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## Binding of human Cdc123 to eIF2 $\gamma$

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## SUMMARY

Eukaryotic initiation factor 2 (eIF2) plays a key role in protein synthesis and in its regulation. The assembly of this heterotrimeric factor is facilitated by Cdc123, a member of the ATP grasp family that binds the  $\gamma$  subunit of eIF2. Notably, some mutations related to MEHMO syndrome, an X-linked intellectual disability, affect Cdc123-mediated eIF2 assembly. The mechanism of action of Cdc123 is unclear and structural information for the human protein is awaited. Here, the crystallographic structure of human Cdc123 (Hs-Cdc123) bound to domain 3 of human eIF2 $\gamma$  (Hs-eIF2 $\gamma$ D3) was determined. The structure shows that the domain 3 of eIF2 $\gamma$  is bound to domain 1 of Cdc123. Moreover, the long C-terminal region provides a link between the ATP binding site on Hs-Cdc123 and Hs-eIF2 $\gamma$ D3. A thermal shift assay shows that ATP is tightly bound to Cdc123 whereas the affinity of ADP is much smaller. Yeast cell viability experiments, western blot analysis and two-hybrid assays show that ATP is important for the function of Hs-Cdc123 in eIF2 assembly. These data and recent findings allow us to propose a refined model to explain the mechanism of action of Cdc123 in eIF2 assembly.

The heterotrimeric initiation factor 2 (eIF2) has a central role in eukaryotic and archaeal translation initiation (Hinnebusch, 2014; Schmitt et al., 2020). It is composed of three subunits  $\alpha$ ,  $\beta$ ,  $\gamma$ . The  $\gamma$  subunit forms the core of the heterotrimer and binds GTP.  $\alpha$  and  $\beta$  subunits are bound to  $\gamma$  but do not interact with each other (Schmitt et al., 2010). In canonical eukaryotic translation initiation, eIF2:GTP specifically binds the methionyl-initiator tRNA and carries it to the small ribosomal subunit to form, in the presence of other initiation factors, a pre-initiation complex (PIC) able to scan the mRNA to search for a start codon in a correct nucleotidic context. Once the start codon has been found, eIF2 is released from the PIC in its GDP-bound form. Final steps of translation initiation allow the recruitment of the large ribosomal subunit and the formation of a ribosome competent for elongation (Kazan et al., 2023). Specific binding of the initiator tRNA to eIF2-GTP and the control of the nucleotide state of the factor are crucial for efficient translation initiation (Dubiez et al., 2015; Hinnebusch, 2014). Once eIF2-GDP is released from the PIC, the guanine nucleotide exchange factor eIF2B catalyzes recycling of eIF2-GDP to eIF2-GTP to allow additional rounds of translation initiation (Pavitt, 2018). However, stress conditions, such as amino acid deprivation, viral infection or increase of the load of unfolded proteins in the endoplasmic reticulum, trigger phosphorylation of the  $\alpha$  subunit of eIF2 at the Serine 52 residue (S51 in yeast) by eIF2 $\alpha$ -specific kinases (Wek, 2018). Phosphorylated eIF2 $\alpha$  tightly binds eIF2B and prevents its recycling activity (Krishnamoorthy et al., 2001). As a consequence, the global availability of eIF2-GTP decreases leading to a global attenuation of protein synthesis. Importantly, translation of some mRNAs (most of them containing short open reading frames upstream from the main one, uORFs) coding for regulatory proteins is activated by the low levels of eIF2:GTP:Met-tRNA<sub>i</sub><sup>Met</sup> and allows the cell to respond to stress. This specific response called ISR (integrated stress-response) is crucial to recover cellular homeostasis and deregulation of ISR are linked to a wide range of human diseases (Wek, 2018).

In eukaryotes, the assembly of the heterotrimeric protein eIF2 is facilitated by the Cdc123 protein (Perzmaier et al., 2013). Indeed, Cdc123 interacts with eIF2 $\gamma$  (Bieganowski et al., 2004; Chen et al., 2023; Ho et al., 2002) and the association of eIF2 $\gamma$  to eIF2 $\alpha$  and eIF2 $\beta$  was shown to depend on

