Inhibition of Smurf2 E3 ubiquitin ligase by heclin and its analogues enhances HIF-1α expression and transcriptional activity in normoxia or hypoxia

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ABSTRACT

Hypoxia-inducible factors (HIFs) act as transcription factors and play an essential role in cellular and systemic responses to low oxygen environments. HIF-1α expression is induced in acute hypoxia typically through failure of its ubiquitination and degradation mediated by Von Hippel-Lindau (VHL). Previously we discovered a non-canonical mechanism where the SMAD specific E3 ubiquitin protein ligase 2 (Smurf2) promotes the ubiquitination of HIF-1α and reduces HIF-1α level in HCT116 colorectal cancer cells. Smurf2 is a HECT-type ubiquitin ligase known to interact with Smad proteins, leading to their ubiquitination and proteasomal degradation. Overexpression of Smurf2 decreased HIF-1α expression in HCT116 and SW480 colorectal cancer cells under hypoxia as well as in RCC4 VHL-deficient kidney renal clear cell carcinoma cells under normoxia. Treatment with MG132 at least partially rescued the expression of HIF-1α following Smurf2 overexpression, indicating involvement of proteasome-dependent degradation in Smurf2-mediated HIF1α destabilization. Knockdown of SMURF2 increased HIF-1α expression under normoxia in HCT116 and RCC4 cells. To investigate the effect of Smurf2 inhibition, we tested a selective reversible inhibitor of HECT E3 ubiquitin ligases, heclin, along with its analogues, PYR-41, C646 and 4E1RCat. Treatment with heclin increased HIF-1α expression in HCT116 under hypoxia as well as in SW480 and RCC4 cells under normoxia in a dose-dependent manner. PYR-41 elevated the level of HIF-1α level in HCT116 cells under hypoxia, in RCC4 cells under normoxia and in SW480 cells under both normoxia and hypoxia. To examine the effect on HIF transcriptional activity, we performed luciferase reporter assay using plasmids containing the hypoxia response element (HRE) from the PGK1 promoter and the VEGF promoter, respectively. PYR-41 induced transcriptional activity on PGK1-HRE in both HCT116 and SW480 cells. 4E1RCat robustly activated transcription on both VEGF-HRE and PGK1-HRE in HCT116 and SW480 cells. In summary, Smurf2 targets HIF-1α for ubiquitination and degradation independently of oxygen concentration and inhibition of Smurf2 to stimulate HIF activity under normoxia or hypoxia may be beneficial in pathological circumstances featuring anemia and hypoxemia.

RESULT

HECT-type E3 ubiquitin ligase inhibitor heclin treatment enhances HIF-1α expression

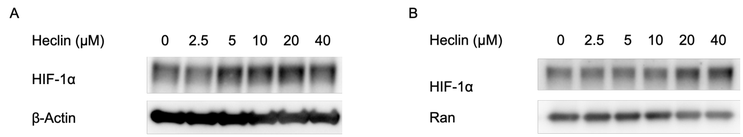

Figure 1. Induction of HIF1α level by heclin in colorectal cancer cells. (A) HCT116 cells were treated at indicated doses for 6h in hypoxia (0.5% O_2). (B) SW480 cells were treated at indicated doses for 6h in normoxia (21% O_2).

Analogue 4E1RCat induces HIF-1α transcriptional activity in HRE luciferase reporter assay

