Supplementary Fig 1. **A.** Dose dependence of N803-OH to T-hydro in U2OS cells. U2OS cells expressing dox-inducible 344-826 V5 HIF-1α were treated with and without T-hydro (at doses indicated) in the presence of MG132 for 3h. Extracts were then analysed by immunoblotting (IB) using the indicated antibodies. While 10 μM inhibited, 2.5 and 1 μM T-hydro had little or no effect on N803-OH immunoreactivity. **B.** Comparison of HIF-1α induction in U2OS cell extracts following additions of T-hydro (10 μM) every 25 min for 4h, or 4h treatment with DFO (100 μM), MMOG (1 mM) and MG132 (25 μM). **C.** Dose dependence of HIF-1α induction by T-hydro in U2OS cells. Cell extracts were prepared following additions of T-hydro (at doses indicated) and treated as in B. Morphological changes were observed in cells treated with 20 and 40 μM T-hydro.

Supplementary Fig 2. **A.** Extracted ion chromatograms illustrating the effect of T-hydro 10 μM on the abundance of the hydroxylated OH(N) and unmodified N485 peptide ions from Rabankyrin-5. **B.** Analysis of FIH protein levels in HEK 293 cells expressing the Rabankyrin-5/HIF-1α CAD fusion and subjected to a 3h peroxide exposure as in A.

Supplementary Fig 3. Pre-treatment of cells with either ascorbate or N-acetyl cysteine does not prevent the inhibition of FIH activity by peroxide. **A.** U2OS cells were incubated with ascorbate (Asc, 30 μM) for 90 min prior to T-hydro (10 μM) exposure for 20 min. Hypotonic extracts were prepared and FIH activity assayed *in vitro* by incubation with RRL GAL 775-826 substrate and subsequent IB for N803-OH (left panel). NP-40 extracts prepared in parallel were immunoblotted for normoxic HIF-1α level as readout for efficacy of the ascorbate pre-treatment (right panel). **B.** U2OS cells were incubated with N-acetyl cysteine

(NAC, 5 mM) for 20 h prior to T-hydro (10 μM) exposure, preparation of extracts and assay of FIH activity as in A.

Supplementary Fig 4. Dependence of FIH activity in Jurkat cells to peroxide concentration. Jurkat cells were incubated with T-hydro (at doses indicated) or H_2O_2 (10 μM) for 20 min prior to preparation of hypotonic extract and assay of FIH activity *in vitro*. FIH activity was assessed by incubation with RRL GAL 775-826 substrate and subsequent IB for N803-OH.

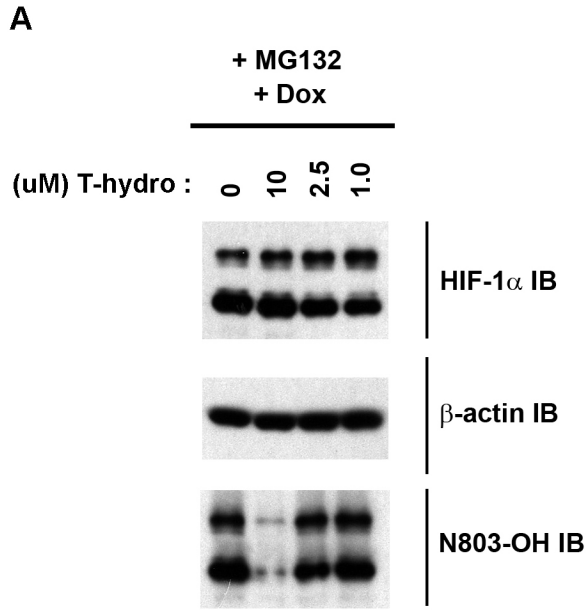
Supplementary Fig 5. Treatment of cells with iron chelators protects FIH from inhibition by peroxide. FLAGFIH-expressing cells (Cockman *et al*. 2006 *Proc Natl Acad Sci USA* **103**: 14767-14772) were treated for 24 h with either dox alone, or in combination with desferrioxamine (DFO, 100 μM) or 2,2' Dipyridyl (100 μM). Where indicated, cells were exposed to T-hydro (10 μM) applied in 2 bolus additions 40 min apart at the end of the time course. IB of extracts for HIF-1α confirmed the efficacy of the 2,2' Dipyridyl and DFO treatments. FLAGFIH was then immunopurified and FLAG eluate tested for N803-OH activity using RRL GAL 775-826 substrate and IB of reaction mixtures. * indicates a non-specific band.

