AACR-NCI-EORTC
Annual Meeting, Oct 11th-16th, 2023
Abstract #B090

Partially Open Conformation Of The G323E Mutated HIF-2*α* PASB Domain Captured By X-ray Crystallography

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INTRODUCTION

Under hypoxic conditions, vital cell processes such as angiogenesis, erythropoiesis, cell proliferation and metabolism are regulated by a group of transcription factors known as hypoxia-inducible factors (HIFs). These proteins belong to the helix-loop-helix family of transcription factors and require heterodimerization to function. Constitutively expressed HIF-1β protein forms a heterodimer with one of the oxygen-labile counterparts: HIF-1α, HIF-2α, or HIF-3α. The HIF-αs are degraded under normoxic conditions via ubiquitination by the von Hippel Lindau (VHL) E3 ubiquitin ligase. They become available for dimerization under either hypoxia or VHL dysfunction. Certain cancer types take advantage of this HIF-regulated transcription for survival and growth, as exemplified by VHL-deficient clear cell renal cell carcinoma (ccRCC).

Inhibitors aimed at disruption of the HIF-1β:HIF-2α heterodimer have advanced into clinical development, including belzutifan (approved by the Food and Drug Administration for adult patients with VHL disease who require therapy for RCC among other indications) and AB521 (Arcus Biosciences). This class of small molecules functions by disrupting HIF heterodimerization via binding to the internal cavity of the HIF-2α Per-Arnt-Sim (PAS) B domain.[1] A mutation within this internal cavity, G323E, was reported in a patient undergoing treatment with HIF-2α inhibitor.[2] This mutation restored the ability of HIF-1β to heterodimerize with HIF-2α in presence of the inhibitor.

To better understand how the G323E mutation in the HIF-2α PASB domain affects protein-inhibitor interactions, we identified a set of compounds capable of binding to the mutated recombinant protein and inhibiting HIF-2α activity. We used X-ray crystallography to examine the structure of the mutant HIF-2α PASB domain in complex with a novel small molecule inhibitor active *in vitro* against the G323E mutant form.

METHODS

- HIF-1β and HIF-2α PASB domains were co-expressed in *E. coli* at 16 °C overnight. The heterodimer was purified to homogeneity using Nickel affinity and size exclusion chromatography (SEC).
- Thermal shift assay (TSA) was performed per manufacturer's protocol to monitor direct binding of compounds to the target using Protein Thermal Shift Dye Kit (ThermoFisher Sci).
- HiBit tagged HIF-2α G323E mutant clone was introduced into HEK293 luciferase reporter cell line using manufacturer's guidelines (CLS-007L, Qiagen). This cell line was used to measure luciferase activity following ONE-Glo Luciferase Assay instructions (E6110, Promega).
- Crystals were obtained using Hampton Research sparse matrix screens in a hanging drop format at 19 °C. Data were collected at the LBNL ALS501 beamline.

