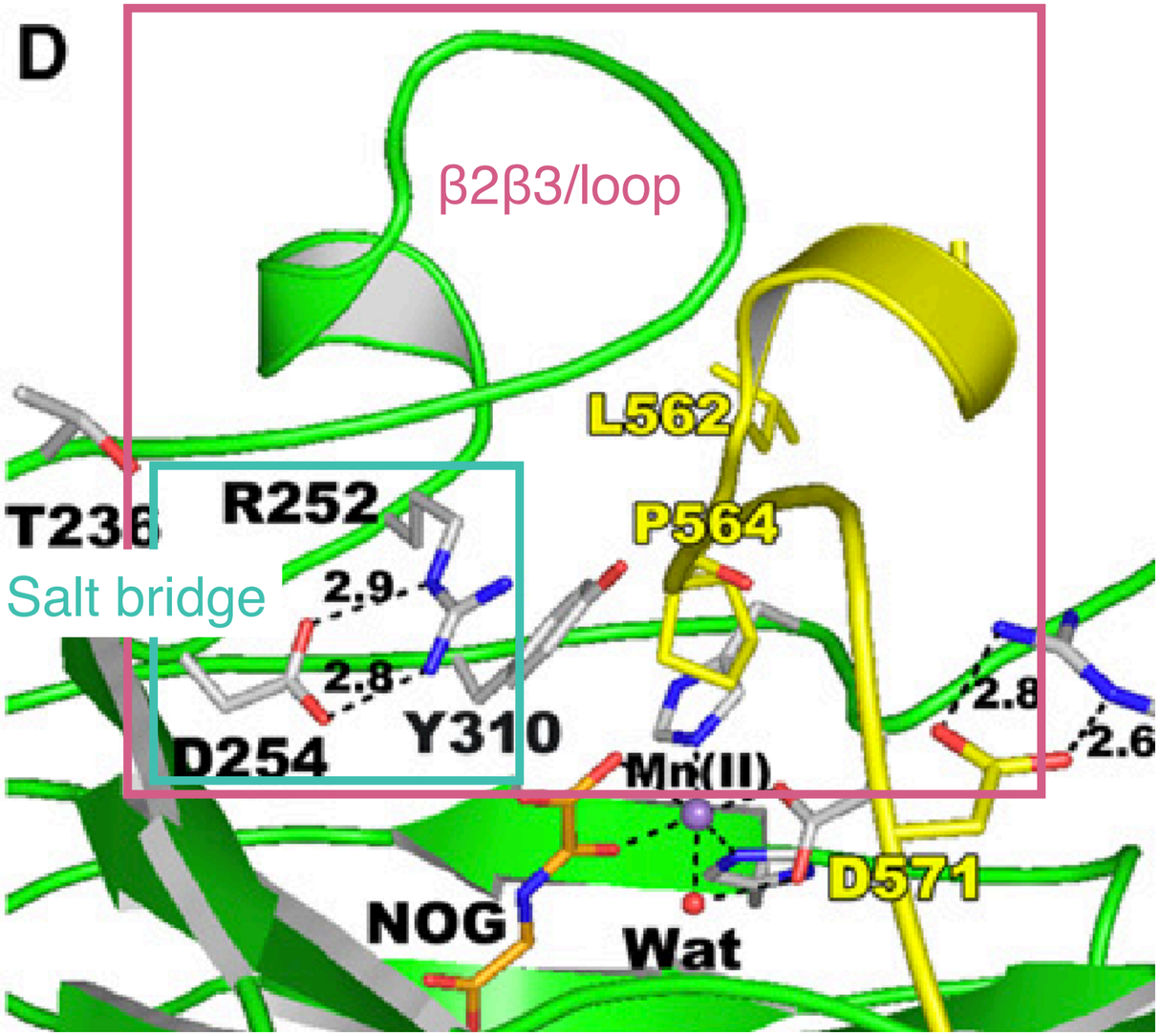
NOG (Orange) and Mn(II) (Purple) are substituited for 2OG and Fe(II) as non-reactive analogs.

CODD (HIF1α peptide including Pro-564, Yellow) is used as a substrate.

The tPHD fold comprises four α helices and ten β strands of which eight form a double-stranded β helix (DSBH, dark blue).

Three of four α helices (α1, α2, and α3) pack along the major β sheet and stabilize the DSBH.

β2β3/loop anchored by a salt bridge



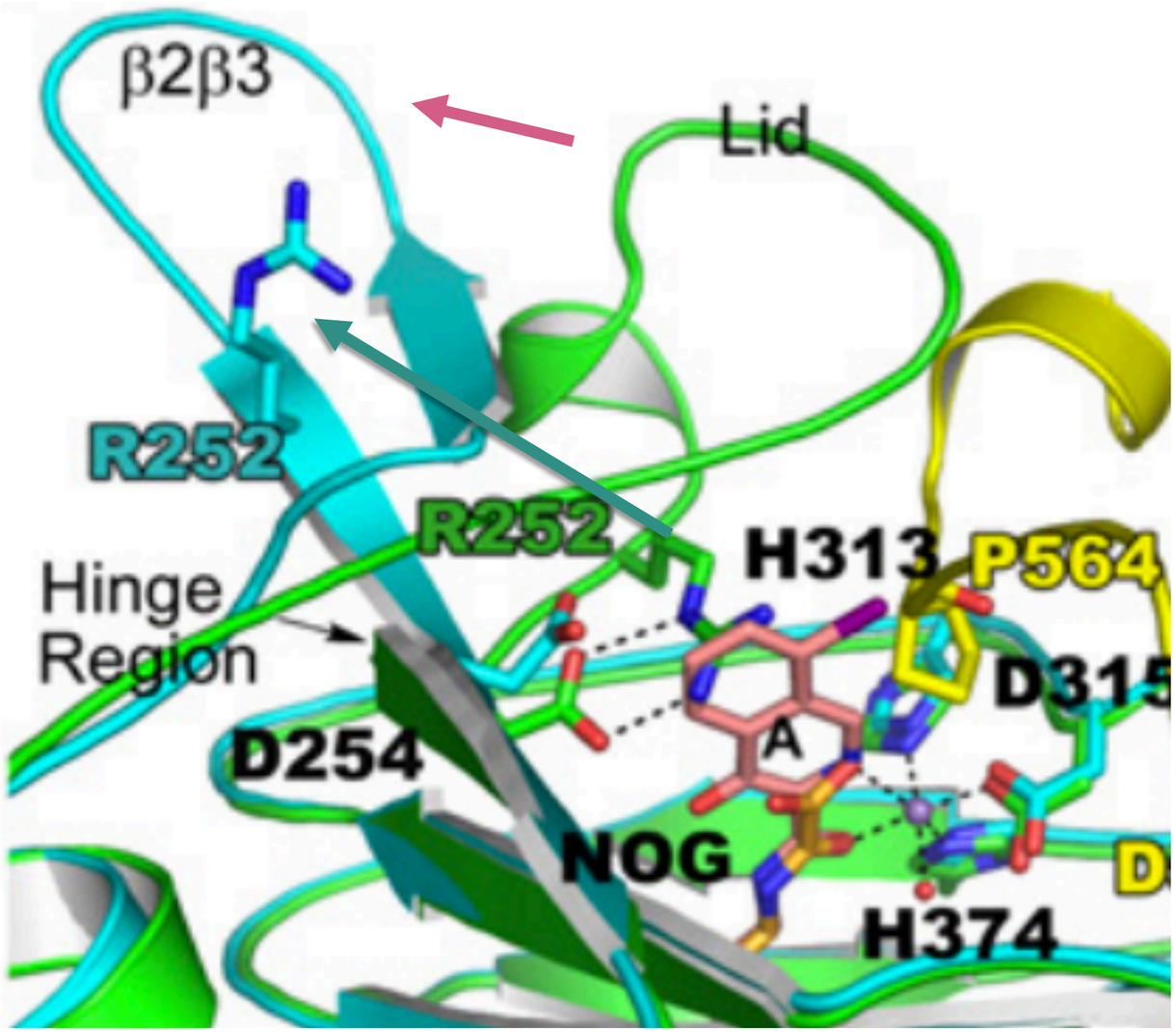
The β2β3/loop of PHD envelops the CODD substrate and stabilizes the substrate-enzyme complex.

→ HIFα stably binds PHD.

B2β3/loop semmed to be anchored by a salt bridge of R252 and D254.

Chowdhury, R., *et al.*, (2009)

The β2β3/loop is important for substrate binding.



PHD2.CODD (Green)

・R252 and D254 form a salt bridge.

PHD2.Fe(II).inhibitor (Cyan)

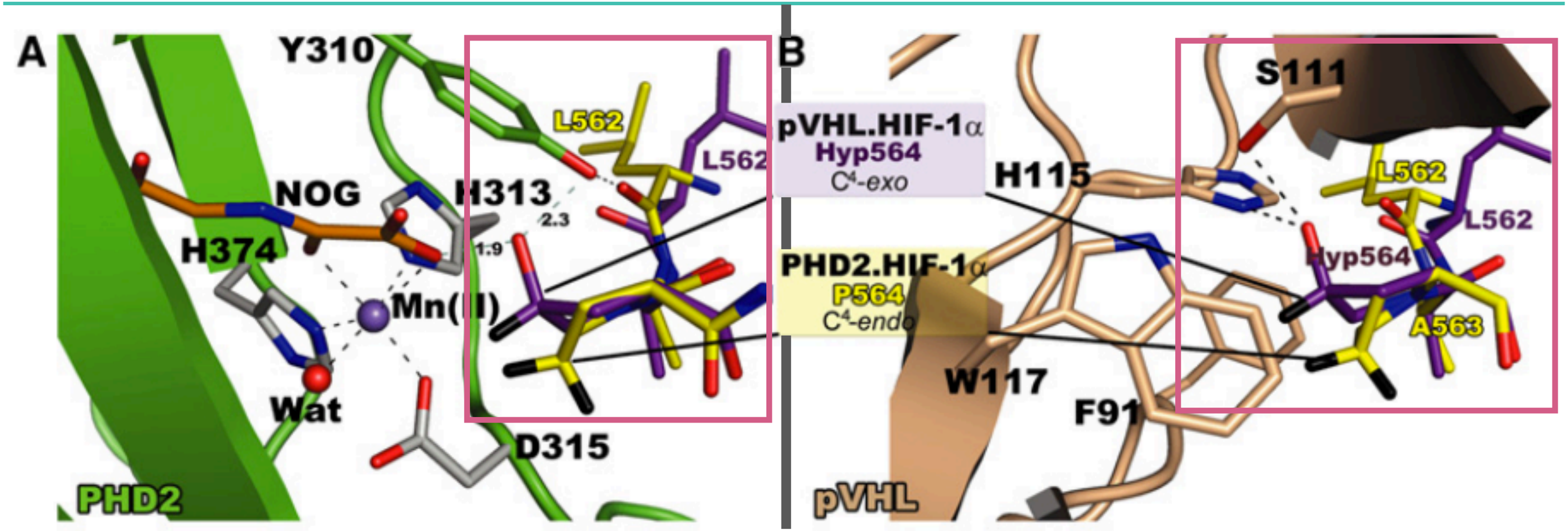
・The **inhibitor**(salmon) seemed to prevent the salt bridge formation.

PHD2 R252 and D254 variants couldn't hydroxylate CODD Pro.

→ The β2β3/loop stabilized by the salt bridge of D254 and R252 contributes much to substrate binding

Chowdhury, R., *et al.*, (2009)

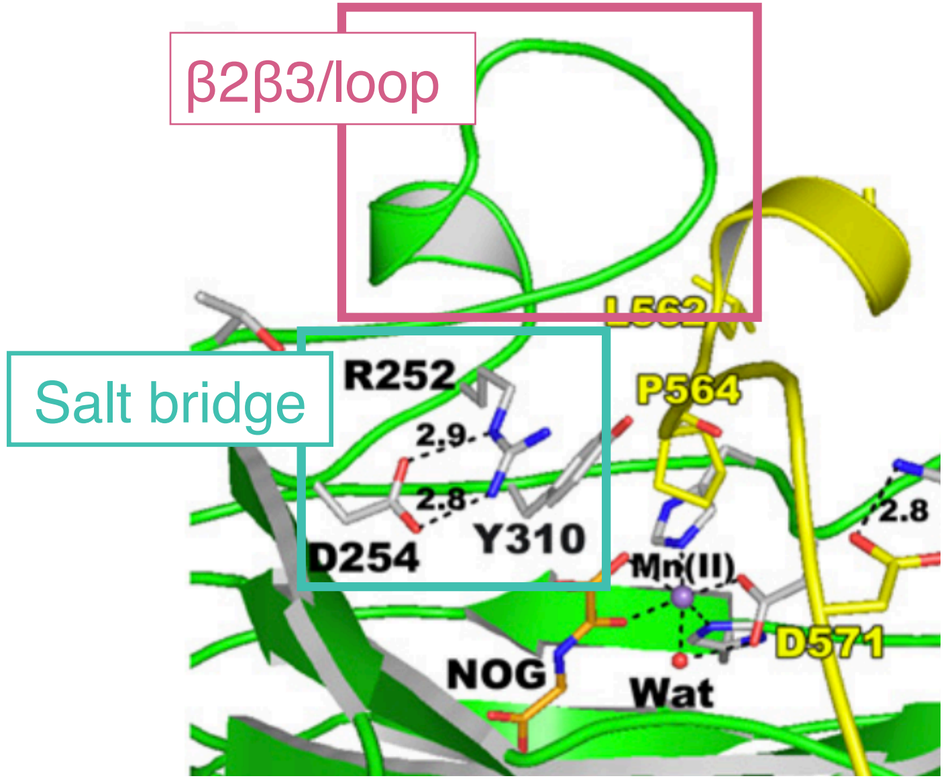
The conformational change of Pro564 by hydroxylation