the interaction of the C-terminal domain (D3) of eIF2 $\gamma$  with Cdc123 in yeast and human (Panvert et al., 2015; Perzlmaier et al., 2013; Vanselow et al., 2022). Moreover, the C-terminal region of *S. cerevisiae* Cdc123 (the last 30 residues) is indispensable for its *in vivo* function (Panvert et al., 2015). In yeast, human and plants, mutations in Cdc123 lower the availability of functional eIF2 and dysregulate protein synthesis triggering ISR (Chen et al., 2023; Perzlmaier et al., 2013; Vanselow et al., 2022; Young-Baird et al., 2020). Notably, mutations related to MEHMO syndrome (Mental deficiency, Epilepsy, Hypogonadism, Microcephaly and Obesity), an X-linked intellectual disability, were found in the gene encoding eIF2 $\gamma$  in mammals (*EIF2S3*) (Gregory et al., 2019; Kotzaeridou et al., 2020; Moortgat et al., 2016; Skopkova et al., 2017). One mutation (eIF2 $\gamma$ -I222T) impairs the binding of eIF2 $\beta$  to eIF2 $\gamma$  (Borck et al., 2012). Another impairs the binding of the initiator tRNA to eIF2 (eIF2 $\gamma$ -I259M) (Young-Baird et al., 2019). Some other mutations are located close to the GTP binding site in eIF2 $\gamma$  domain 1 (Kotzaeridou et al., 2020). The P432S mutation, located in the domain 3 of eIF2 $\gamma$  is additionally related to hypopituitarism and glucose dysregulation (Gregory et al., 2019). The mechanism for the pathological effect of this mutation has not been described yet. Finally, a mutation corresponds to a 4-nucleotide deletion near to the 3' end of the *EIF2S3* coding region that changes the C-terminal amino acid sequence of eIF2 $\gamma$  from G<sub>462</sub>VTIKPTVDD to G<sub>462</sub>VTSQQ (Moortgat et al., 2016). This frameshift mutation (eIF2 $\gamma$ -I465Sfs\*4 also abbreviated eIF2 $\gamma$ -fs) impairs the Cdc123-assisted assembly of eIF2 (Young-Baird et al., 2020) but the mechanism remains to be described. Overall, the data suggest that Cdc123-assisted eIF2 assembly can be a new node of translation regulation from yeast to human. Notably, *CDC123* is part of the essential genes in human, which only include about 10% of all human genes (Wang et al., 2015).

The crystallographic structure of *Schizosaccharomyces pombe* Cdc123 (Sp-Cdc123) showed that the protein belongs to the ATP grasp family. Binding of ATP was indeed observed in the structure and Cdc123 variants in the ATP binding pocket suggested that ATP was important for the Cdc123 function (Chen et al., 2023; Panvert et al., 2015; Perzlmaier et al., 2013; Vanselow et al., 2022). On the other hand, the crystallographic structure of Sp-Cdc123 bound to the domain 3 of eIF2 $\gamma$  from *Saccharomyces cerevisiae* (Sc-eIF2 $\gamma$ D3 or simply Sc- $\gamma$ D3) was determined (Sp-Cdc123:Sc-eIF2 $\gamma$ D3).

This structure showed how Sc-eIF2 $\gamma$ D3 is bound to domain I of Sp-Cdc123 in yeast. However, the C-terminal tail of Cdc123 was not visible in the structure, leaving the essential function of this region unexplained. A docking of the eIF2 $\alpha\gamma$  structure onto Cdc123, combined with genetic and biochemical experiments, allowed the authors to propose a model for the role of Cdc123 in the assembly of eIF2 $\gamma$  to eIF2 $\alpha$  (Panvert et al., 2015). Despite these recent findings based on the structure of a heterologous complex, the mechanism of action of Cdc123 remains unclear and structural information for the human protein is still awaited.

In the present study, we determined the crystallographic structure of human Cdc123 (Hs-Cdc123) bound to human eIF2 $\gamma$ D3 (Hs-eIF2 $\gamma$ D3) domain. The overall structure of the human complex is similar to that of the yeast complex. However, the present structure shows how the long C-terminal region of Hs-Cdc123 interacts with Hs-eIF2 $\gamma$ D3 and is connected to the ATP binding site. We also used a thermal shift assay to study the binding of ATP and ADP to Cdc123 from yeast and human. The results show that ATP is tightly bound to Cdc123 whereas the affinity of ADP is much smaller. Using yeast cell viability experiments, western blot analysis and two-hybrid assays, we show that ATP is important for the function of Hs-Cdc123 in eIF2 assembly. These data and recent findings (Chen et al., 2023; Vanselow et al., 2022) allow us to propose a refined model to explain the mechanism of action of Cdc123 in eIF2 assembly.