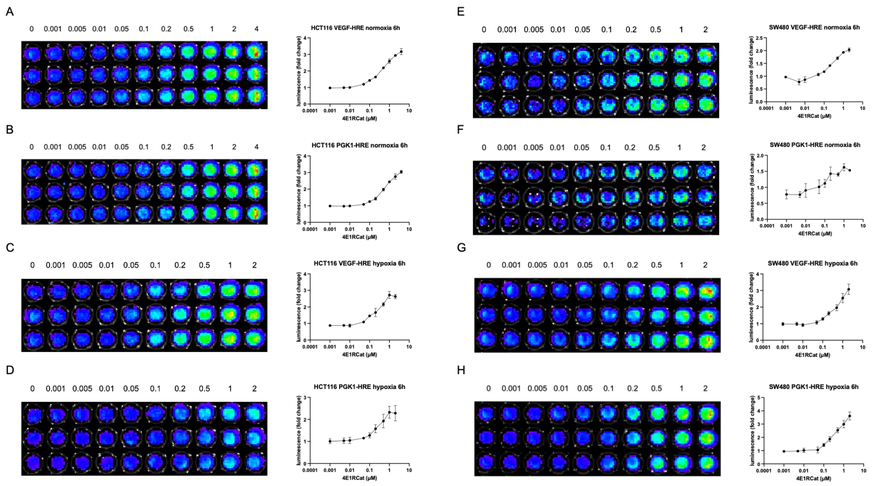

Figure 2. 4E1RCat induces HIF-1α activity on HRE. (A-D) HCT1116 and (E-H) SW480 cells were stably transfected with (A, C, E, G) VEGF-HRE or (B, D, F, H) PGK1-HRE and treated with 4E1RCat for 6h in (A, B, E, F) normoxia or (C, D, G, H) hypoxia.

Treatment with 4E1RCat at low doses elevated HIF-1α expression level in normoxia

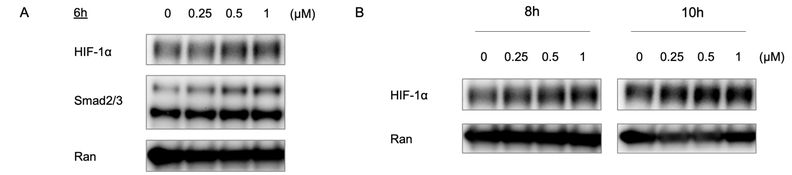

Figure 3. 4E1RCat increases HIF-1α expression in HCT116 cells under normoxia. Cells were treated with 4E1RCat at indicated doses for (A) 6 hours, (B) 8 or 10 hours.

4E1RCat upregulates the transcription of the HIF-1α target gene *EPO (erythropoietin)*

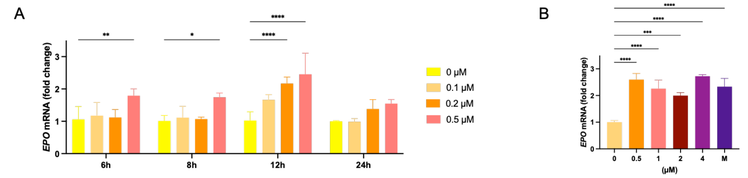

Figure 4. 4E1RCat increases EPO mRNA expression in HCT116 cells. Cells were treated for indicated durations in (A) normoxia or (B) hypoxia. Extracted RNA was analyzed by real-time PCR. M: MG132 (5μM).

PYR-41 increases the expression level of HIF-1α in different cell lines

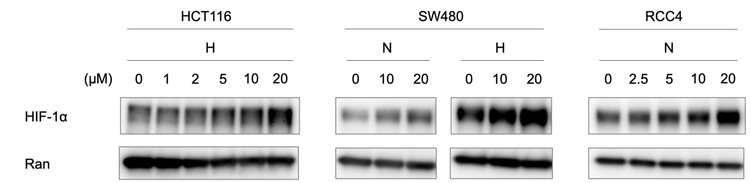

Figure 5. PYR-41 increases HIF-1α expression in HCT116 under hypoxia, in SW480 under normoxia and hypoxia, and in RCC4 under normoxia. N: normoxia; H: hypoxia.

MODEL

CONCLUSIONS

1. 4E1RCat enhances HIF-1α expression and induces its transcriptional activity in normoxia and hypoxia at very low doses (in nanomolar range).
2. PYR-41 increases HIF-1α expression level in different cell lines under normoxia or hypoxia.
3. Inhibition of Smurf2 with small molecules has therapeutic potentials in circumstances demanding HIF activation.

FUTURE DIRECTIONS

1. Effect on HIF-1α targets, EPO protein level and HIF-2α.
2. Mechanisms involving Smurf2.
3. Combination therapies.
4. Window chamber model for *in vivo* effect.
5. Effect on erythroid differentiation into reticulocytes and red blood cells.
6. Reticulocytes after *in vivo* administration.

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