Supplementary Fig 6. U2OS cells were transfected with a CA9-HRE luciferase (Wykoff *et al*. 2000 Cancer Res. **60**: 7075-7083) or control luciferase plasmid together with a pCMV β-galactosidase plasmid. At 20h post transfection, cells were incubated for 3 h either in normoxia or 1 % O2 and treated with or without T-hydro (10 μM, added every 25 min). Whole cell extracts were assayed for luciferase activity and β-galactosidase activity (to

normalise for transfection efficiency). The luciferase counts from replicate experiments (n=3) are displayed, with error bars to represent the standard deviation (upper panel). Extracts were also assayed by immunoblotting and a representative immunoblot is displayed (lower panel).

Supplementary Fig 7. Cysteine oxidation does not appear to be the mechanism of inhibition of FIH by peroxide. **A**. Treatment of cells with peroxide results in an oxidative modification of cysteine. FLAGFIH-expressing cells were treated with and without dox for 24 h and, where indicated, exposed to T-hydro 10 μM applied in 2 bolus additions at 40 min intervals. Cells were then lysed in buffer either with or without dimedone (according to manufacturer's instructions, see below), FLAGFIH immunopurified and analysed by IB with anti-cysteine sulfenic acid antibody (Cys-SOH, Millipore) that recognises proteins containing cysteine sulfenic acid derivitised with dimedone. Anti-Cys-SOH signal co-migrating with the immunopurified FLAGFIH is detected in a dox-dependent, peroxide-dependent and dimedone-dependent manner, providing evidence of a peroxide-induced cysteine oxidation in FIH. **B**. Mutation of individual cysteine residues in FIH does not prevent inhibition by peroxide. U2OS cells were engineered to express dox-inducible FLAGFIH mutants with each of the three cysteine residues mutated individually to serine (denoted C216S, C226S and C236S). Cells were treated with dox and, where indicated, exposed to T-hydro (10 μM) applied in 2 bolus additions at 40 min intervals. Cells were then lysed, FLAGFIH WT and cysteine mutant proteins immunopurified and eluted with FLAG peptide. A representative coomassie stain of a set of extracts and FLAG eluates confirms the efficacy of the purification procedure (lower panel). The FLAG eluates were then tested for N803-OH activity using GAL 775-826 RRL substrate and IB of reaction mixtures (upper panel). In all