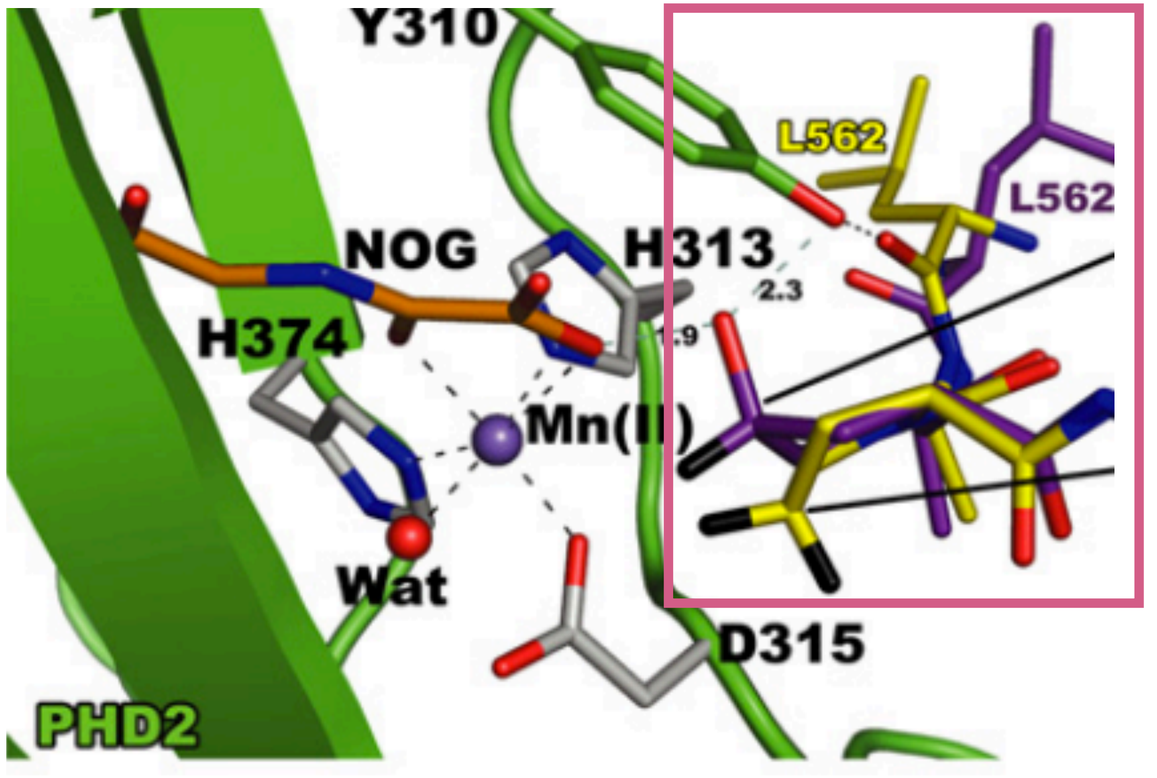


Pro-564_{CODD} : C^4 *endo* → Hyp-564_{CODD} : C^4 *exo* conformtion.
Endo to *Exo* switch might contribute to the release of the product from PHD active site.
VHL seemed to recognize *exo* conformation of Hyp-564_{CODD} but cannot recognize *endo* conformation of Pro-564_{CODD}.

Chowdhury, R., *et al.*, (2009)

Summary of structural analysis of PHD2





The β2β3/loop anchored by a salt bridge contribute much to the stable substrate binding.

Endo to *exo* conformational switch is important for release of product and recognition by VHL-E3 ligase.

Chowdhury, R., *et al.*, (2009)

Summary of structural analysis of PHD2

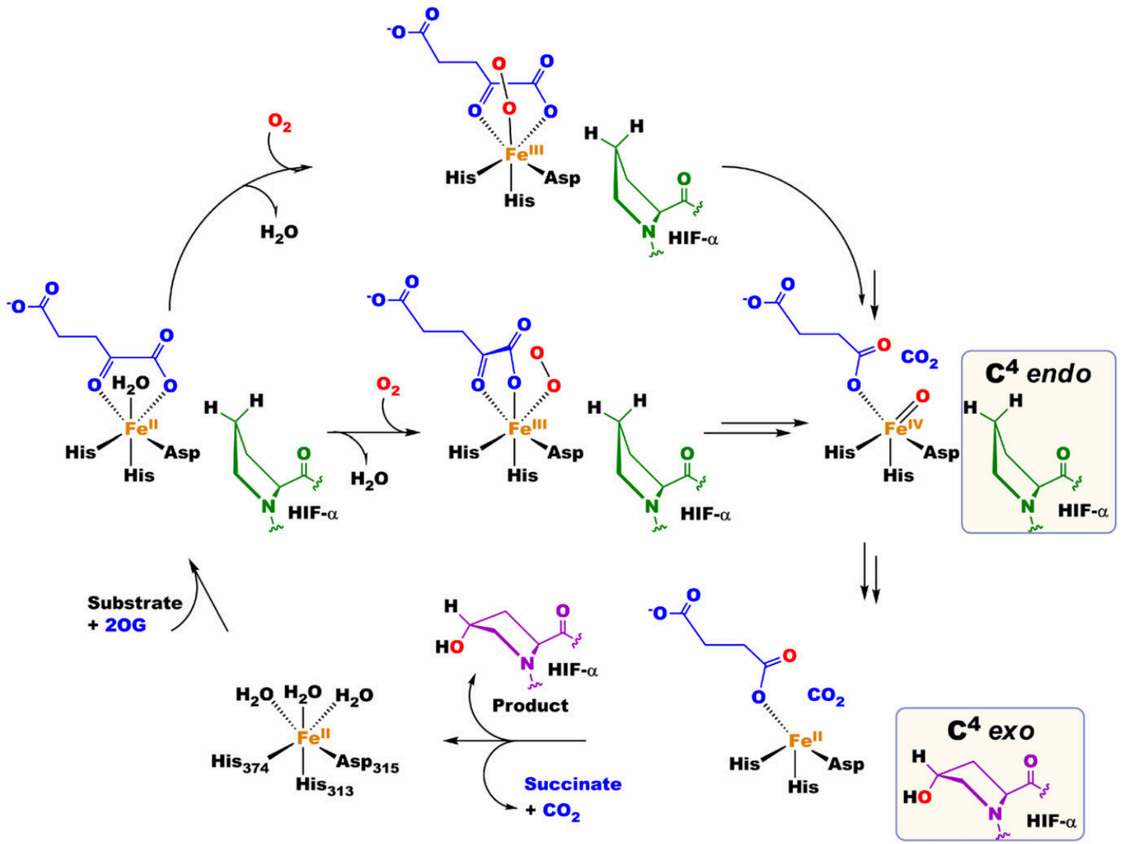


Figure 4. Outline Catalytic Cycle for PHD2 Showing the Proposed Ferryl and Other Intermediates, Highlighting (Boxed) the Proposed Switch in the Pro-564 Ring Conformation

Work with other 2OG oxygenases suggests it is possible that oxygen binding occurs either *trans* to His-313$_{PHD2}$ or *trans* to His-374$_{PHD2}$ (Zhang et al., 2002) (see Figure 3C).

The comformation of the β2β3/loop of PHD2 is important to enclose the Pro-564 region of HIFα.

The salt bridge comprised of R252 and D254 stabilizes the β2β3/loop conformation.

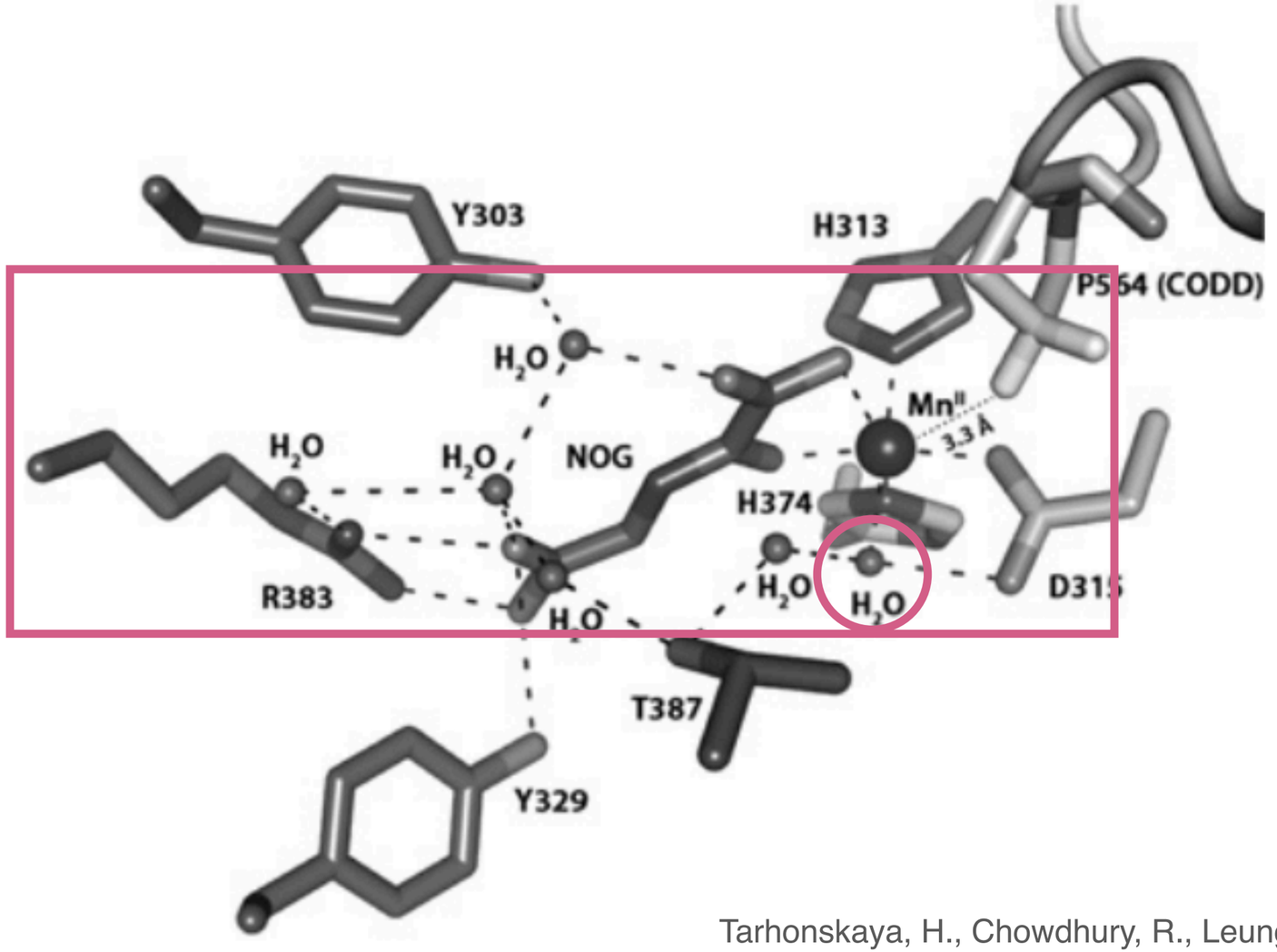
The Pro-564 ring conformation switches *endo* to *exo* by hydroxyation.

Chowdhury, R., *et al.*, (2009)

Contents

- Introduction
  - The Novel Prize in Physiology or Medicine 2019
  - Mechanism of hypoxia response
- Basic characteristics of PHD
- Structural analysis of PHD2
- Contribution of residues of PHD2 to O_2 dependent reactivity
- Summary

2OG is stabilized by R383

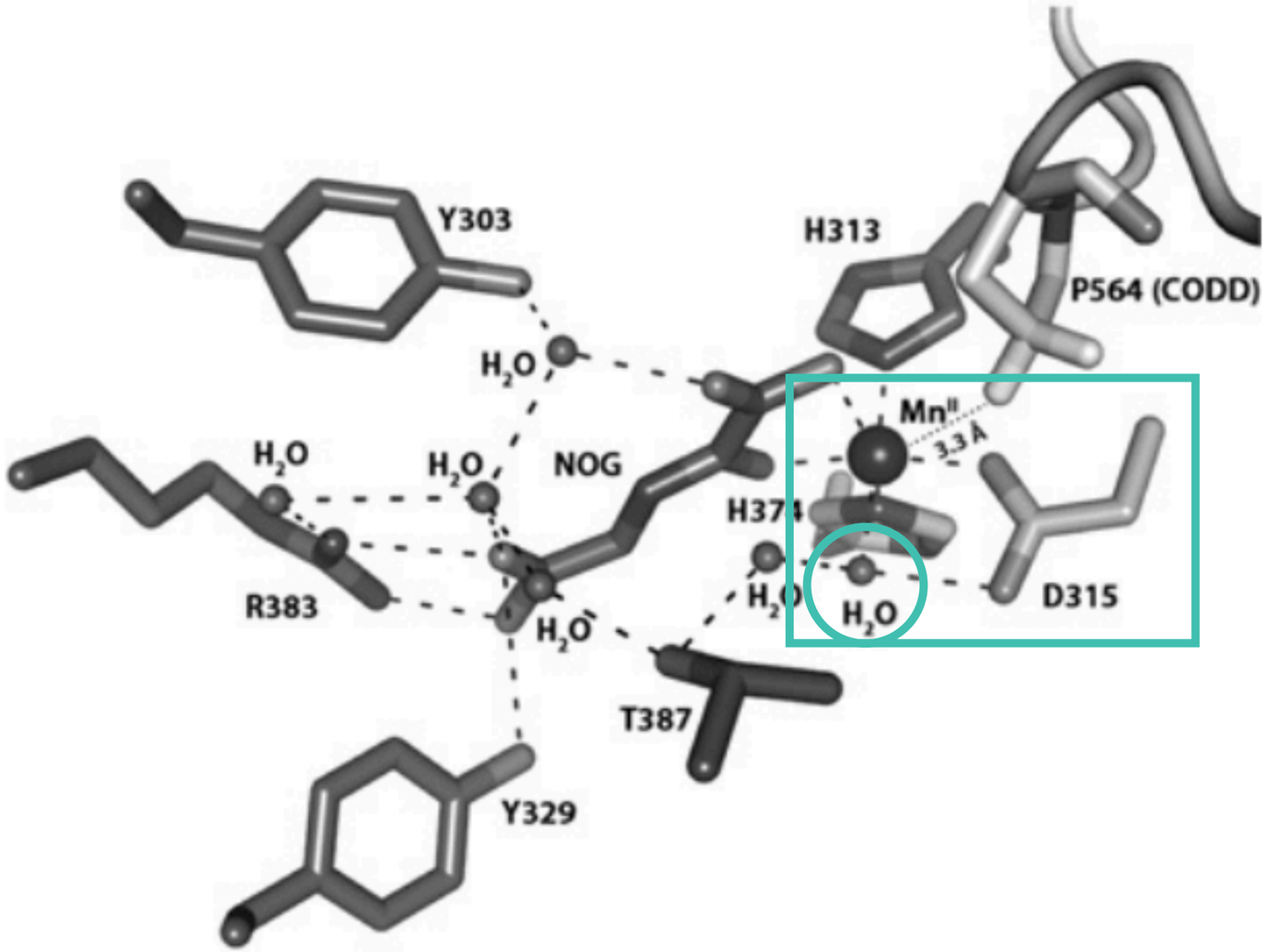


Salt bridge between R383 and 2OG stabilize the 2OG/Fe(II) complex

The stability of 2OG/Fe(II) complex inhibit a **rearrangement of 2OG and water** to bring water close to Pro.