RESULTS

HIF-2α G323E and HIF-1β PASB Domains Form a Stable Heterodimer

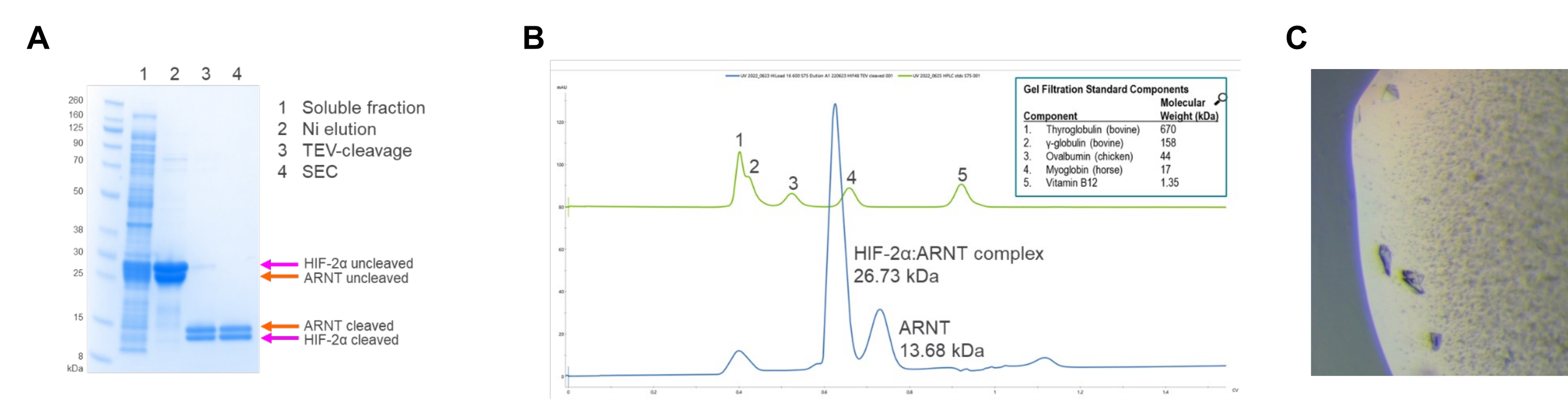


Figure 1. Purification of the stable HIF-2α G323E HIF-1β PASB heterodimer (A) SDS PAGE gel of the protein complex following nickel elution, tobacco etch virus (TEV) tag removal, and final SEC pool. **(B)** Chromatogram of the elution profile of HIF-2α: HIF-1β PASB complex (blue). The elution profile is consistent with 1:1 complex ratio of HIF-2α and HIF-1β. The green trace represents the elution profile of the gel filtration standards. **(C)** Image of the HIF-2α: HIF-1β PASB crystals from the optimized screen used in the data collection.

Direct Binding of Compounds to the HIF-2*α* G323E PASB Protein Observed by TSA

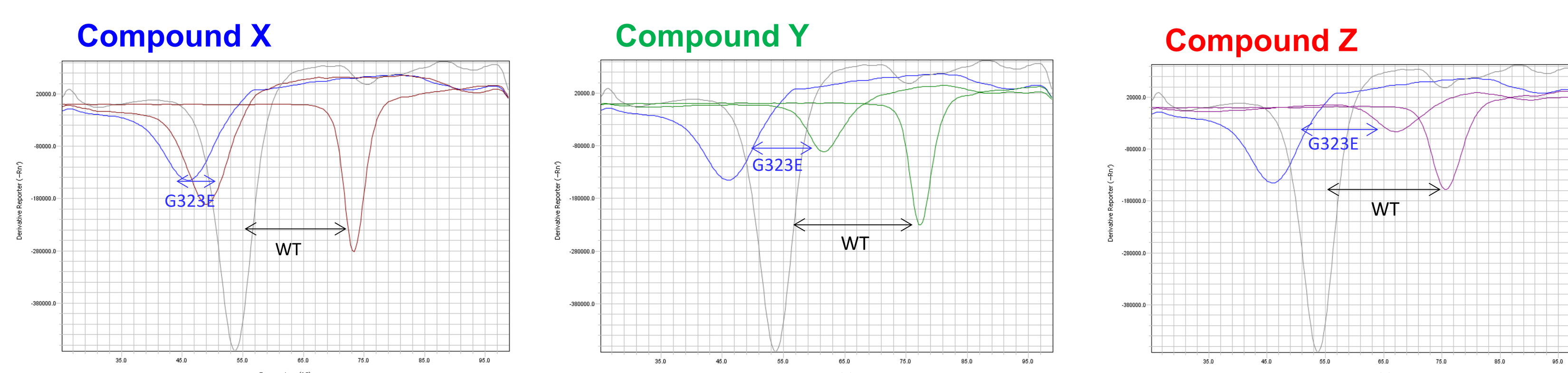
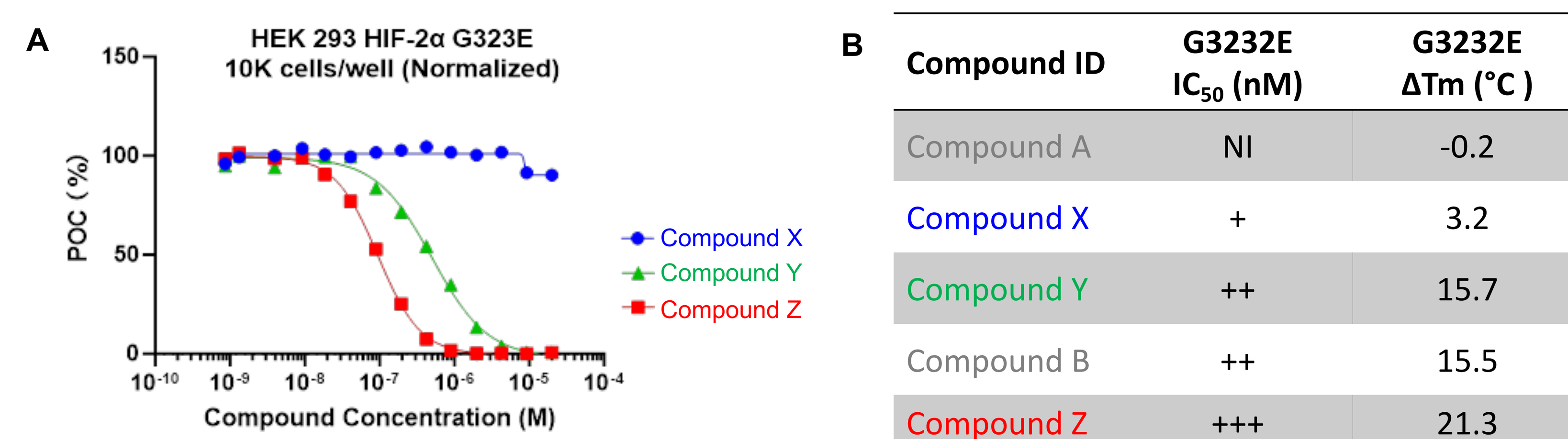