cases (C216S, C226S and C236S), mutation of the cysteine residue does not ablate FIH activity, nor prevent the inhibition by peroxide.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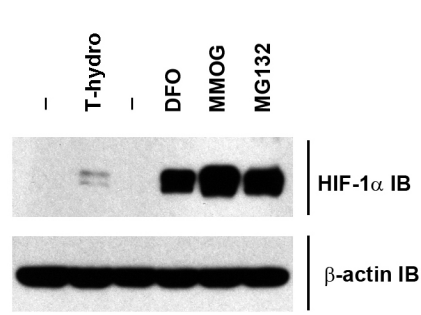
+ MG132
+ Dox
(uM) T-hydro :
0
10
2.5
1.0
HIF-1α IB
β-actin IB
N803-OH IB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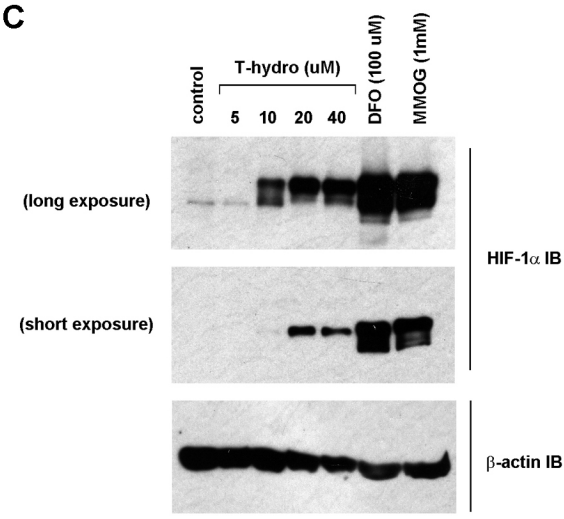
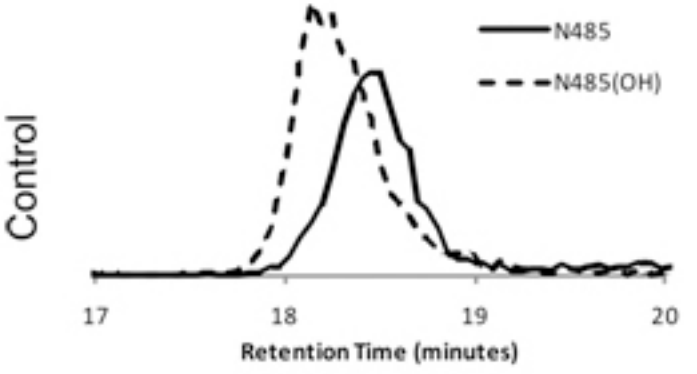
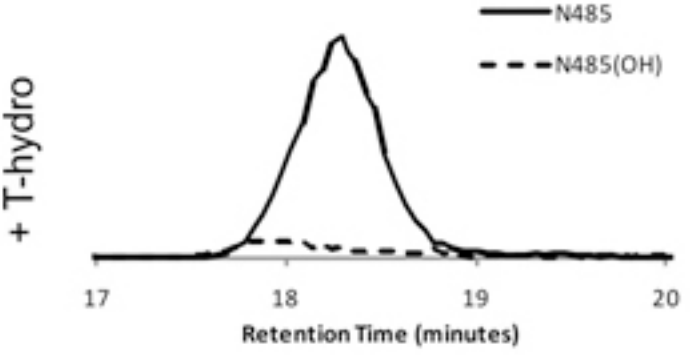
T-hydro
–
DFO
MMOG
MG132
HIF-1α IB
β-actin IB


C
control
T-hydro (uM)
5
10
20
40
DFO (100 uM)
MMOG (1mM)
(long exposure)
(short exposure)
HIF-1α IB
β-actin IB