Tarhonskaya, H., Chowdhury, R., Leung, I.K.H., Loik, N.D., McCullagh, J.S.O., Claridge, T.D.W., Schofield, C.J., and Flashman, E. (2014). Biochem. J. 463, 363–372.

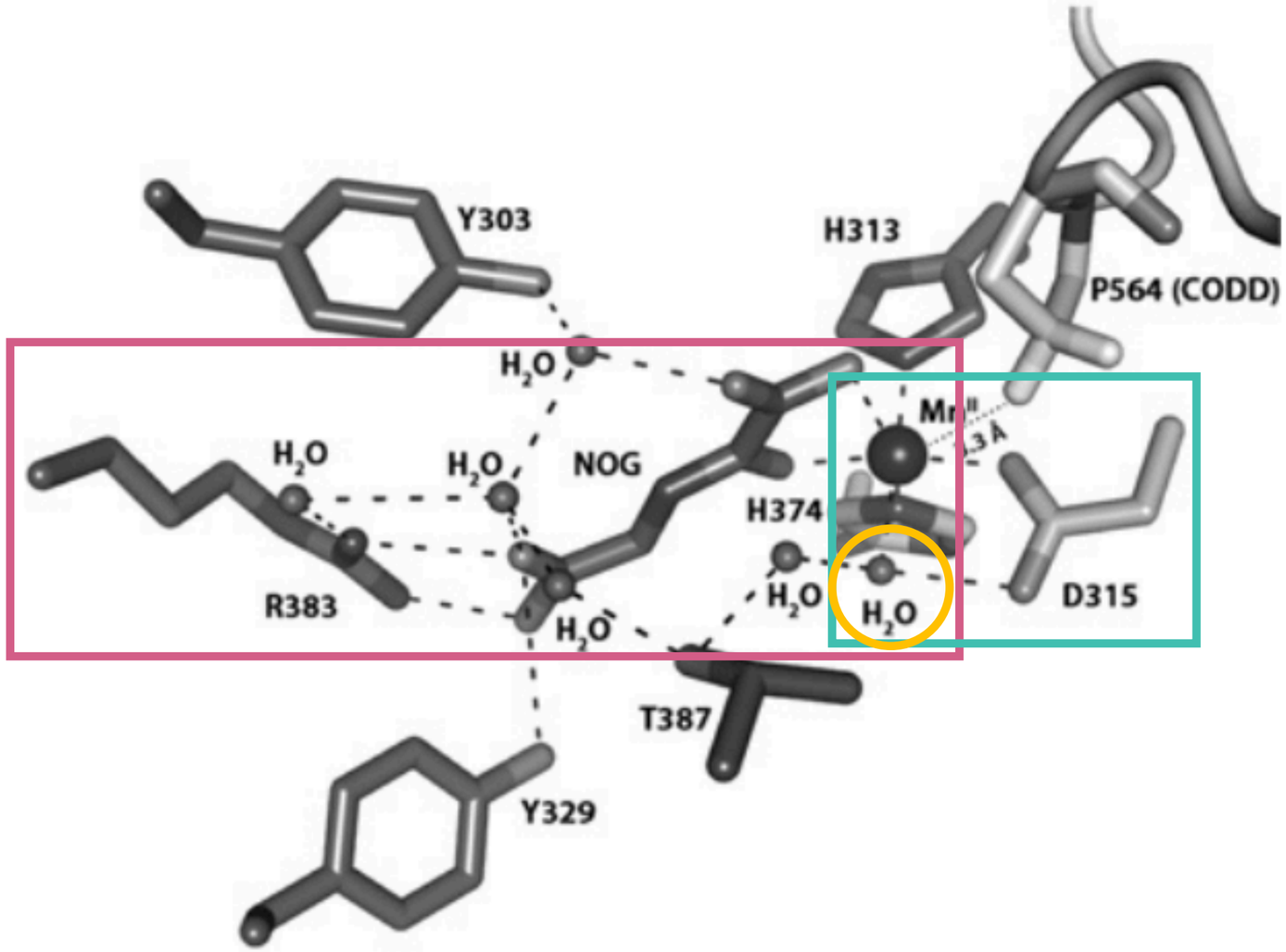
The metal-bound water is stabilized by Asp-315.



Hydrogen bond between D315 and metal ligated water stabilize the water-metal coordination, which inhibits **O_2-H_2O exchange**.

Tarhonskaya, H,. *et al.*(2014)

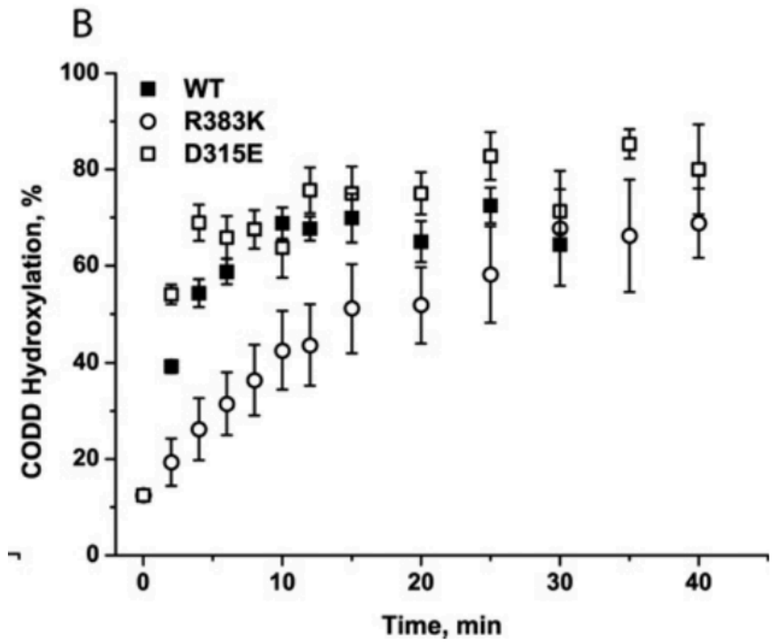
Question II

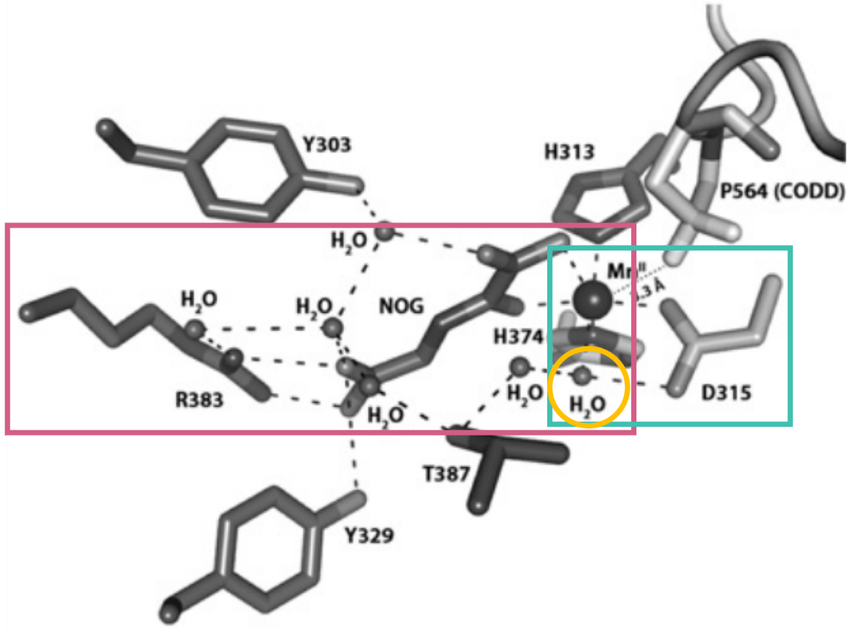


How do these residue contribute to the O_2 dependent reactivity?

Tarhonskaya, H,. *et al.*(2014)

CODD hydroxylation of PHD2 variants





Y303F and Y329F (2OG): No significant change

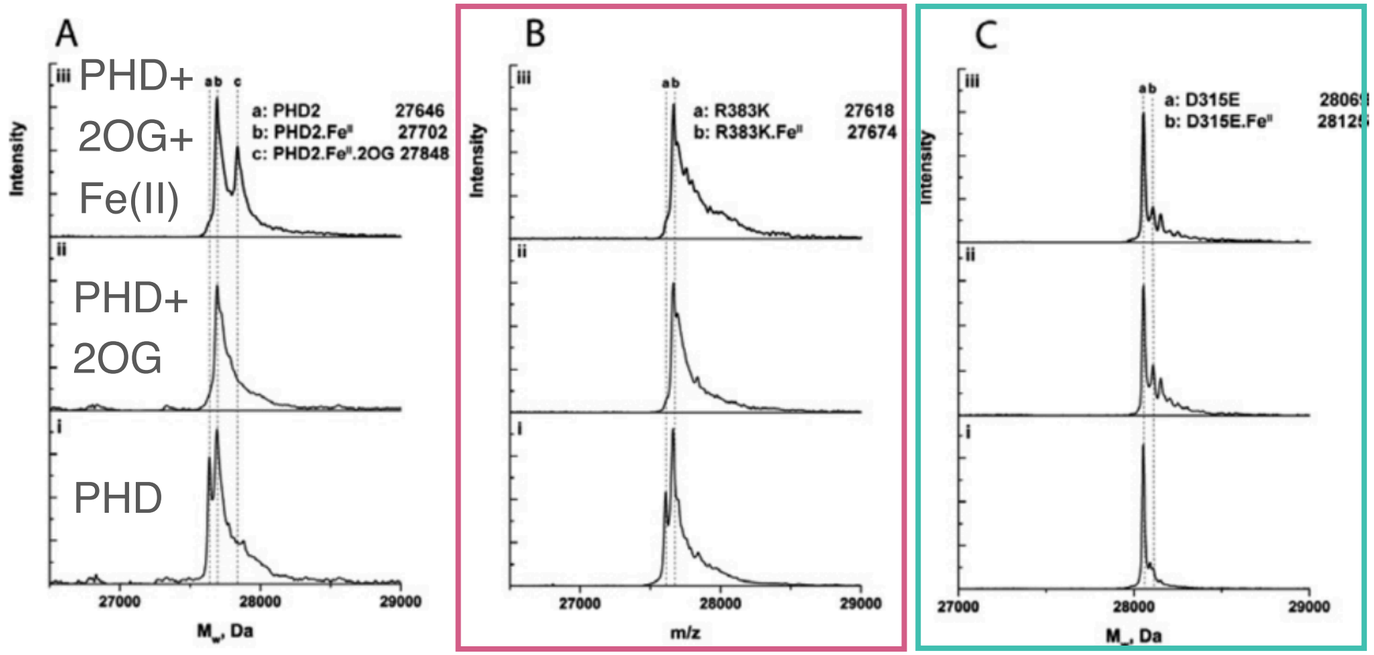
H313D, D315H, H374E and H374R (Fe(II)): Loss of catalytic activity

R383K (2OG): Moderately decrease

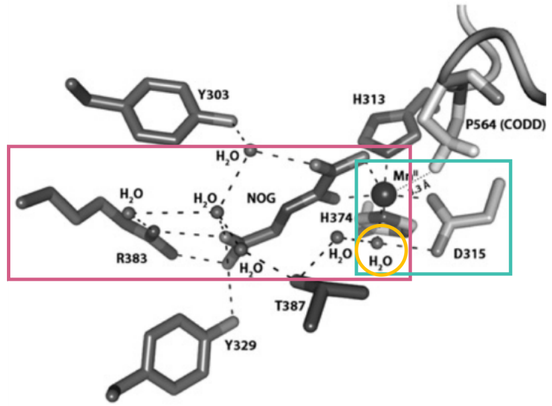
D315E (Fe(II)): Moderately increase

Tarhonskaya, H,. *et al.*(2014)

Binding Fe(II) and 2OG to the PHD2 variants



Complex	Ligand	K_d (μM)	Method
WT·Fe(II)·2OG	2OG	5 ± 3	UV–visible
R383K·Fe(II)·2OG	2OG	550 ± 150	UV–visible
R383K·Fe(II)·2OG·CODD	2OG	105 ± 5	UV–visible
WT·Mn(II)·2OG	2OG	0.9 ± 0.1	NMR [29]
R383K·Mn(II)·2OG	2OG	$>10{,}000$	NMR
R383K·Mn(II)·2OG·CODD	2OG	430 ± 30	NMR
R383K·Zn(II)·2OG	2OG	110 ± 20	NMR
R383K·Zn(II)·2OG·CODD	2OG	< 10	NMR
WT·Mn(II)	Mn(II)	0.6 ± 0.4	NMR
R383K·Mn(II)	Mn(II)	0.6 ± 0.2	NMR
D315E·Mn(II)	Mn(II)	240 ± 30	NMR



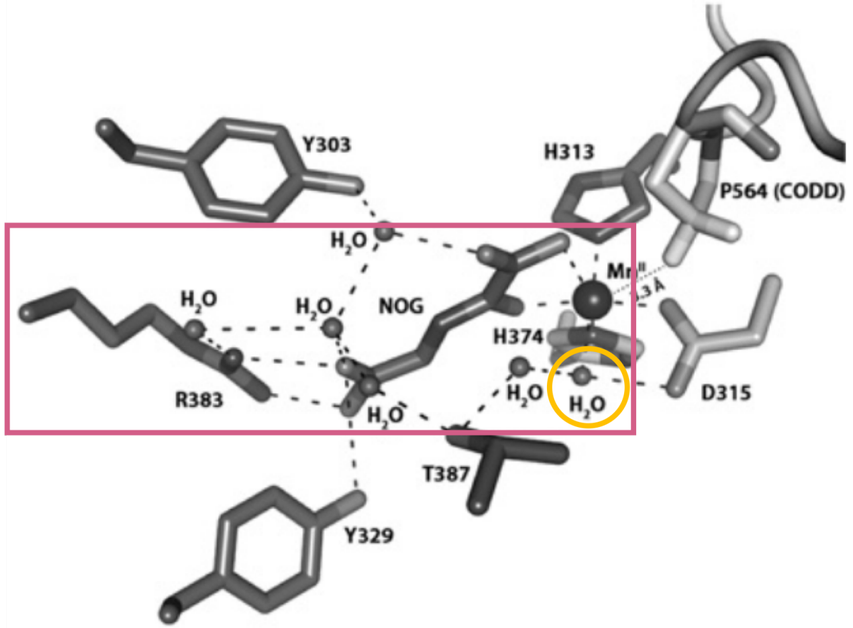
R383K: Reduced affinity for 2OG and no change in the affinity for Fe(II)
K_d for 2OG with CODD is lower than without CODD.