Figure 2. Thermal shift derivative curves for three representative compounds, labeled "Compound X, Y, Z" demonstrate the ability of both wild type (WT) and mutant HIF-2α G323E PASB to bind the corresponding inhibitors with variable affinity. Gray line represents the thermal melt trace for the WT protein in apo form, and the blue line represents the thermal melt trace for the mutant protein in apo form. Red, green, and purple traces represent the thermal melt traces for either WT or mutant protein incubated with Compounds X, Y, and Z, respectively. The differences in thermal melt temperatures (ΔTm) between apo and compound-bound proteins are tabulated below.

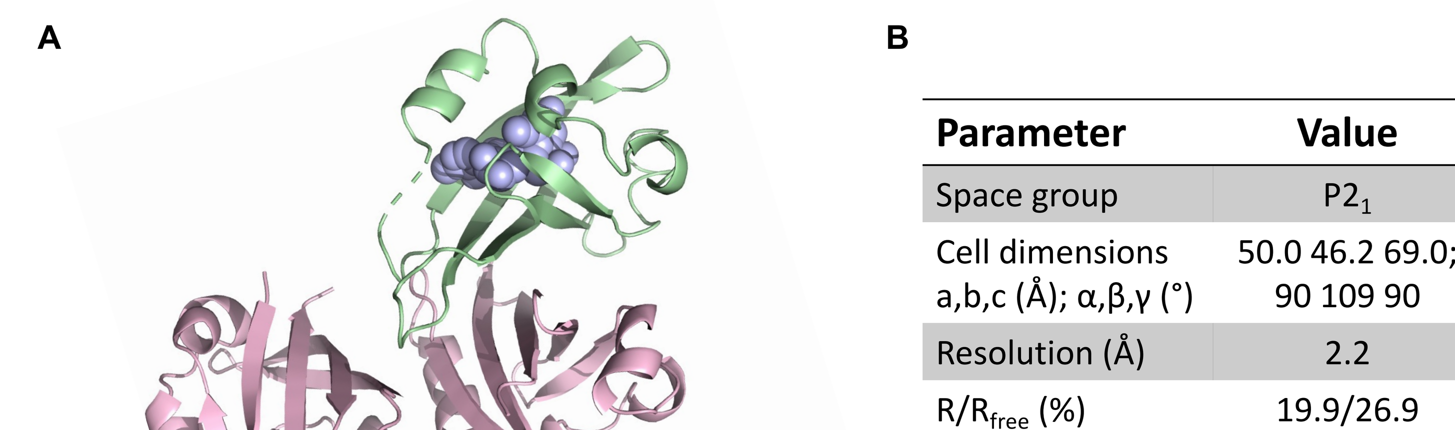
Disruption of the Full Length HIF-2α G323E: HIF-1β Heterodimer Confirmed in Cells



Compound ID	G3232E IC_{50} (nM)	G3232E ΔTm (°C)
Compound A	NI	-0.2
Compound X	+	3.2
Compound Y	++	15.7
Compound B	++	15.5
Compound Z	+++	21.3

Figure 3. Rank ordering of compounds is retained between the cellular assay and the biochemical direct binding assay. (A) A set of representative compound IC_{50} measurements in HEK293 reporter assay confirms heterodimer disruption. **(B)** A table summarizing ΔTm values described in Figure 2 and IC_{50} rankings for the representative compounds (+++ - low nM; ++ - high nM; + - µM; NI – no inhibition).