A

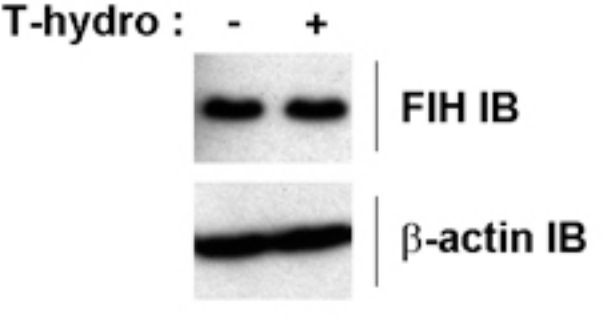


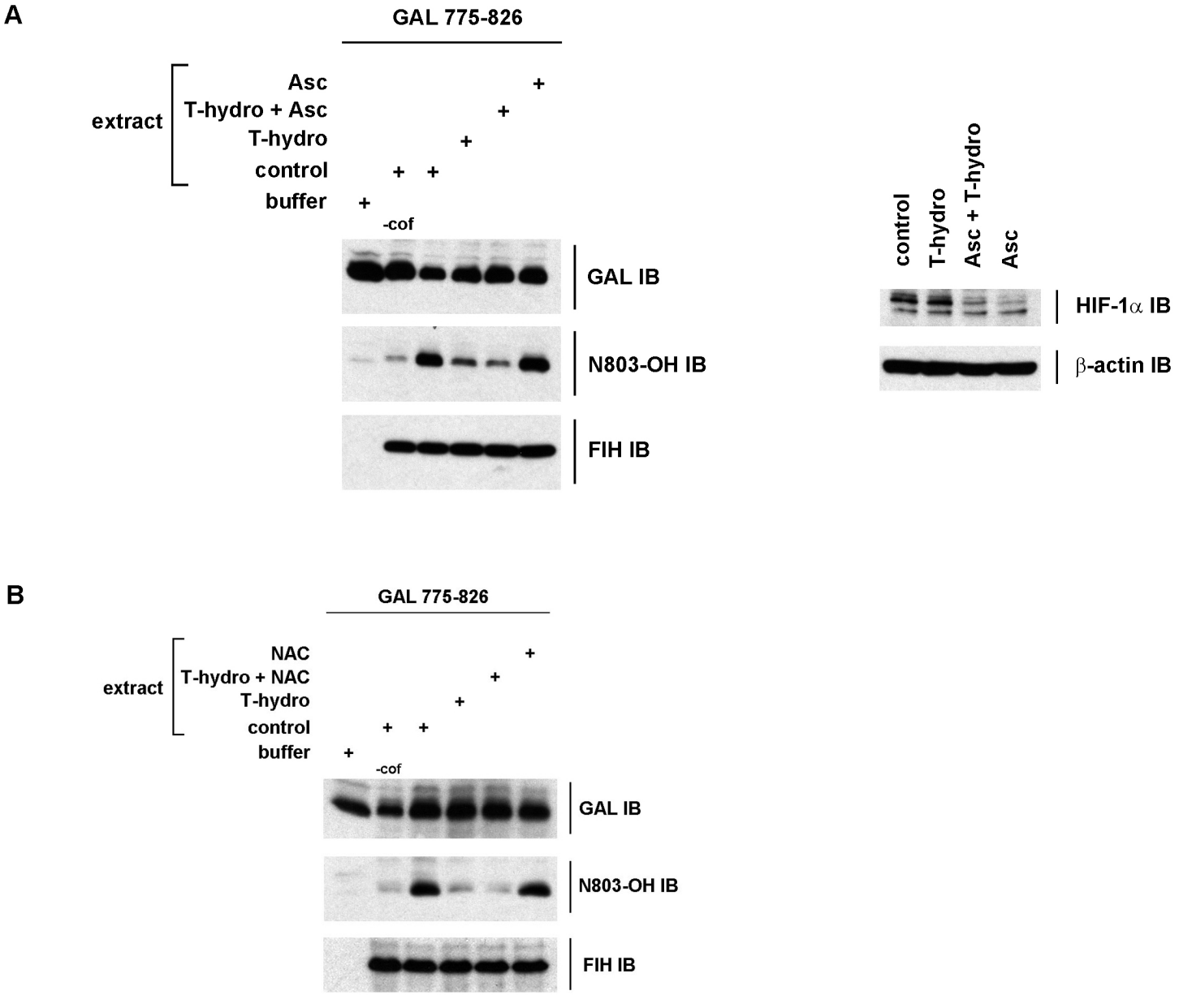
Unmodified: 45%
OH(N): 55%

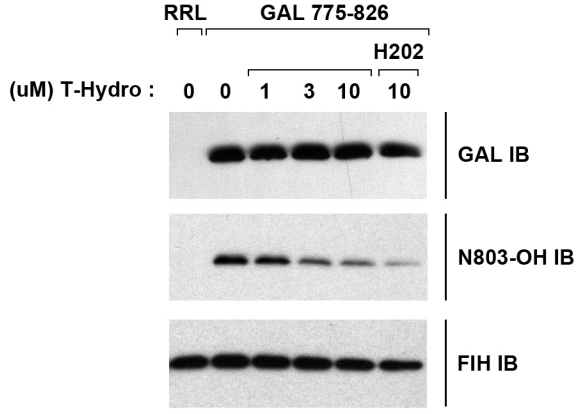


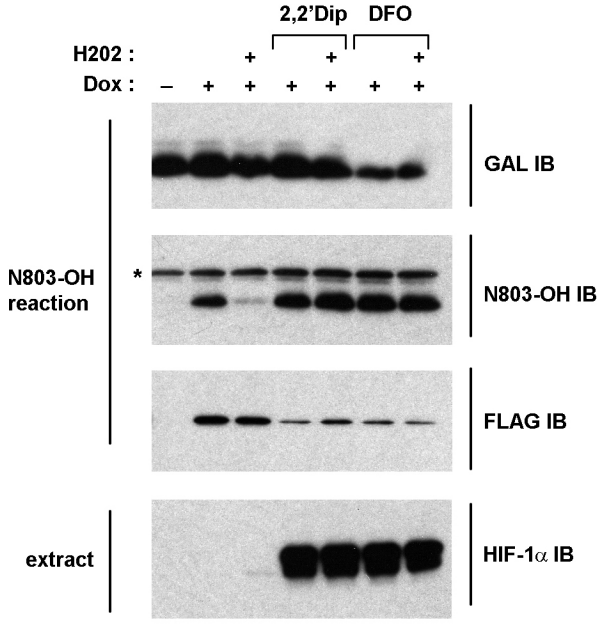
Unmodified: 93%
OH(N): 7%

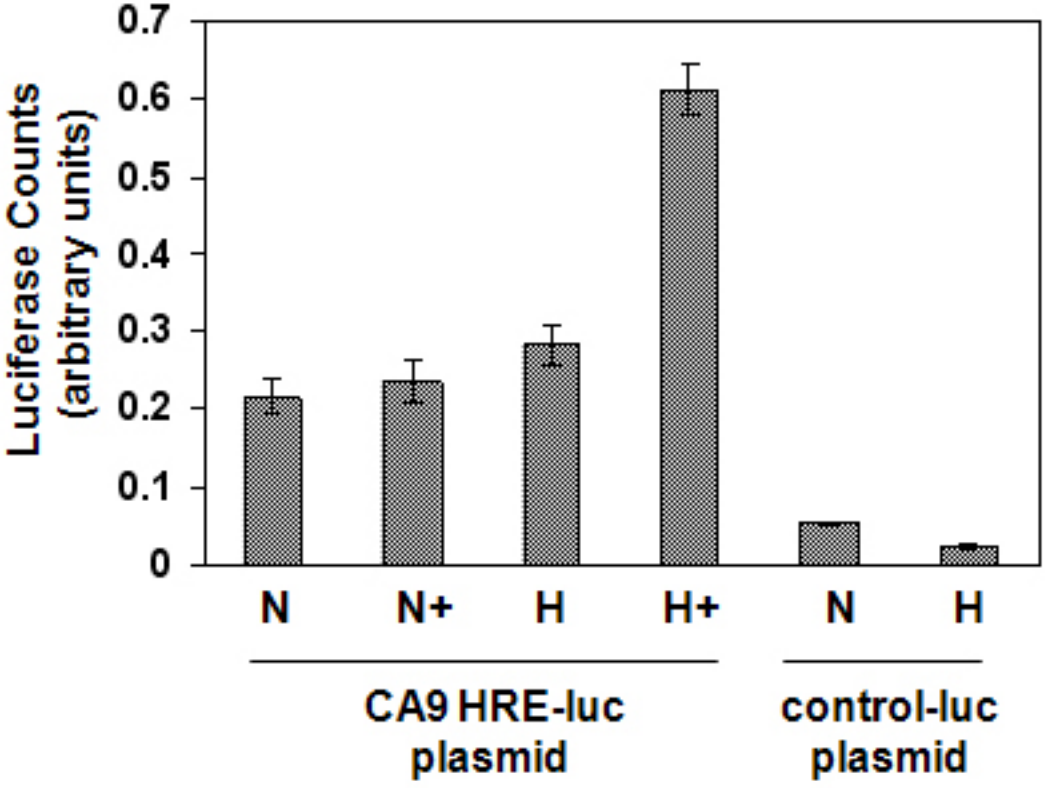
B

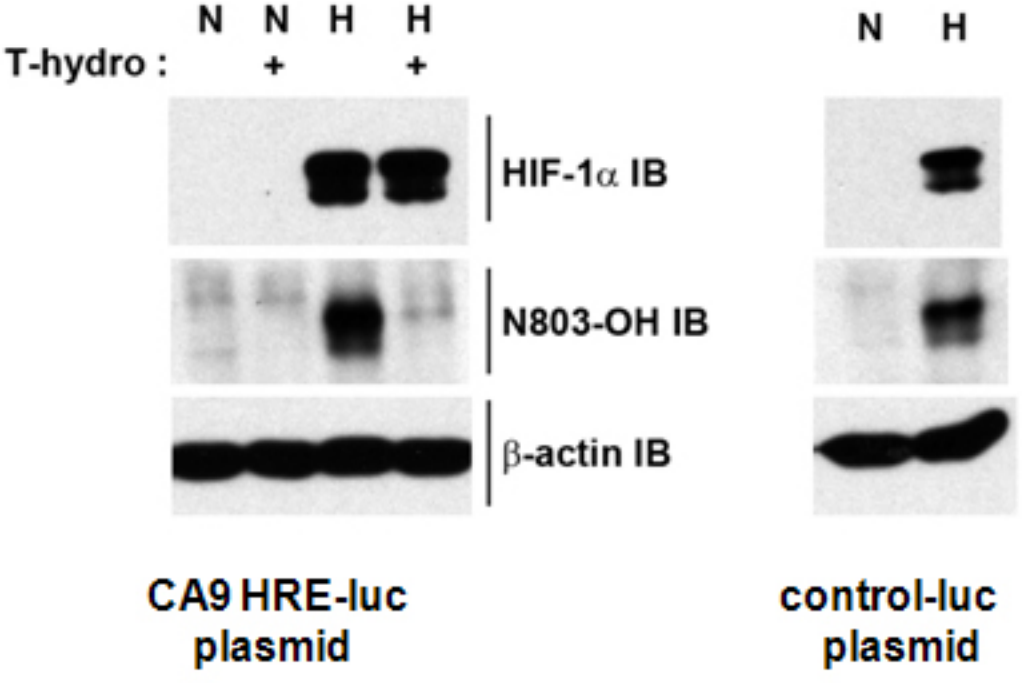