## Materials and Methods

### *Production and purification of Cdc123*

The coding sequence of Hs-Cdc123 was amplified by PCR using a Hs-Cdc123 cDNA plasmid (Vanselow et al., 2022) and cloned into NdeI and NotI restriction sites of pET15b1pa. The resulting plasmid, pET15b-Hs-Cdc123 allows expression of an N-terminally tagged version of Hs-Cdc123. The plasmid was transformed into *E. coli* BL21 Rosetta pLacI-Rare (Merck, Novagen). 1 L cultures were in 2xTY containing 50 µg/mL of ampicillin and 34 µg/mL of chloramphenicol. Expression was induced by adding 1 mM of IPTG in an overnight 37°C culture. After induction, the cultures were continued for 5 hours at 18°C. For Hs-Cdc123, cells corresponding to 1 L of culture were disrupted by sonication in 40 mL of buffer A (10 mM HEPES pH 7.5, 500 mM NaCl, 3 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM benzamidine). The crude extract was loaded onto a column (5 mL) containing Talon affinity resin (Clontech) equilibrated in buffer A. After washing the column with buffer A supplemented with 10 mM imidazole, the tagged protein was eluted with buffer A supplemented with 125 mM imidazole. The eluted protein was diluted to reach a 200 mM NaCl concentration and then loaded onto a Q-Sepharose column (10 mm x 4 cm; GE-Healthcare) equilibrated in buffer A1 (10 mM HEPES pH 7.5, 3 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM benzamidine) supplemented with 200 mM NaCl. A gradient from 200 mM NaCl to 1 M NaCl was used for elution (200 mL at a flow rate of 2.5 mL/min). The recovered protein was finally loaded onto a Superdex 200 10/300 (GE-Healthcare) equilibrated in buffer A1 supplemented with 200 mM NaCl. Hs-Cdc123 was finally concentrated to 10 mg/mL using centricon 30. The same protocol was used to purify Sp-Cdc123 (Panvert et al., 2015) and Sc-Cdc123 (Supplementary Figure 1A).

### *Production and purification of the human Hs-Cdc123:Hs-γD3 complex*

The gene coding for human eIF2γ was first PCR amplified from cDNA and cloned into the NdeI-XhoI restriction sites of the pColaDuet-1-ORF2. The resulting plasmid was then modified using standard QuikChange™ site-directed mutagenesis (Stratagene) to produce a gene fragment coding for the γD3 domain (residues 362-471) of human eIF2γ. The corresponding plasmid was named pColaDuet-

1-ORF2-H-eIF2 $\gamma$ D3. pColaDuet-1-ORF2-H-eIF2 $\gamma$ D3 and pET15b-Hs-Cdc123 were co-transformed into *E. coli* BL21 Rosetta pLacI-Rare (Merck, Novagen) and selected onto LB plates containing 50  $\mu$ g/mL of ampicillin, 34  $\mu$ g/mL of chloramphenicol and 25  $\mu$ g/mL kanamycin. 1 L cultures were in 2xTY containing the desired antibiotics. Expression was induced by adding 1 mM of IPTG when OD<sub>650</sub> nm reached 0.8. After induction, the cultures were continued overnight at 18°C. Cells corresponding to 1 L of culture were disrupted by sonication in 40 mL of buffer A. The crude extract was loaded onto a column (5 mL) containing Talon affinity resin (Clontech) equilibrated in buffer A. After washing the column with buffer A supplemented with 10 mM imidazole, the tagged protein was eluted with buffer A supplemented with 125 mM imidazole. The eluted protein was diluted in buffer A1 to reach 200 mM NaCl concentration and then loaded onto a Q-Sepharose column (10 mm x 4 cm; GE-Healthcare) equilibrated in the same buffer. A gradient from 200 mM NaCl to 1 M NaCl was used for elution (200 mL at a flow rate of 2.5 mL/min). This step allowed us to separate the Hs-Cdc123: $\gamma$ D3 complex from the large excess of Hs-Cdc123 isolated after Talon affinity purification. The recovered Hs-Cdc123: $\gamma$ D3 complex was finally loaded onto a Superdex 200 10/300 column (GE-Healthcare) equilibrated in buffer A containing 200 mM NaCl to polish purification of the complex. Finally, Hs-Cdc123: $\gamma$ D3 was concentrated to 10 mg/mL using centricon 30 before crystallization (Supplementary Figure 1A).

To facilitate structure resolution, we also produced and purified Hs-Cdc123: $\gamma$ D3 containing selenomethionine. The selenomethionylated proteins were expressed in autoinducible medium as described (Studier, 2005). After cultivation, the selenomethionylated protein complex was purified as described for native Hs-Cdc123: $\gamma$ D3.