HIF-2α G323E: HIF-1β PASB Co-crystal Structure Reveals Compound Binding Mode



Parameter	Value
Space group	$P2_1$
Cell dimensions a,b,c (Å); α,β,γ (°)	50.0 46.2 69.0; 90 109 90
Resolution (Å)	2.2
R/R_{free} (%)	19.9/26.9

Figure 4. The contents of the asymmetric unit of the HIF-2α G323E: HIF-1β PASB co-crystal structure. (A) One molecule of HIF-2α G323E PASB (green) and two molecules of HIF-1β PASB (pink) are present in the asymmetric unit (shown in ribbon representation). General placement of the inhibitor in the HIF-2α G323E PASB cavity is indicated by light blue spheres. **(B)** Table of symmetry parameters and statistics for the co-crystal structure.

Size and Shape of the HIF-2*α* PASB Internal Cavity is Altered in the Mutant Protein

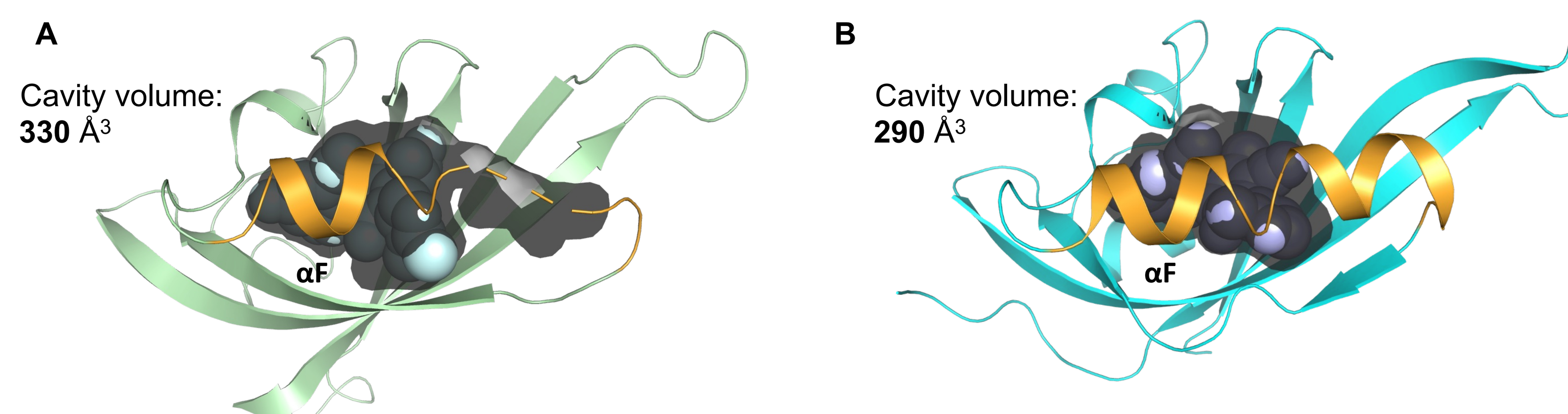


Figure 5. Comparison of the HIF-2α PASB internal cavity between the G323E and WT protein. (A) The structure of HIF-2α G323E PASB (green) highlighting the cavity where the compound binds. Protein and ligand representations as in Figure 4; pocket surface rendered in dark gray. Helix αF (orange) is partially disordered in the mutant structure; this disorder contributes to the change in the size and shape of the cavity. **(B)** The structure of HIF-2α WT PASB (cyan). Ordered αF helix observed.

Disorder in αF Helix Consistent with the Predicted Open HIF-2α PASB Conformation[3]