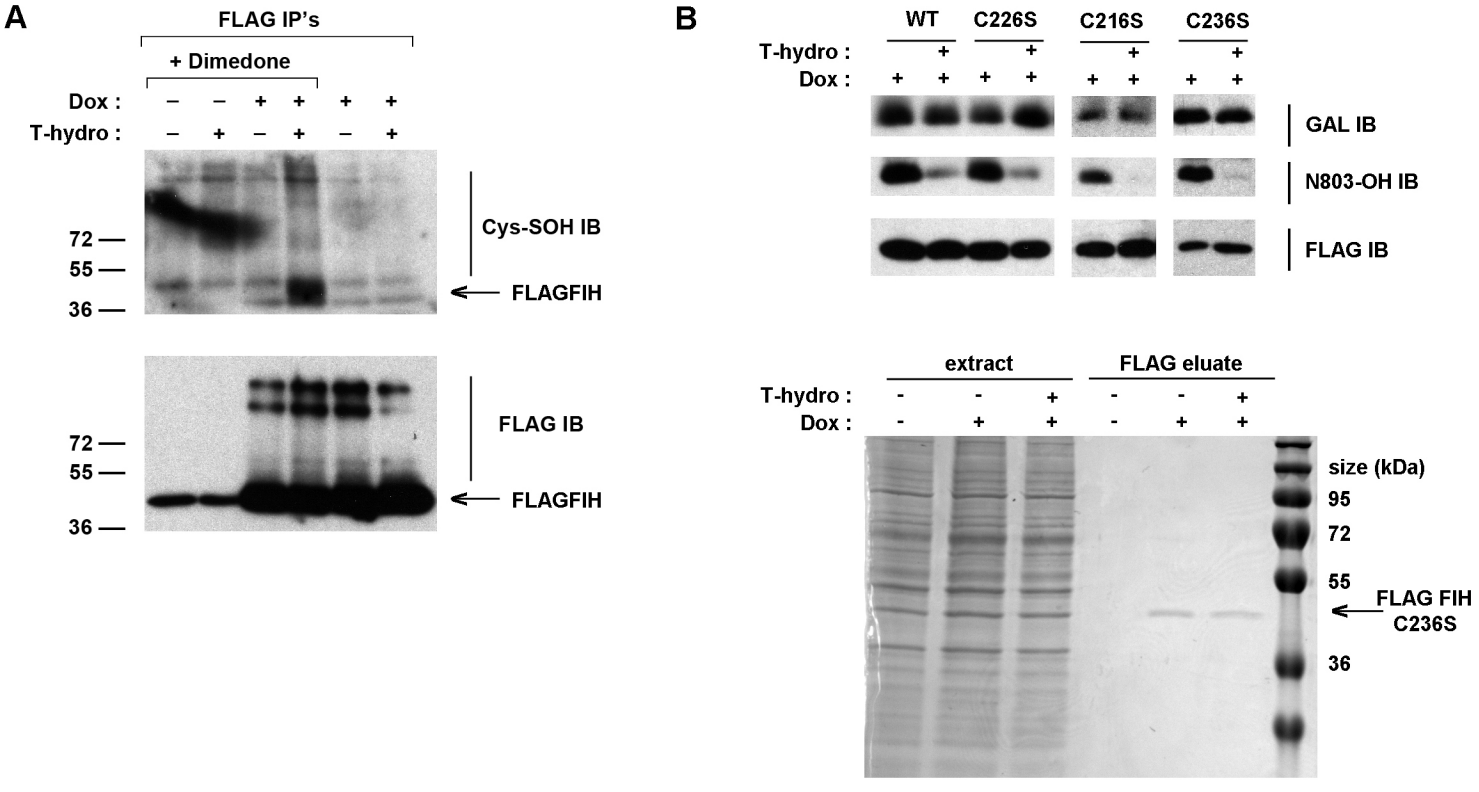

A
GAL 775-826
extract
Asc
T-hydro + Asc
T-hydro
control
buffer
-cof
GAL IB
N803-OH IB
FIH IB
control
T-hydro
Asc + T-hydro
Asc
HIF-1α IB
β-actin IB
B
GAL 775-826
extract
NAC
T-hydro + NAC
T-hydro
control
buffer
-cof
GAL IB
N803-OH IB
FIH IB


RRL
GAL 775-826
H202
(uM) T-Hydro :
0
0
1
3
10
10
GAL IB
N803-OH IB
FIH IB


2,2'Dip
DFO
H202 :
Dox :
– + + + + + +
GAL IB
N803-OH reaction
*
N803-OH IB
FLAG IB
extract
HIF-1α IB


Luciferase Counts
(arbitrary units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N
N+
H
H+
N
H
CA9 HRE-luc
plasmid
control-luc
plasmid


N
N
H
H
T-hydro :
+
+
N
H
HIF-1α IB
N803-OH IB
β-actin IB
CA9 HRE-luc
plasmid
control-luc
plasmid


A
FLAG IP's
+ Dimedone
Dox :
T-hydro :
72
55
36
Cys-SOH IB
FLAGFIH
FLAG IB
B
WT
C226S
C216S
C236S
GAL IB
N803-OH IB
FLAG IB
extract
FLAG eluate
size (kDa)
95
72
55
36
FLAG FIH
C236S