D315E: The stability of PHD2.Fe(II) complex was decreased.

Tarhonskaya, H,. *et al.*(2014)

The K_m for 2OG and CODD and k_{cat} values of PHD2 variants

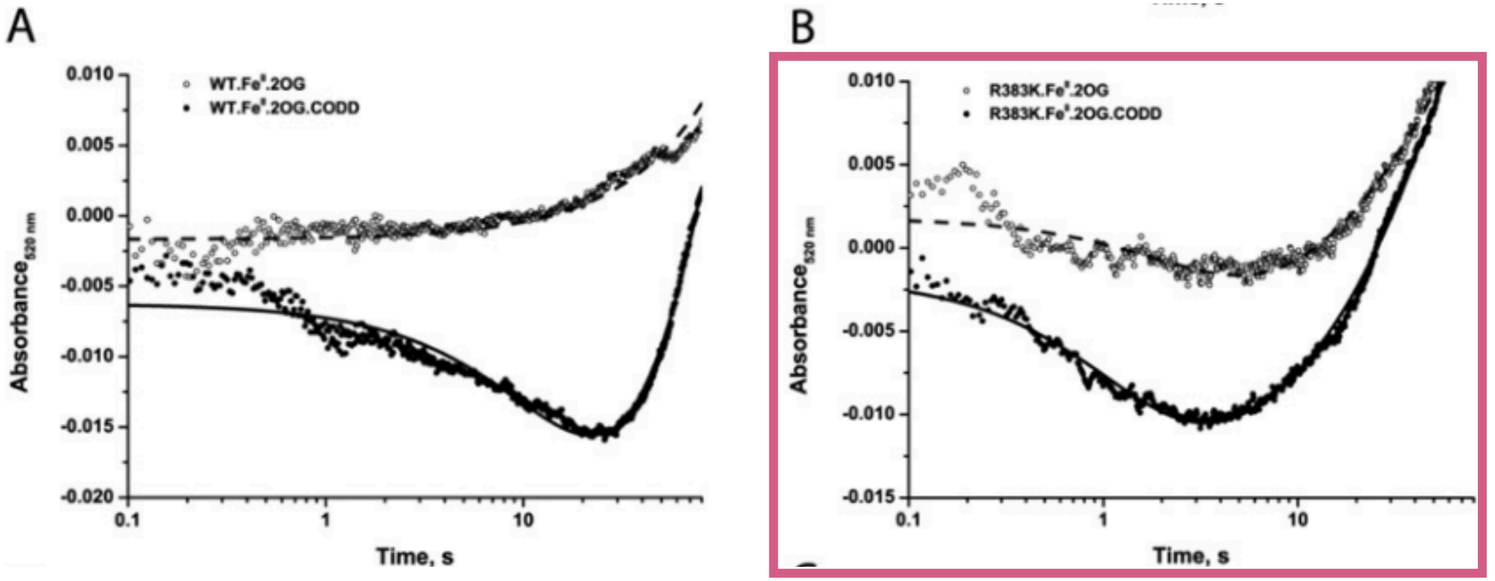
PHD2	K_m(2OG) (μM)	K_m(CODD) (μM)	k_{cat} (s^{-1})
WT	13 ± 2	9 ± 3	0.060 ± 0.007
R383K	45 ± 10	18 ± 4	0.017 ± 0.001
Y303F	37 ± 4	8 ± 2	0.027 ± 0.001
Y329F	21 ± 4	8 ± 2	0.072 ± 0.002
D315E	20 ± 7	11 ± 3	0.099 ± 0.007

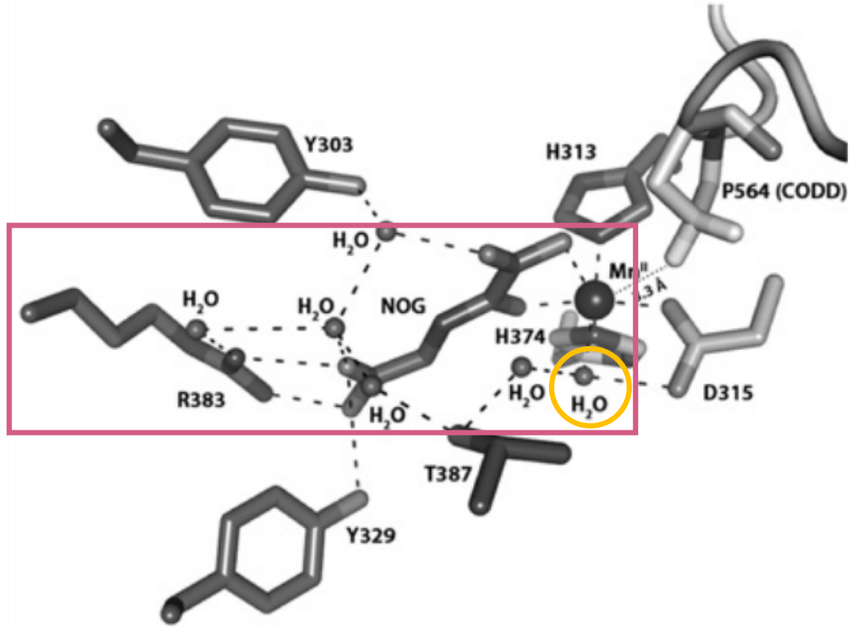


R383K K_m(2OG): ~3-fold higher, Km(CODD): ~2-fold higher, k_{cat}: lower
R383 contribute much to the stability of 2OG/Fe(II) complex and some to substrate dependent reactivity.

D315E K_m(2OG): slightly higher, Km(CODD): No change, k_{cat}: slightly higher
D315 does not contribute to both of them.

Accumulation of Fe intermediate rearranged in chelation





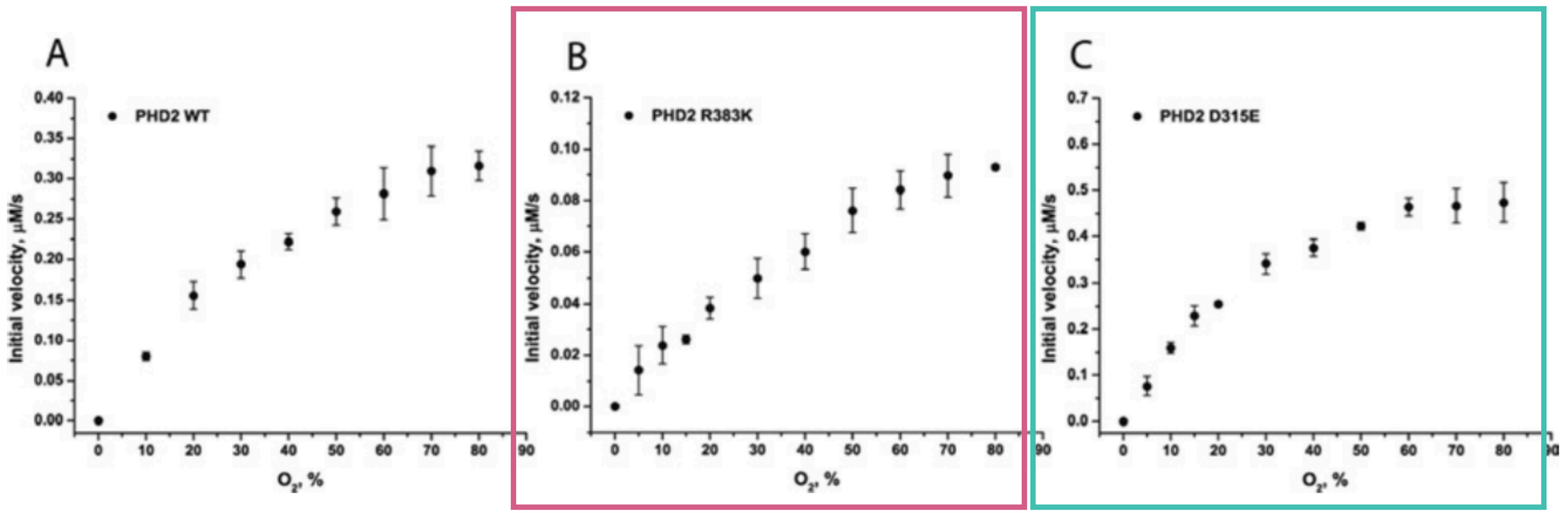
R383K: faster than WT with CODD

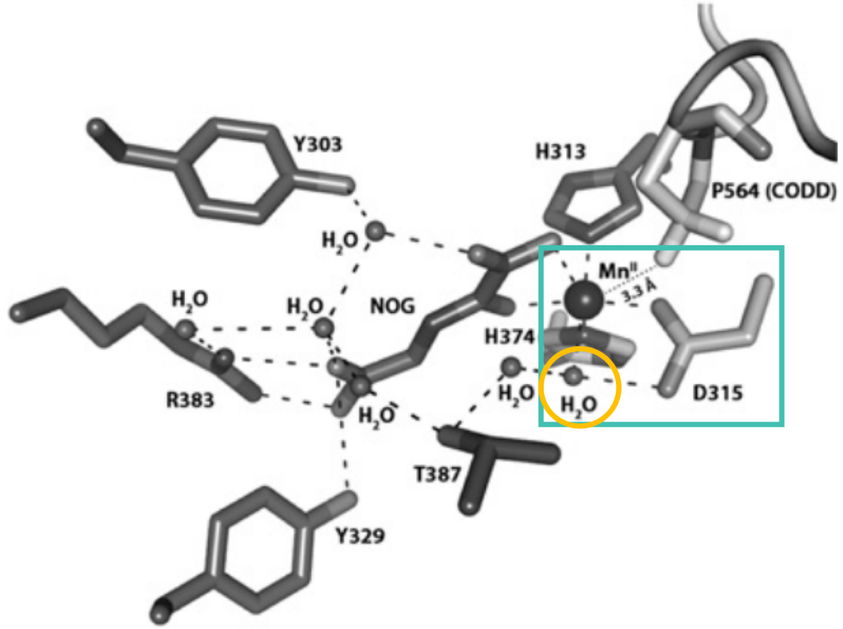
No rearrangement without CODD

→ R383 contributes to the stability of the 2OG/Fe(II) complex

Tarhonskaya, H,. *et al.*(2014)

O_2-dependency of PHD2 WT and variants



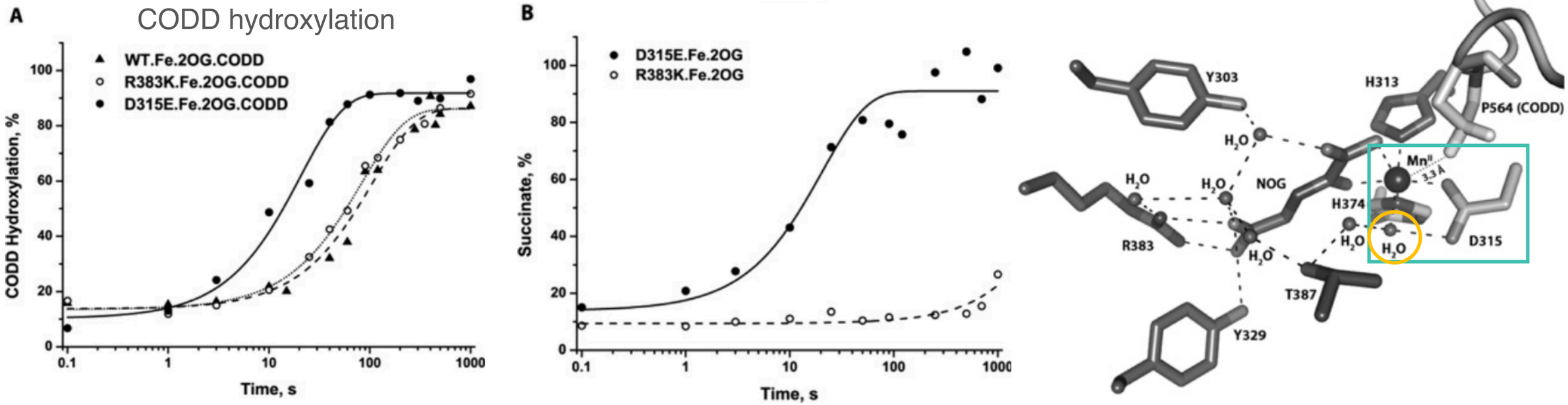


R383K $K_m(O_2)$: Couldn't be determined (estimated >450 μM, WT estimated >450 μM)
D315E $K_m(O_2)$: 200±30 μM lower than WT and R383K

→ D315 contributes much to the high K_m for O_2

Tarhonskaya, H,. *et al.*(2014)

CODD hydroxylation and uncoupled 2OG decarboxylation



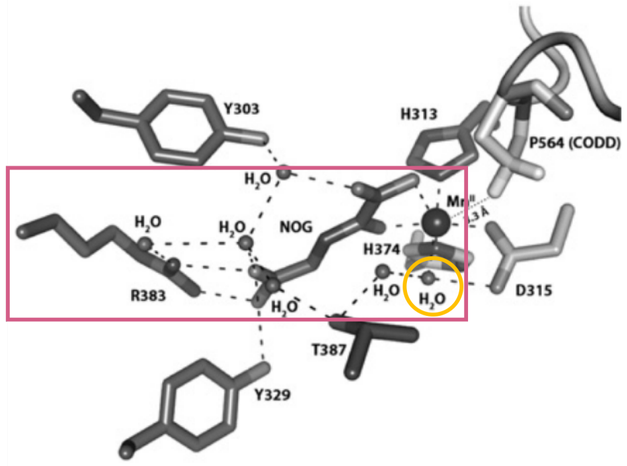
R383K: CODD hydroxylation and production of succinate is similar to WT

→ The 2OG/Fe(II) complex in R383K without is as stable as WT.