### **Crystallization and structure determination**

Crystallization trials were performed at 4°C with or without 1 mM ATP and 5 mM MgCl<sub>2</sub>. Sitting drops were made with a Mosquito robot (TTP Labtech) and standard commercial kits (Hampton Research and Qiagen) by mixing 1:1 Hs-Cdc123: $\gamma$ D3 w/wo 1 mM ATP and 5 mM MgCl<sub>2</sub> and the precipitating solution. Crystals for native or selenomethionylated Hs-Cdc123: $\gamma$ D3 were rapidly obtained in many different conditions containing PEG3350 (PEG/ION and Index Hampton Research). Before



data collection, the crystals were rapidly soaked into a solution containing the precipitating agent supplemented with 25 to 30% of glycerol. Two X-ray crystallographic datasets collected at the Soleil Synchrotron (Saint Aubin France) on the Proxima-2 beamline were used in this study (Table 1). One dataset was collected from a crystal obtained with selenomethionylated Hs-Cdc123: $\gamma$ D3 with no added ATP-Mg<sup>2+</sup> and a second dataset was collected from a crystal of Hs-Cdc123: $\gamma$ D3 obtained in the presence of 1 mM ATP and 5 mM Mg<sup>2+</sup>. Data were processed with XDS (Kabsch, 2010), scaled with AIMLESS (Evans and Murshudov, 2013), analyzed with CCP4 programs (Winn et al., 2011) and finally processed for anisotropy in STARANISO (Tickle et al., 2016). All these steps were performed in AUTOPROC (Vonnrhein et al., 2018). Crystals belong to the P2<sub>1</sub> space group. However, there is a two-fold NCS axis yielding a strong pseudo P2<sub>1</sub>2<sub>1</sub>2 symmetry. The structure of Hs-Cdc123: $\gamma$ D3 complex was solved by molecular replacement with PHASER (McCoy et al., 2007) using two separate ensembles, one corresponding to Sp-Cdc123 and the second one to the Sc- $\gamma$ D3 domain (Panvert et al., 2015, PDB ID Code 4ZGQ). For each ensemble, a solution with a high Z-score value was found (Z=8.9 and Z=16.7). The molecular replacement 2mFo-DFc electron density map was of good quality and allowed us to build a first model of Hs-Cdc123: $\gamma$ D3. To facilitate the refinement process, we also calculated an experimental map using the automated SAD phasing procedure with a partial model in Phaser-EP (McCoy et al., 2007; Winn et al., 2011). Coordinates and associated B factors were then refined through several cycles of manual adjustments with Coot (Emsley et al., 2010) and positional refinement with PHENIX (Adams et al., 2010). A small fraction of twinning was evidenced and refined in PHENIX. Moreover, residual electron density showed that some ATP-Mg<sup>2+</sup> was present in the selenomethionylated Hs-Cdc123: $\gamma$ D3 crystals. Therefore ATP-Mg<sup>2+</sup> was positioned in the model and the occupancy of the molecule was refined. In the final models, regions 50-68, 294-299 and 311-318 of Hs-Cdc123 and regions 384-390 and 466-470 of Hs- $\gamma$ D3 belonging to long mobile loops could not be traced in the electron density. Statistics for data collection and refinement are given in Supplementary Table 1.

## Binding of ATP to Cdc123 using fluorescence-based thermal shift assays

Experiments were done in 96-well plates using a Bio-Rad CFX-96 Real-Time PCR system. Reaction mixtures (25  $\mu$ L) were assembled in buffer A (10 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 2 mM  $MgCl_2$ , 10 mM 2-mercaptoethanol) and contained Cdc123 (0.15 mg/mL), 5x SYPRO™ orange (Invitrogen) and various concentrations of ATP- $Mg^{2+}$  (0.1  $\mu$ M to 6.4 mM). Temperature was increased by 0.5°C every 60 seconds. Fluorescence curves as a function of temperature in the 10°C-70°C range were processed as described (Pantoliano et al., 2001; Redhead et al., 2015), by non-linear least square fitting of the following equation 1 to the experimental datapoints (Supplementary Figure 1B), using Origin (OriginLab) or Prism (GraphPad):

$$F(T) = F_u + m_u(T - T_m) + \frac{F_f - F_u + (T - T_m)(m_f - m_u)}{1 + \exp\left(-\frac{\Delta H_u}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) + \frac{\Delta Cp_u}{R}\left(\ln\left(\frac{T}{T_m}\right) + \frac{T_m}{T} - 1\right)\right)}$$

$F(T)$  is the measured fluorescence in arbitrary units at the temperature  $T$ ;  $F_u$  and  $F_f$  are the fluorescence at the melting temperature  $T_m$  with the protein in the unfolded and folded states, respectively;  $m_u$  and  $m_f$  are coefficients reflecting temperature dependence of the fluorescence of the dye bound to the unfolded and folded states, respectively;  $\Delta H_u$  and  $\Delta Cp_u$  are the enthalpy and heat capacity, respectively, of protein unfolding at a reference temperature chosen as  $T_m$  in the absence of ligand and  $R$  is the gas constant. Usually, all parameters were allowed to vary in the fitting procedure. In the case of Sc-Cdc123, that had a lower melting temperature than the two other orthologues,  $m_f$  was poorly defined for curves recorded at low ATP concentrations and was therefore kept at a constant value of 0 throughout, consistent with the shape of the fluorescence curves at high ATP concentration. In the case of the *Schizosaccharomyces pombe* protein (Sp-Cdc123), in addition to the main fluorescence transition that occurred around 52°C, a minor fluorescence increase was observed around 80°C. This suggested the existence of an additional minor denaturation step. Only the main transition was considered for  $T_m$  calculations (Supplementary Figure 1C).