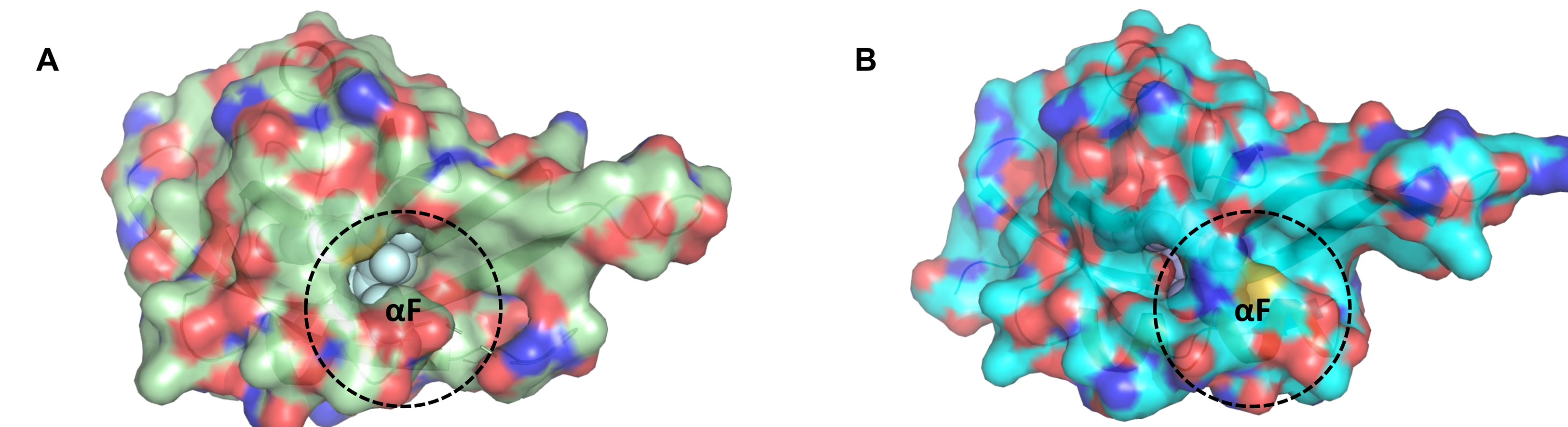


Figure 6. Comparison of the HIF-2α PASB protein surface between G323E and WT. (A) Surface representation for G323E PASB domain colored by atom type (carbon atoms are in green, oxygen – in red, and nitrogen – in blue) Disorder in the helix αF allows for solvent access to the cavity in the G323E structure (dashed circle). **(B)** Surface representation for the WT PASB colored by atom type (carbon atoms are in cyan) with the dashed circle highlighting the location of the fully-enclosed cavity.

Steric Incompatibility Between E323 and L296 May Play a Role in the Observed Helix αF Disorder

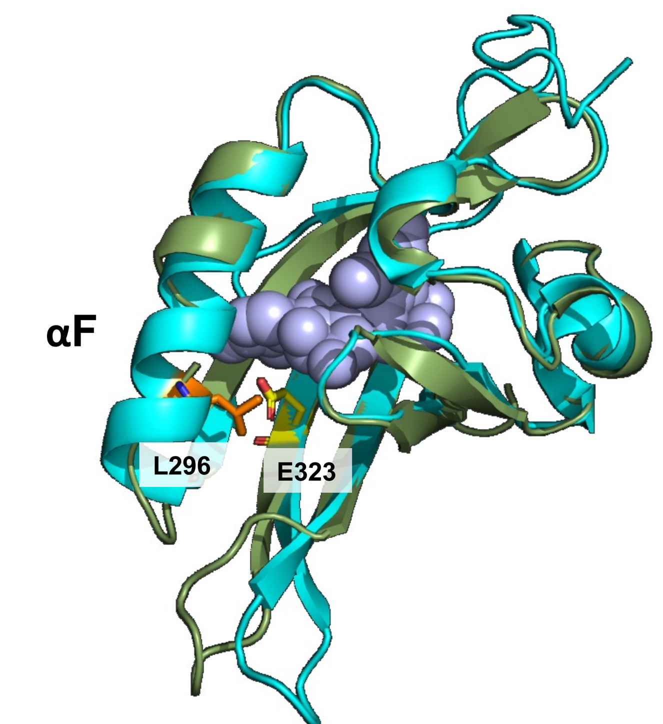


Figure 7. Superposition of the WT and G323E HIF-2α PASB domains. WT structure is in cyan (PDBID 7W80)[1] and mutant structure is shown in green. Based on the superposition, G323E mutation appears to create a steric hindrance with the αF helix residue L296 (orange), resulting in the partial disorder of this helix in our mutant structure. This observation is consistent with the report by *Key et al.*[3] mapping one of the main compound entry points into the HIF-2α cavity via the flexible αF helix.

CONCLUSIONS

G323E mutated HIF-2α PASB protein was purified to homogeneity and characterized biophysically. Binding to G323E HIF-2α PASB recombinant protein by a set of compounds was observed using TSA, and the compound-mediated disruption of heterodimerization with HIF-1β was further characterized in a cellular context.

HIF-2α G323E: HIF-1β PASB complex co-crystal structure was determined in presence of an active compound. Contrary to expectation, this structure reveals that the HIF-2α internal cavity is larger and solvent accessible for the G323E protein as compared to the WT.

Solvent accessibility is achieved through partial disorder in the helix αF, likely a consequence of steric incompatibility between E323 and L296, the latter being located on the αF helix.

This partially disordered "open" conformation has been previously described to be part of the entry way into the HIF-2α cavity,[3] and has now been captured crystallographically in this work.

REFERENCES

1. Ren X. et al. *Mol. Pharm.* **2022.** 102 (6) 240-247
2. Courtney K et al. *Clin Cancer* Res. **2020.** 26(4) 793-803
3. Key et al. *JACS* **2009.** 131, 17647–17654

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