D315E: CODD hydroxylation is faster than WT and production of succinate without CODD increase.

→ The uncouple reaction proceed, which means the stability of 2OG/Fe(II) complex is lost.

Summary of kinetic analysis of PHD2 variants R383K

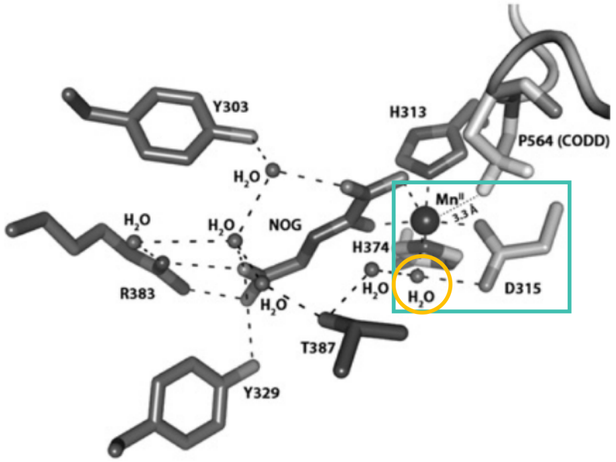


Tarhonskaya, H,. *et al.*(2014)

Compare to WT	R383K	D315E
CODD hydroxylation	Decrease	Increase
Binding Fe(II)	Same	Decrease (flexibility increase)
Binding 2OG	Decrese (higher w/ than w/o CODD)	Decrease
K_m (2OG)	~3-fold higher	Slightly higher
K_m (CODD)	No major change	No major change
K_m (O_2)	–	Lower
Fe(IV)=O accumulation	Faster	–
Oxidation of 2OG without CODD	Little increase	Much increase

R383 contributes to stabilize 2OG at the active site.
The rearrange of chelation in R383K is faster than for WT, but no changes in the kinetics of product accumulation (hydroxylated CODD and succinate) was observed.
→ The rearrangement of the 2OG-binding mode may not be a rate-determining step in PHD2-catalysed hydroxylation. However, R383 contributes much to the stability of 2OG/Fe(II) complex.

Summary of kinetic analysis of PHD2 variants D315E



Tarhonskaya, H,. *et al.*(2014)

Compare to WT	R383K	D315E
CODD hydroxylation	Decrease	Increase
Binding Fe(II)	Same	Decrease (flexibility increase)
Binding 2OG	Decrese (higher w/ than w/o CODD)	Decrease
K_m (2OG)	~3-fold higher	Slightly higher
K_m (CODD)	No major change	No major change
K_m (O_2)	–	Lower
Fe(IV)=O accumulation	Faster	–
Oxidation of 2OG without CODD	Little increase	Much increase

D315 stabilizes the Fe(II) at the active site and the iron-ligated water by a hydrogen bond.
CODD hydroxylation by D315E is faster than WT.
Oxidation of 2OG uncoupled to CODD hydroxylation by D315E was observed unlike WT.
→ The bond of the metal-co-ordinated water to metal is weakened, facilitating water release and accelarating O_2 activation.
This is an important factor in the slow O_2 activation of PHD2 catalysis.

Summary

Oxygen sensor

O_2 dependent reactivity

2OG & Substrate dependency

Binding substrate & Release of product

Slow O_2-H_2O exchange

Stable 2OG/Fe(II) complex

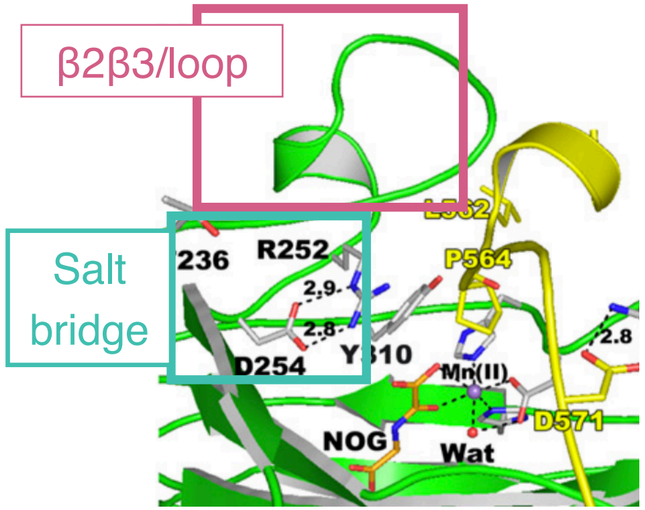
Stabilization of substrate by β2β3/loop

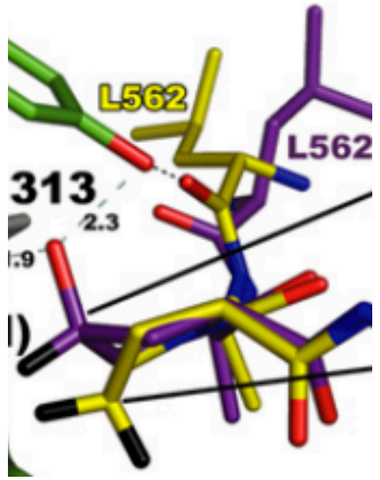
Hydrogen bond of metal coordinated water with D315

Salt bridge formed by 2OG and R383

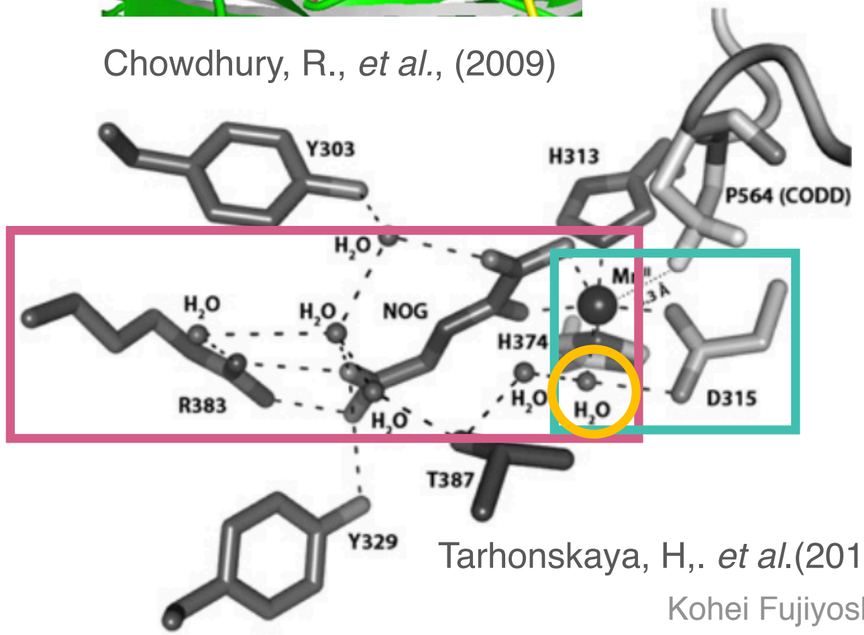
Endo to *exo* switch by hydroxylation

$$v([S],[2OG],[O_2]) \simeq \frac{k_{cat} \cdot [E]_T \cdot [O_2]}{K_m} \left(K_m \gg [O_2] \right)$$





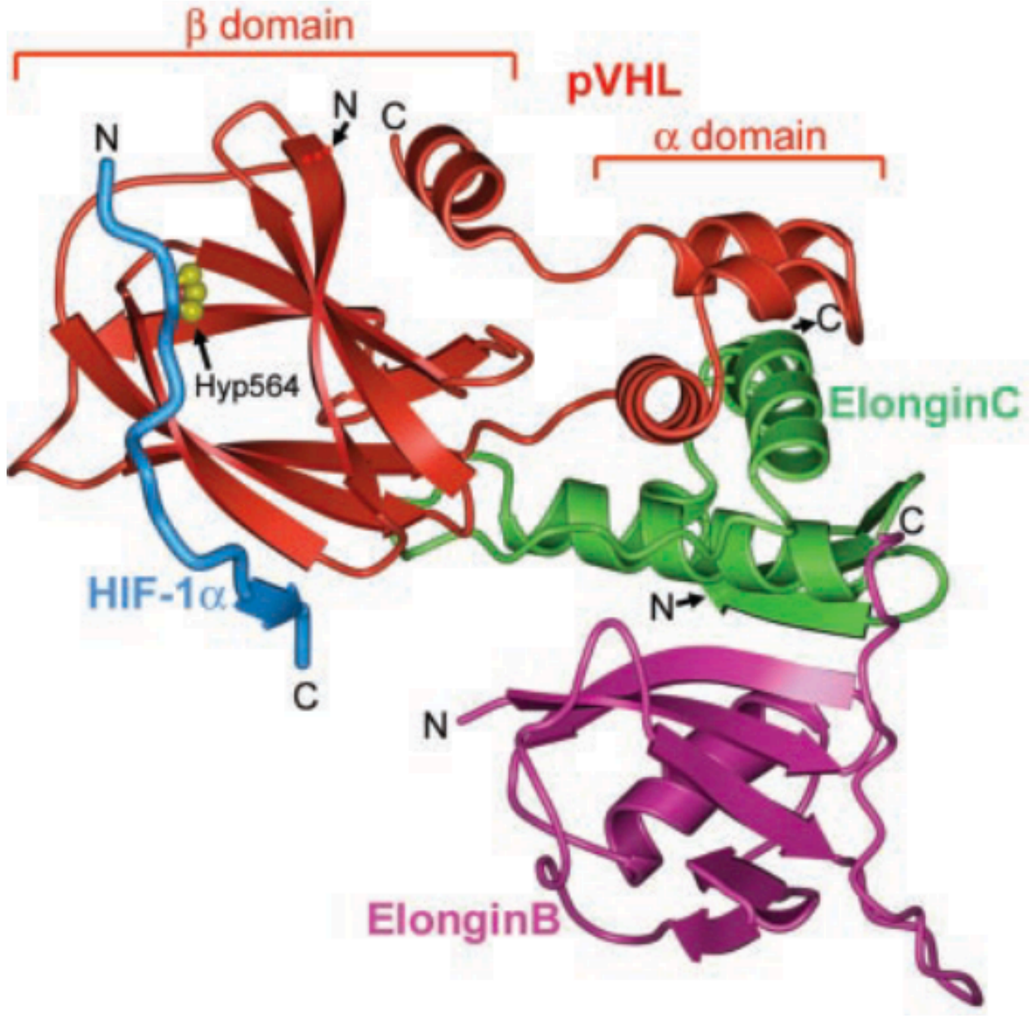
Chowdhury, R., *et al.*, (2009)

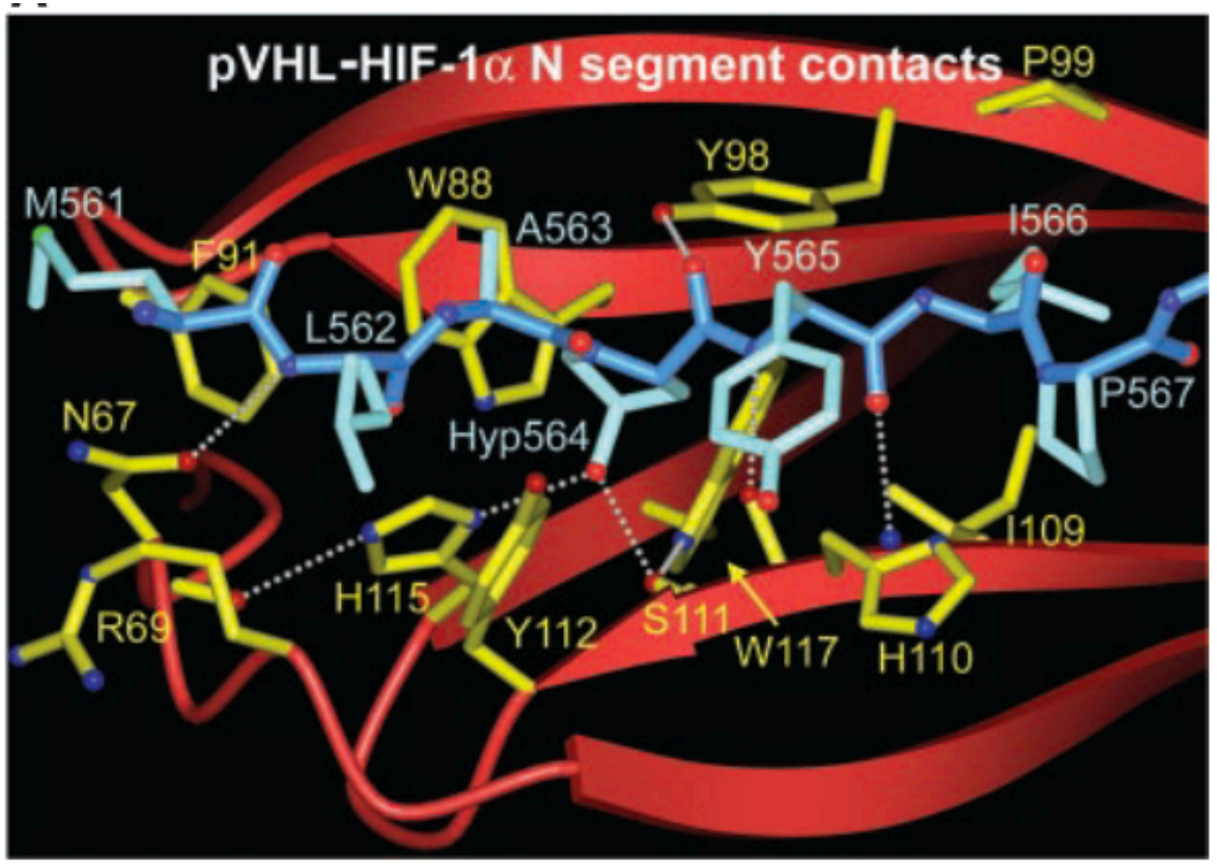


Tarhonskaya, H,. *et al.*(2014)