The obtained  $T_m$  values were then used to estimate the dissociation constant of the studied enzyme-ligand couple as described (Cimpmperman et al., 2008; Matulis et al., 2005; Pantoliano et al.,

2001; Redhead et al., 2015; Zubrienè et al., 2009). Briefly, the equilibrium constant for protein unfolding at a temperature  $T$  can be expressed as:

$$K_u = \exp\left(-\frac{\Delta H_u}{R}\left(\frac{1}{T} - \frac{1}{T_r}\right) + \frac{\Delta Cp_u}{R}\left(\frac{T_r}{T} - 1 + \ln\left(\frac{T}{T_r}\right)\right)\right)$$

where  $\Delta H_u$  and  $\Delta Cp_u$  are the enthalpy and heat capacity, respectively, of protein unfolding at a reference temperature  $T_r$  chosen as  $T_m$  in the absence of ligands. Moreover, the binding constant of the ligand at temperature  $T$  can be expressed as:

$$K_b = \frac{1}{K_d} \exp\left(\frac{\Delta H_b}{R}\left(\frac{1}{T_o} - \frac{1}{T}\right) - \frac{\Delta Cp_b}{R}\left(-\frac{T_o}{T} + 1 + \ln\left(\frac{T_o}{T}\right)\right)\right)$$

where  $\Delta H_b$  and  $\Delta Cp_b$  are the enthalpy and heat capacity, respectively, of binding of the ligand to the protein unfolding at a reference temperature  $T_o$  chosen as 298K here, and  $K_d$  is the dissociation constant of the protein:ligand complex at  $T_o$ . The total concentration of ligand in the assay  $[L_t]$  necessary to raise the melting temperature to a temperature  $T$  can be expressed as (equation 2):

$$[L_t] = (K_u - 1)\left(\frac{[P_t]}{2K_u} + \frac{1}{K_b}\right)$$

where  $[P_t]$  is the total protein concentration in the assay.

For estimating  $K_d$  values, curves simulating the variation of melting temperature as a function of  $[L_t]$  were calculated using equation 2 for various  $K_d$  values and visually compared with the experimental curves. In this procedure,  $\Delta H_u$  and  $\Delta Cp_u$  were set to the mean of the values obtained from the fluorescence curves obtained with the control experiments in the absence of ligands. In some cases, for better fitting the whole data, the  $T_r$  value was slightly adjusted. Each experiment was repeated at least twice, and the means  $\pm$  sd of the melting temperatures were plotted as a function of the logarithm of ligand concentration.  $\Delta H_b$  was set to -3 kcal.mol<sup>-1</sup>, the value measured for ATP binding to Sp-Cdc123 using Isothermal Titration Calorimetry (Panvert et al., 2015) and  $\Delta Cp_b$  to -0.2 kcal.mol<sup>-1</sup>.K<sup>-1</sup>, an average value for protein-ligand interactions (Matulis et al., 2005). Note that when varied in realistic ranges, the values of  $\Delta H_b$  and  $\Delta Cp_b$  did not have a large influence on the  $K_d$  estimates (less than a factor of two).

## Yeast methods

Standard protocols were followed for growth of budding yeast cells, transformation, sporulation and tetrad dissection (Ausubel et al., 2005). Yeast strains are derivatives of W303 and listed in Supplementary Table 2. Preparation of yeast cell extracts, immunoprecipitation of flag-tagged proteins and Western analysis were done as described (Panvert et al., 2015; Vanselow et al., 2022). For detection of the flag and myc epitopes mouse monoclonal antibodies were used (anti-flag M2; anti-myc 9E10). Sc-eIF2 $\beta$  and Sc-eIF2 $\gamma$  were detected by antisera raised in rabbits (Perzmaier et al., 2013). In the yeast-two-hybrid vectors pEG202 and pJG4-5 (Ausubel et al., 2005) the human eIF2 $\alpha$  encoding cDNA was fused to a *lexA*-DNA binding domain sequence (pWS4018) and the human eIF2 $\gamma$  encoding cDNA was fused to the B42 transcriptional activator sequence (pWS4023). The human eIF2 $\alpha$  and eIF2 $\gamma$  cDNAs were obtained from the DNASU plasmid repository. In addition to the regular reporter strain W276, derivatives expressing Hs-Cdc123 wild type (W15327) or the mutant versions Hs-Cdc123-D233A (W15328) and Hs-Cdc123-D246A (W15329) were used. The mutant alleles of the Hs-Cdc123 gene were created by site-directed mutagenesis (QuickChange; Stratagene). To visualize  $\beta$ -galactosidase production following activation of the *lexA*-operator controlled *lacZ* gene in W276 and its derivatives, yeast colonies were overlaid with an X-Gal top agar (0.2 mg/ml X-Gal; 0.5 M sodium phosphate buffer pH 7.0; 0.1% SDS; 6% DMFA; 0.5% Bacto agar).