Appendix

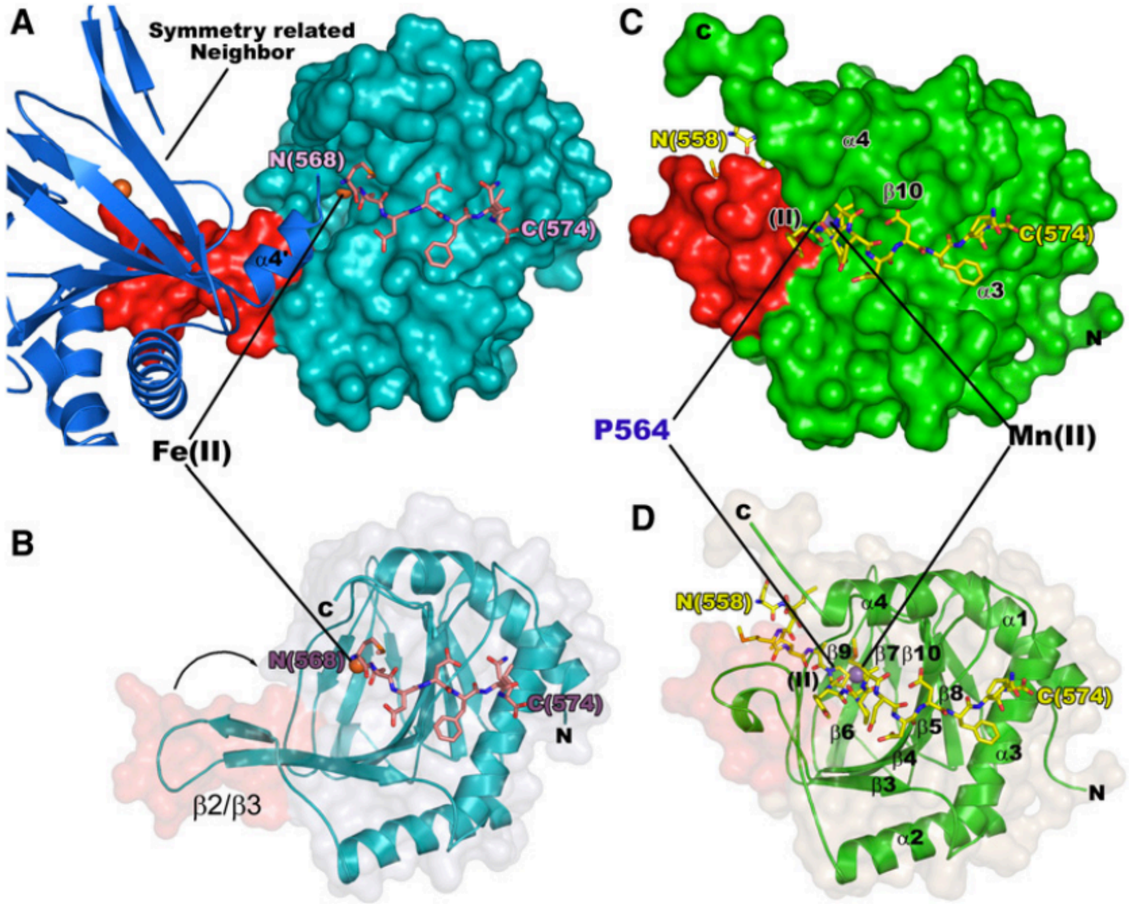
Structure of an HIFα and pVHL complex





Min, J.H., Yang, H., Ivan, M., Gertler, F., Kaelin, W.G., and Pavietich, N.P. (2002). Structure of an HIF-1α-pVHL complex: Hydroxyproline recognition in signaling. Science (80-.). 296, 1886–1889.

Crystallization of tPHD2.HIFα Complex

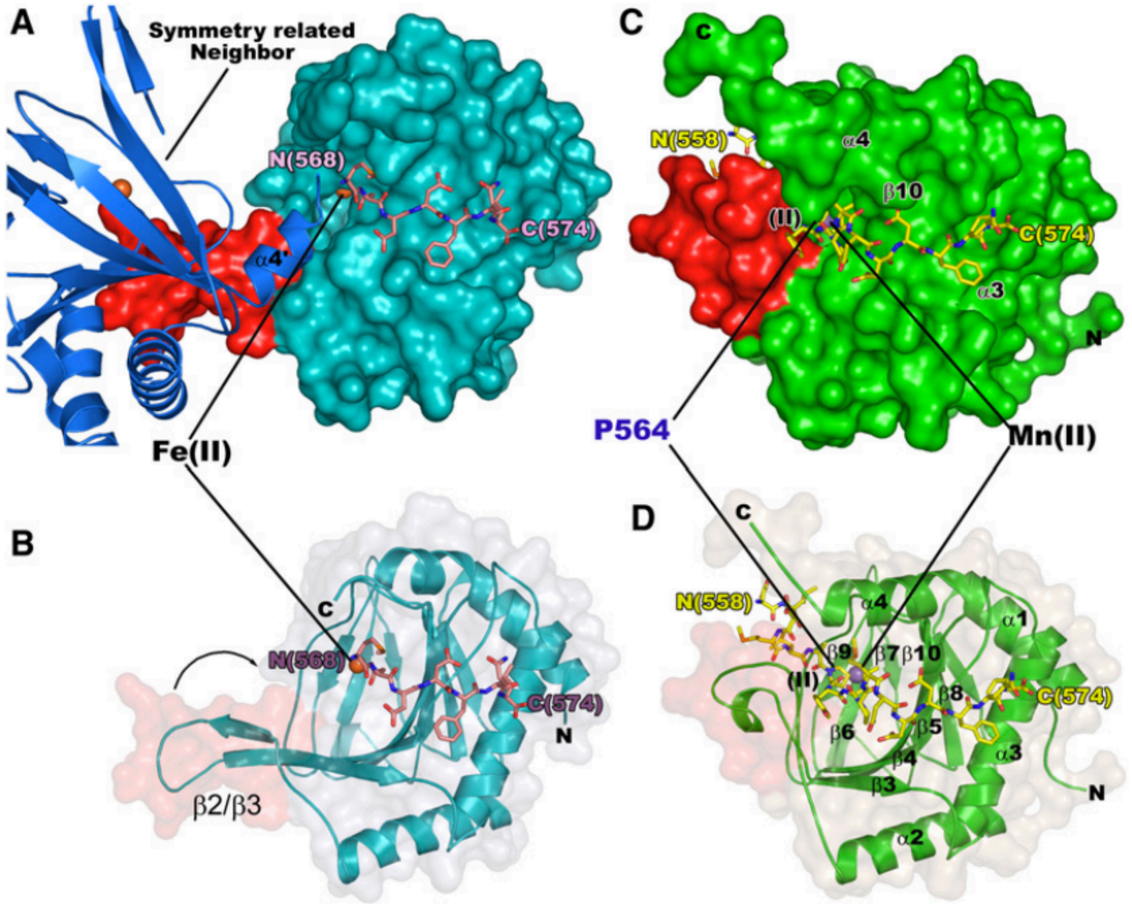


A and B: tPHD2.Fe(II).inhibitor.CODD$_{Hyp564}$ complex (tPHD.Fe(II).A/B)

- It crystallizes in a homotrimeric form.
- The electron density was not observed for hydroxylated Pro-564.
- The reason is that the C-terminal helix (α4) for a symmetry molecule blocks the active site.

Chowdhury, R., *et al.*, (2009)

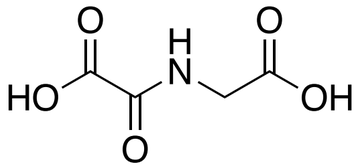
Crystallization of tPHD2.HIFα Complex



C and D:
R398A tPHD2.Mn(II).NOG.HIF-1αCODD$_{556\text{-}574}$ complex (tPHD2.CODD)

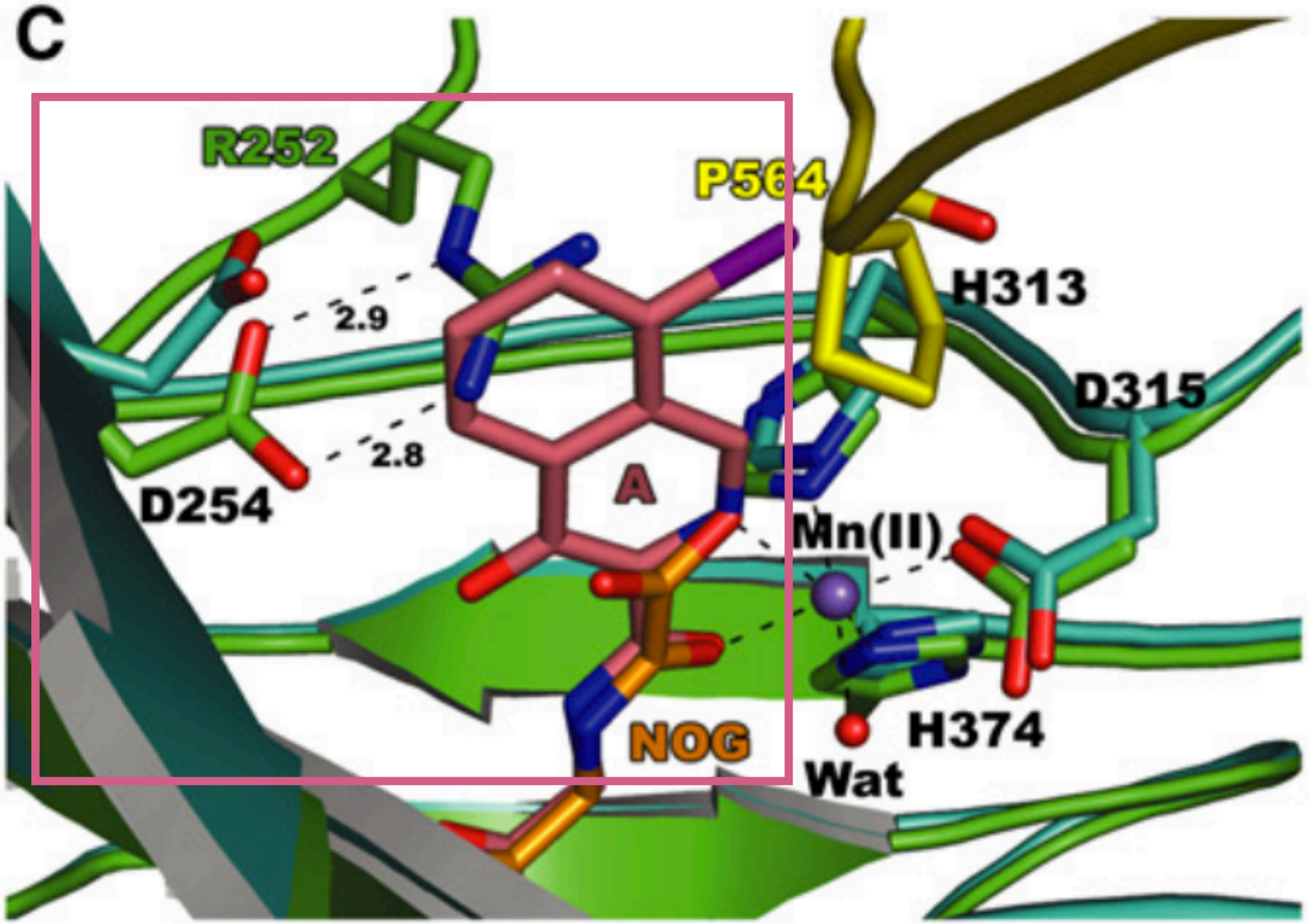
- R398A variant destabilize the homotrimer formation.
- NOG and Mn(II) were substituted for 2OG and Fe(II), respectively.
- The apparent movement of residues 237-254 (β2β3/loop) is proposed to enclose the substrate.