## RESULTS

### Binding of ATP or ADP to Cdc123 using thermal shift assay

In order to study the binding of ATP-Mg<sup>2+</sup> to Cdc123, we used a thermal shift assay. This method is based on the general idea that ligand binding increases protein stability (Pantoliano et al., 2001). Protein denaturation caused by increasing temperature was monitored using the ThermoFluor™ method with an environmentally sensitive fluorescent dye, allowing us to determine the protein melting temperature  $T_m$  (Pinz et al., 2022). This experiment was repeated in the presence of various concentrations of ATP-Mg<sup>2+</sup> (Supplementary Figure 1B). From the analysis of the variations of the melting temperatures as a function of the concentrations of the nucleotide, dissociation constants can be derived ((Cimpmperman et al., 2008; Matulis et al., 2005; Pantoliano et al., 2001; Redhead et al., 2015; Zubrienė et al., 2009); see Materials and methods).

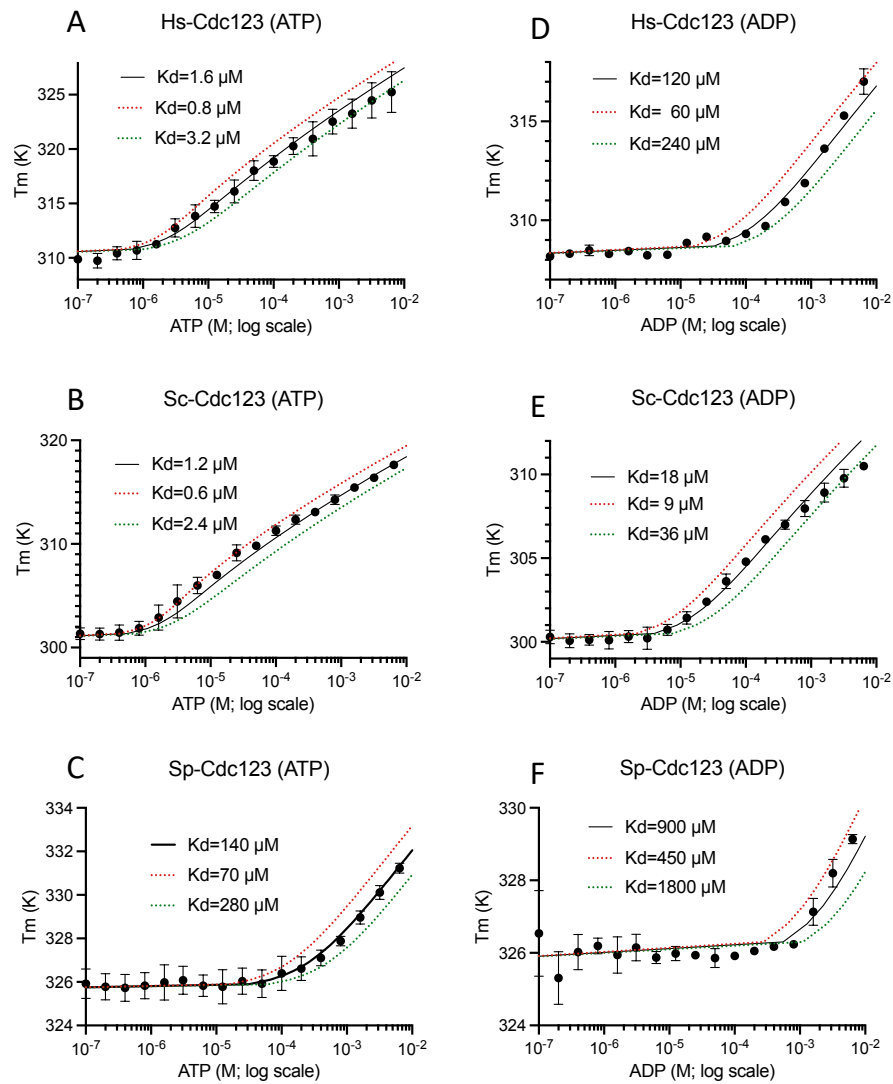
	Estimated $K_d$ ( $\mu$ M) ATP	Estimated $K_d$ ( $\mu$ M) ADP
Hs-Cdc123	1.6 (17°C)	120 (13°C)
Sc-Cdc123	1.2 (18°C)	18 (11°C)
Sp-Cdc123	140 (6°C)	900 (4°C)

**Table 1 :  $K_d$  values of the ATP and ADP nucleotides for the indicated Cdc123 enzymes.**

Values in parentheses show the amplitudes of  $T_m$  variation in the range of nucleotide concentrations used in the thermal shift assay.

A  $K_d$  value of 140  $\mu$ M could be estimated for the Sp-Cdc123:ATP-Mg<sup>2+</sup> complex (Figure 1 and Table 1), in reasonable agreement with the value determined using Isothermal Titration Calorimetry (67  $\mu$ M; (Panvert et al., 2015)). With Sc-Cdc123 and Hs-Cdc123, small concentrations of ATP-Mg<sup>2+</sup> -in the micromolar range- had larger effects of the stabilities of the proteins as compared to Sp-Cdc123. Consistent with these observations, the estimated dissociation constants were 1.6  $\mu$ M and 1.2  $\mu$ M for Hs-Cdc123 and Sc-Cdc123 (Table 1). As compared to ATP-Mg<sup>2+</sup>, larger concentrations of ADP-Mg<sup>2+</sup> were required to observe changes in the melting temperature of the three Cdc123 proteins. Accordingly,

the estimated dissociation constants were systematically increased to values of 120  $\mu\text{M}$  (Hs-Cdc123), 18  $\mu\text{M}$  (Sc-Cdc123) and 900  $\mu\text{M}$  (Sp-Cdc123) (Table1).



**Figure 1: Estimation of  $K_d$  values.**

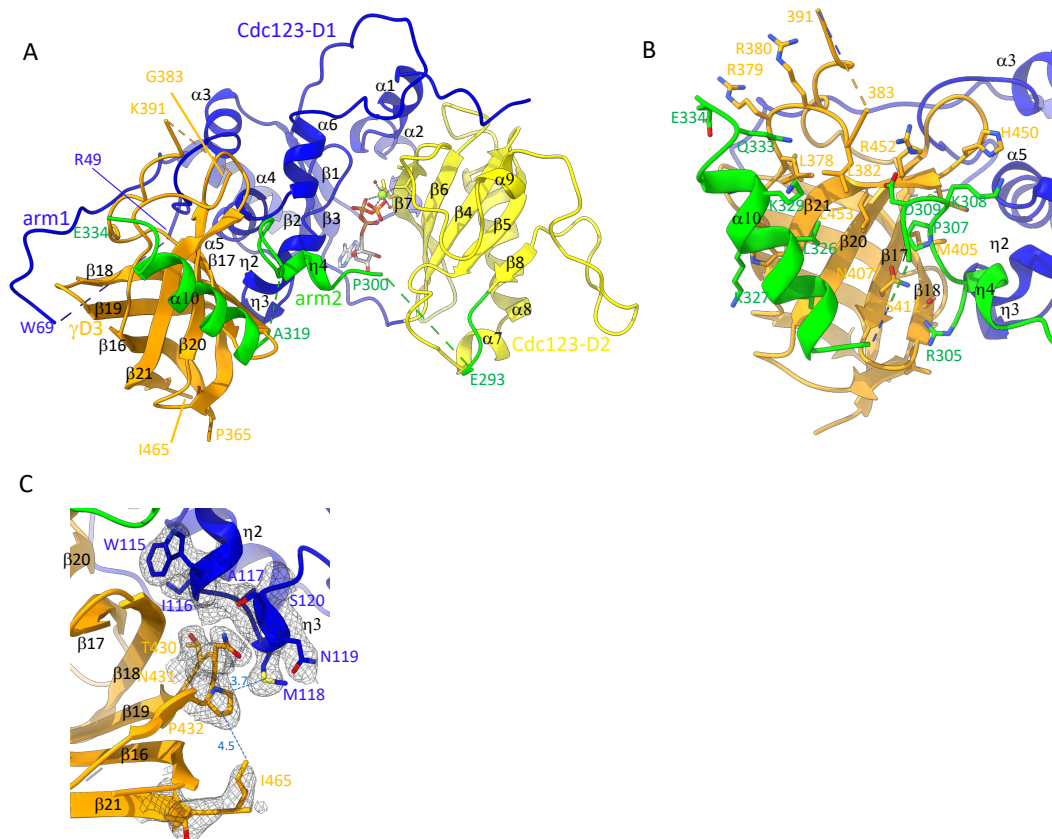
Each panel plots the measured  $T_m$  value (mean  $\pm$  sd from at least two experiments) as a function of the nucleotide concentration in log scale. The best fit to a curve calculated as described in Materials and Methods (Cimpmperman et al., 2008; Matulis et al., 2005; Pantoliano et al., 2001; Redhead et al., 2015; Zubrienė et al., 2009) is drawn with a solid line. The corresponding  $K_d$  value is shown on the panel. Curves obtained with a  $K_d$  value two-fold higher (green) or two-fold lower (red) are also drawn for comparison. Panels A, B, C: ATP binding to Hs-Cdc123, Sc-Cdc123 and Sp-Cdc123, respectively. Panels D, E, F: same for ADP binding. The estimated dissociation constants are shown in Table 1.