N-oxalylglysine (NOG)



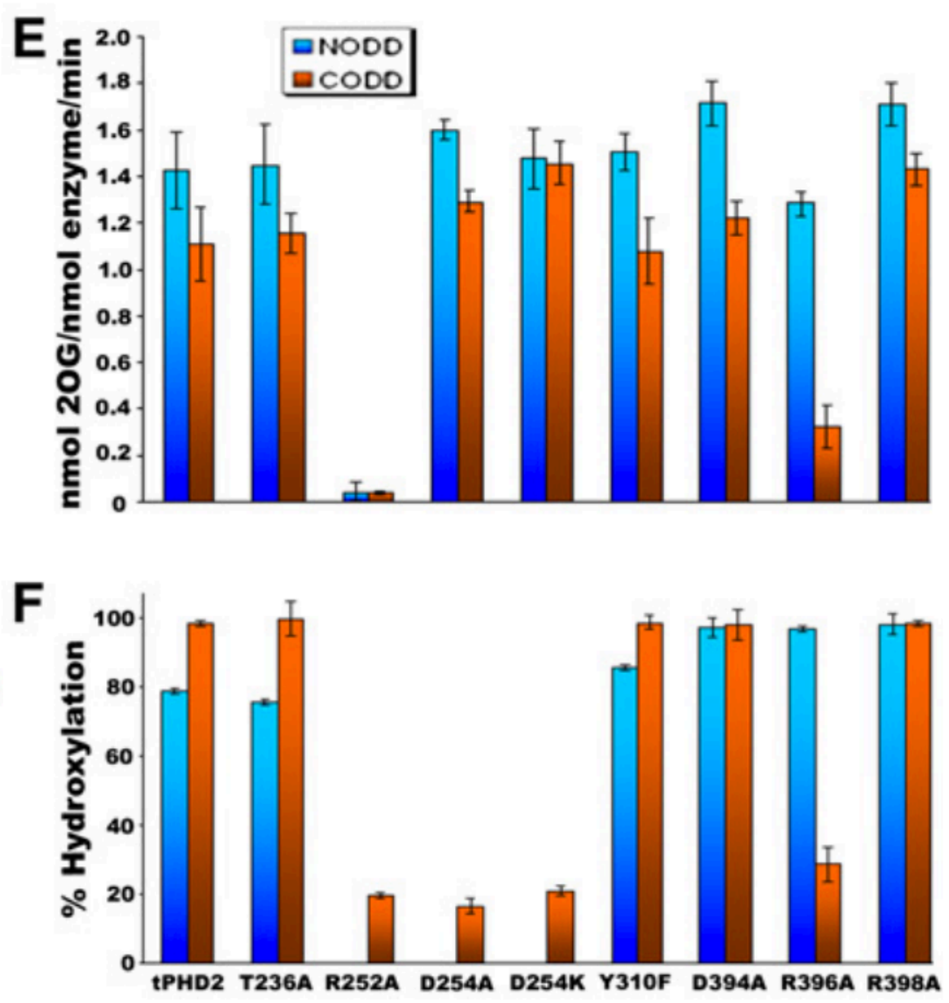
Chowdhury, R., *et al.*, (2009)

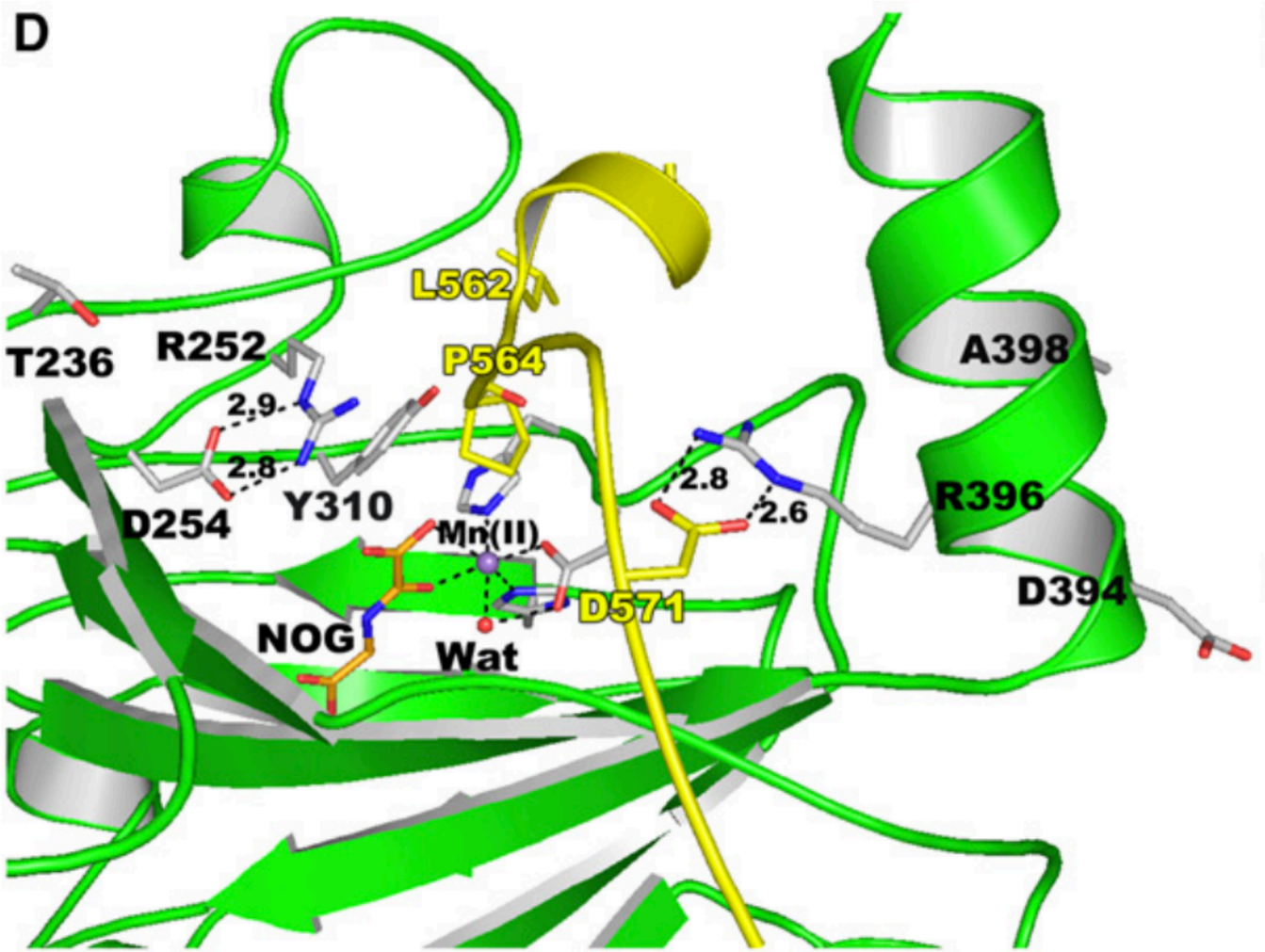
Salt bridge environment



Chowdhury, R., *et al.*, (2009)

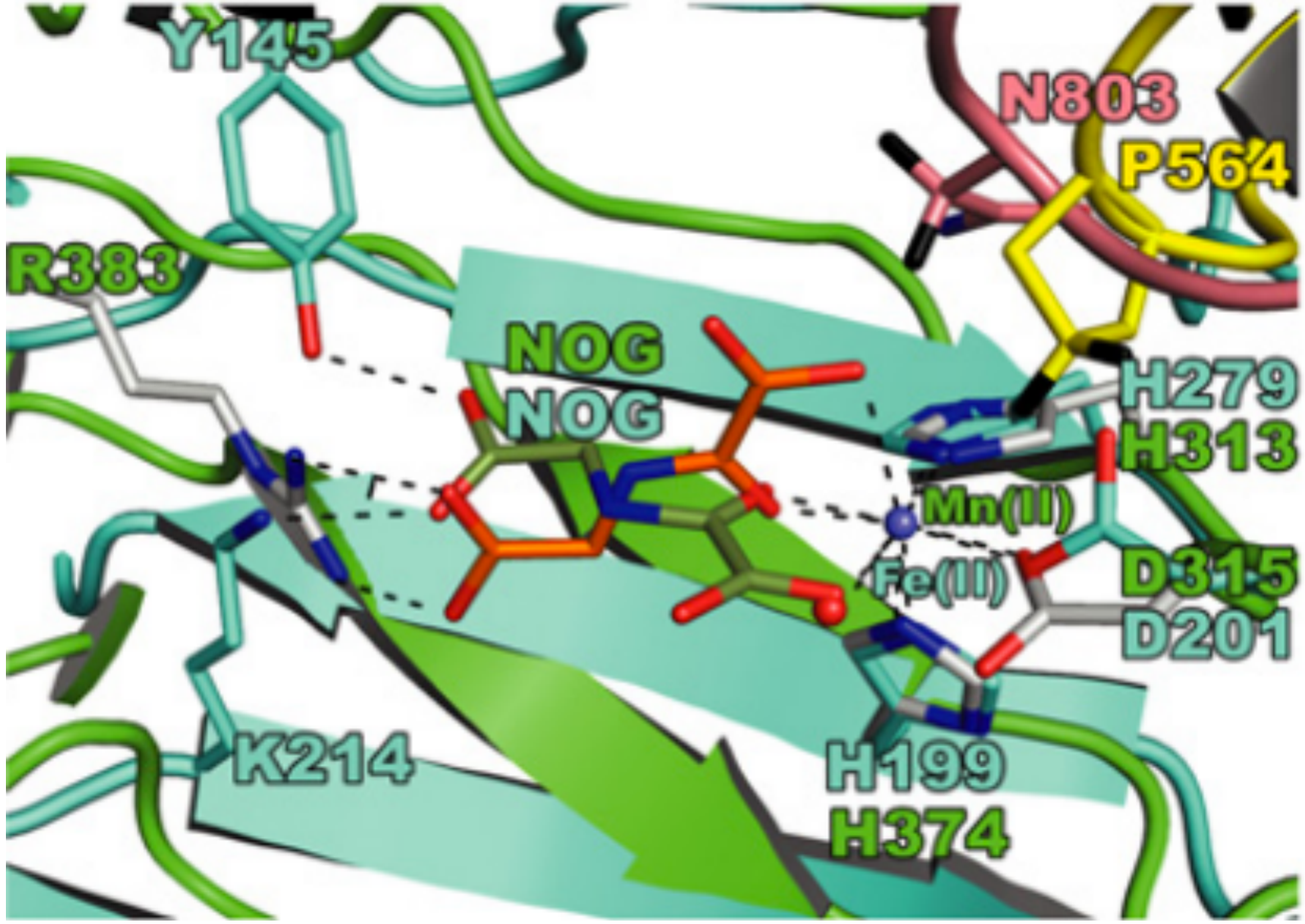
PHD variants forming salt bridge R252 and D254





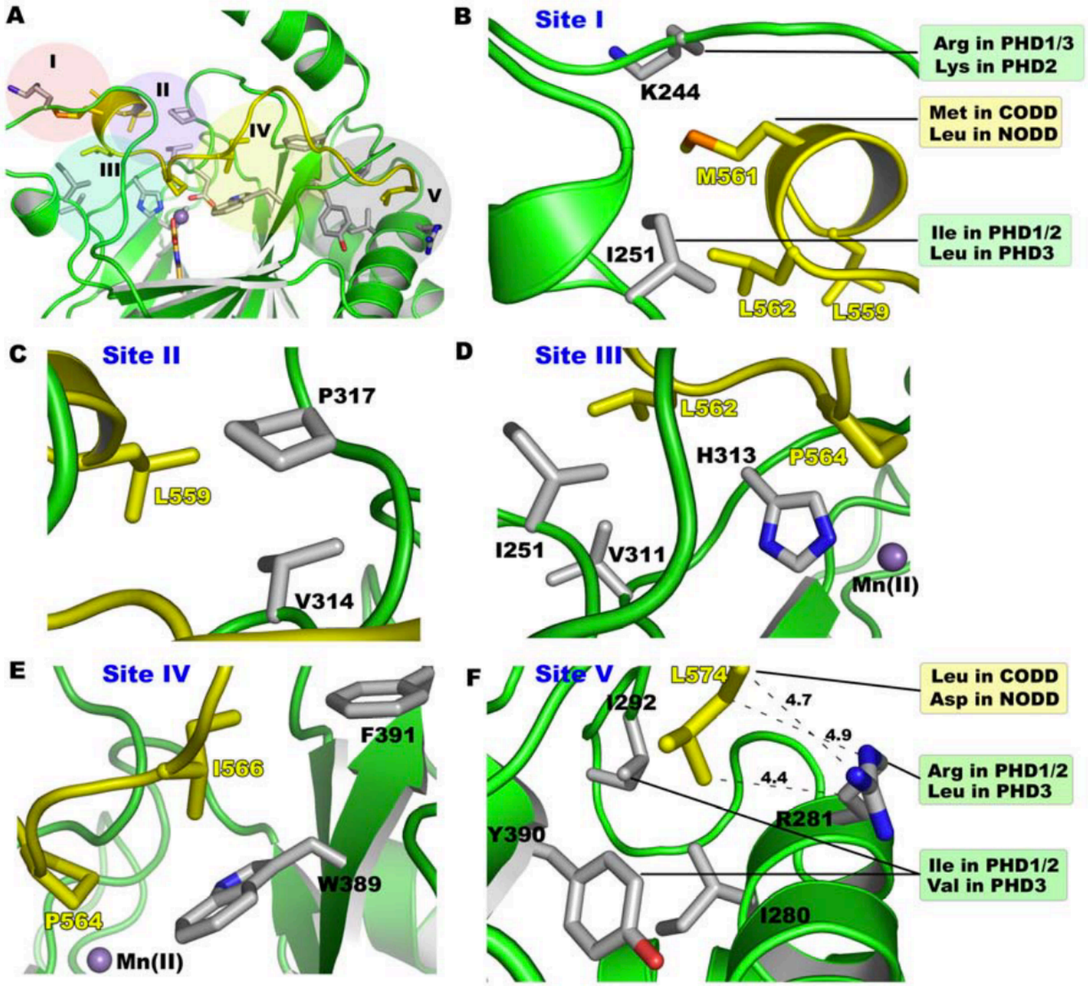
Chowdhury, R., *et al.*, (2009)

PHD and FIH



Chowdhury, R., *et al.*, (2009)

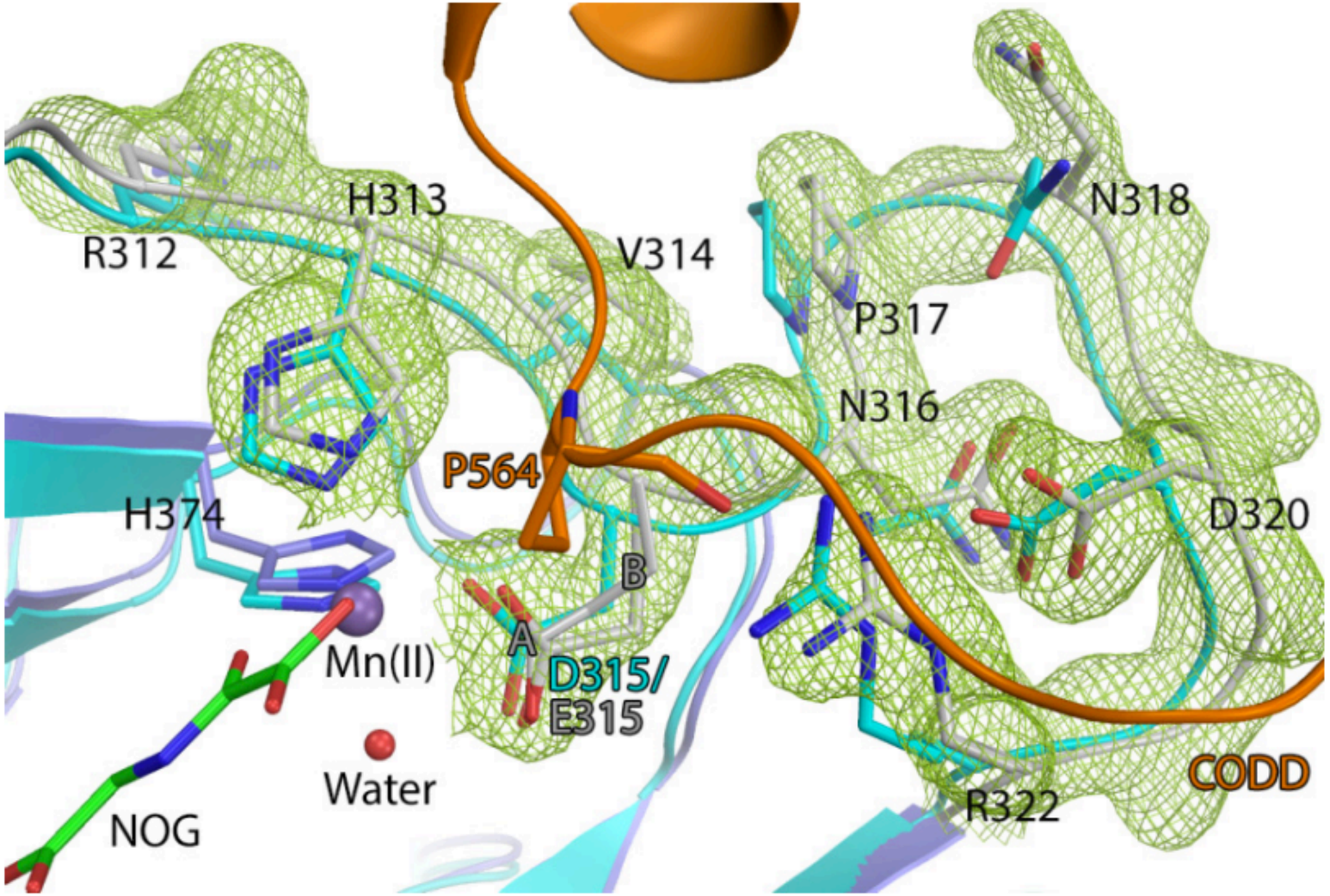
LXXLAP motif



LXXLAP motif of CODD is positioned in hydrophobic site of PHD

Chowdhury, R., *et al.*, (2009)

Binding Fe(II) and 2OG to the PHD2 variants



D315E: The stability of PHD2.Fe(II) complex decreased.

→ It is inconsistant with the high rate of hydroxylation.

Structural analysis of D315E PHD2.Mn(II).Inhibitor complex,

- The flexibility of β2β3 increased
- Two conformation of Glu^{315} side chain were observed
- The bond of metal-water is weaker.

Chowdhury, R., *et al.*, (2009)

2OG turnover and hydroxylation

Table S2. Summary of Results for 2OG Turnover and Hydroxylation of HIF-1α ODDs (NODD and CODD) using tPHD2 and tPHD2 Variants at a 1:25 Enzyme-Substrate Ratio

Classes	Wildtype (wt)/ Variant tPHD2	Equivalent residues in PHD1-3 isoforms			% 2OG turned over relative to tPHD2*		% Hydroxylation	
		PHD1	PHD2	PHD3	CODD	NODD	CODD	NODD
	tPHD2				100.0	100.0	98.5 ± 0.7	78.5 ± 0.7
1. Variants relating to homotrimeric crystal packing								
	Thr236Ala	Arg-220	Thr-236	Arg-57	104.4	101.7	99.5 ± 5.0	75.5 ± 0.7
	Asp394Ala	Lys-378	Asp-394	Glu-216	110.5	120.3	98.0 ± 4.2	97.0 ± 2.8
	Arg398Ala	Ala-224	Arg-398	Glu-220	128.8	119.8	98.5 ± 0.7	98.0 ± 2.8
2. Variants relating to β2/β3 loop								
	Arg252Ala	Arg-236	Arg-252	Arg-74	3.8	3.1	19.5 ± 0.7	n.d.
	Asp254Ala	Arg-238	Arg-254	Arg-76	116.6	112.3	16.5 ± 2.1	n.d.
	Asp254Lys	Asp-238	Asp-254	Asp-76	131.4	103.5	21.0 ± 1.4	n.d.
3. Variants of other residues involved in CODD binding								
	Tyr310Phe	Tyr-294	Tyr-310	Tyr-132	97.5	105.5	98.5 ± 2.1	85.5 ± 0.7
	Arg396Ala	Arg-380	Arg-396	Arg-218	29.3	90.1	28.5 ± 5.0	96.5 ± 0.7

* Activities measured in nmoles of 2OG turned-over/nmoles of enzyme/min (mean ± S.D.) were converted into percentages relative to wt tPHD2 and hence standard deviations are not given.
n.d. = not detected under the experimental conditions.
NODD = HIF-1$\alpha_{395\text{-}413}$; CODD = HIF-1$\alpha_{556\text{-}574}$

Tarhonskaya, H,. *et al.*(2014)

Catalyst of Fe(II)/2OG-dependent oxygenase

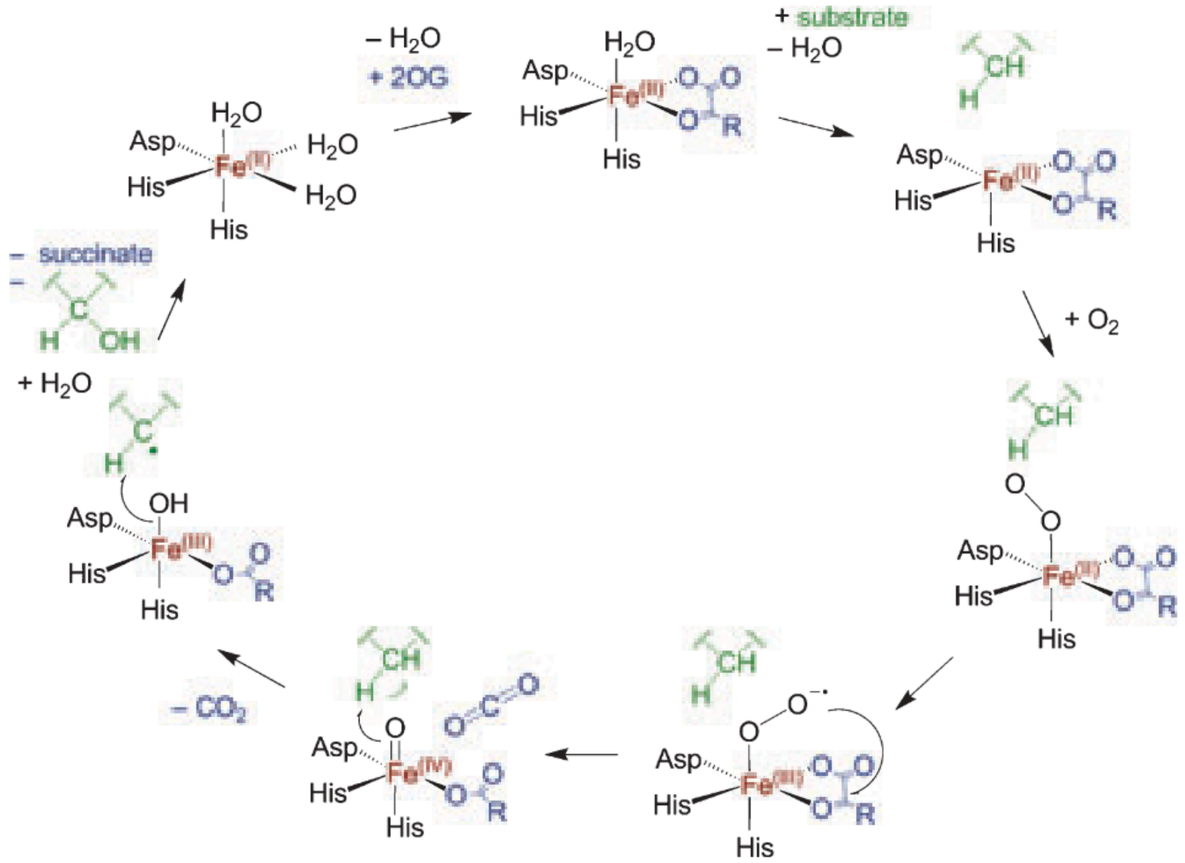
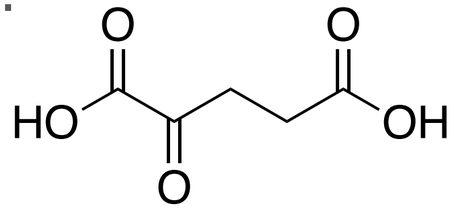


Fig. 1. Proposed general catalytic mechanism for the Fe(II)/2OG oxygenases.

2-oxoglutarate (2OG)

Oxidative decarboxylation of 2OG produces CO_2, succinate and Fe(IV)=O.



Fe(II)

Fe(IV)=O, a reactive intermediate, cleave the substrate C–H bond by hydrogen abstraction to produce Fe(III)–OH and C· radical.

The C· radical reacts with a hydroxyl radical derived from Fe(III)–OH and produces C–OH bond.

Flashman, E., *et al.*, (2010).

The coordination of Fe(II) center

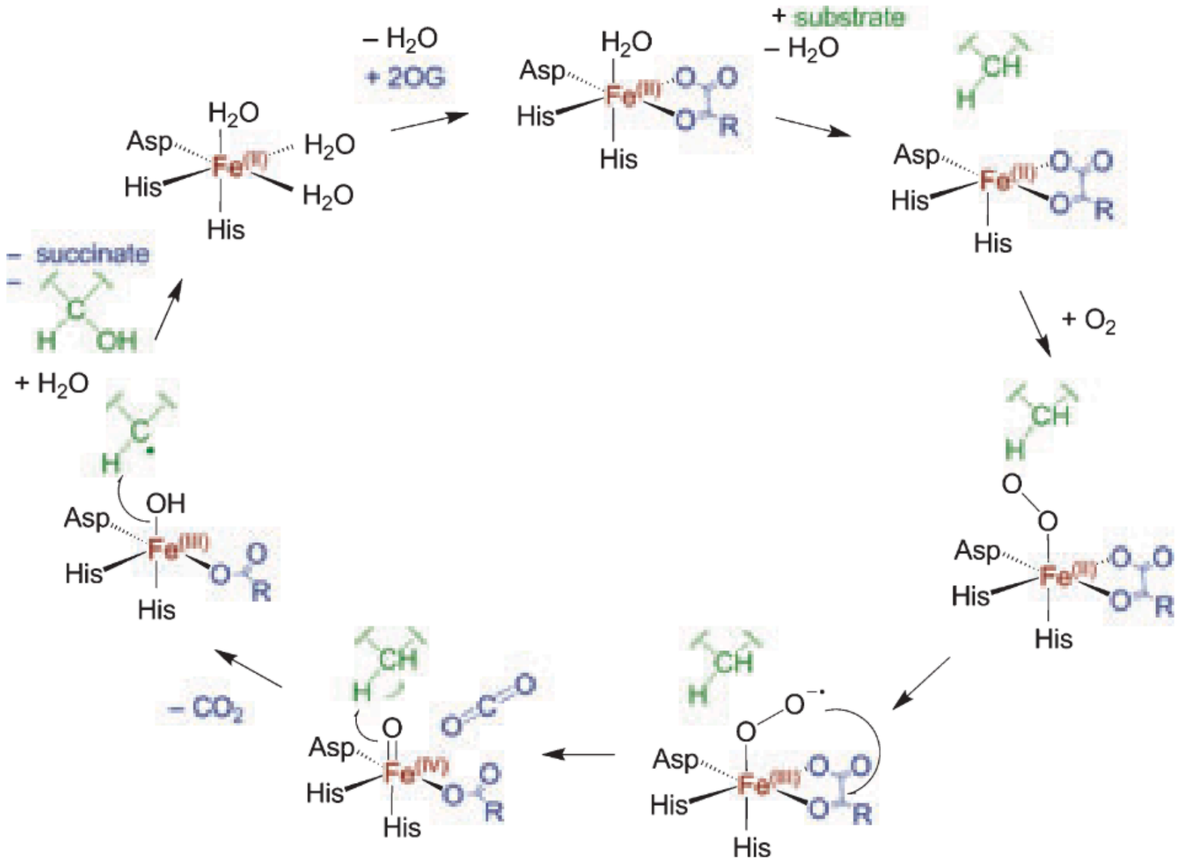


Fig. 1. Proposed general catalytic mechanism for the Fe(II)/2OG oxygenases.

The Fe(II) center of 2OG-dependent oxygenases is normally coordinated by three protein derived ligands two His and one Glu or Asp.

2OG binds to the Fe(II) in a bidentate manner.

The exchange from coordinated water to oxygen occurs when substrate binds to adjacent to the Fe(II) and weaken binding of the water.

Flashman, E., *et al.*, (2010).