## **Crystallographic structure of Hs-Cdc123:eIF2 $\gamma$ D3 complex**

The Hs-Cdc123:eIF2 $\gamma$ D3 complex was first purified by affinity chromatography using an N-terminally tagged version of Hs-Cdc123 to trap Hs-eIF2 $\gamma$ D3 (See Materials and Methods). Additional steps using anion exchange chromatography and molecular sieving allowed us to polish the preparation (Supplementary Figure 1A). Crystals of Hs-Cdc123: $\gamma$ D3 were obtained at 4°C in solutions containing 20% PEG3350 and various additives, in the presence or in the absence of ATP-Mg<sup>2+</sup> (Table 1). The structure was solved by molecular replacement and experimental phasing using a selenomethionine version of the complex. Data were processed with STARANISO to deal with diffraction anisotropy. The structure of Hs-Cdc123: $\gamma$ D3:ATP-Mg<sup>2+</sup> was refined to 2.08 Å resolution and the structure of the selenomethionylated complex was refined to 1.97 Å resolution (Supplementary Table 1). Two molecules related by a pseudo two-fold axis are present in the asymmetric unit. Because the quality of the electron density is slightly better for monomers C and D of Hs-Cdc123 and Hs- $\gamma$ D3, particularly for the long loops of Hs-Cdc123, these two molecules are hereafter used as references. On the other hand, comparison of the structure of the selenomethionylated complex to that of Hs-Cdc123: $\gamma$ D3:ATP-Mg<sup>2+</sup> complex did not reveal major conformational changes (rms value of 0.267 Å for 357 C- $\alpha$  atoms compared). Therefore, the Hs-Cdc123: $\gamma$ D3:ATP-Mg<sup>2+</sup> will be majorily described.

### **Binding of Hs- $\gamma$ D3 to Hs-Cdc123**

Hs-Cdc123 is composed of two domains with ATP-Mg<sup>2+</sup> bound at the interface, as observed in Sp-Cdc123 (Panvert et al., 2015) and in enzymes belonging to the ATP-grasp family (Fawaz et al., 2011) (Figure 2A and Supplementary Figure 2A). D3 of Hs-eIF2 $\gamma$  forms a  $\beta$  barrel highly homologous to its eukaryotic orthologs (Supplementary Figure 3A). It is mainly bound to domain I of Hs-Cdc123 (Figure 2A and 2B).



**Figure 2: Crystallographic structure of Hs-Cdc123:Hs-γD3:ATP-Mg<sup>2+</sup>**

- (A) Overall structure of Hs-Cdc123:γD3:ATP-Mg<sup>2+</sup>. Hs-Cdc123 is shown in blue (residues 1-174), yellow (residues 175-289) and green (C-terminal peptide, residues 290-336). Regions 50-68, 294-299 and 311-318 of Hs-cdc123 are highly mobile and could not be traced in the electron density. ATP is shown with sticks, Mg<sup>2+</sup> ion is shown as a green sphere. Hexacoordination of the Mg<sup>2+</sup> ion is shown with dashed lines (see also Supplementary Figure 4). Hs-γD3 is shown in orange. Secondary structures are labeled as defined for the Sp-Cdc123 structure (Panvert et al., 2015). The figures were made with ChimeraX (Pettersen et al., 2021).
- (B) Close-up view of the binding of the C-terminal region of Hs-Cdc123 to Hs-eIF2γD3. Interacting residues belonging to these regions (as listed in Supplementary Table 3) are in sticks and labeled.
- (C) Close-up view showing the hydrophobic interaction of P432 with M118 and the proximity of I465 from P432. Interactions involving T430, N431 and P432 with residues from the h2-h3 helices of Cdc123 are listed in Supplementary Table 3.

The η2–η3, α6 and the 39-43 loop of Hs-Cdc123 carry residues involved in the interaction with Hs-γD3 (Supplementary Table 3). Overall, the structure of Hs-Cdc123:γD3:ATP-Mg<sup>2+</sup> is similar to the previously determined structure of Sp-Cdc123:Sc-γD3 complex (Panvert et al., 2015) (rmsd between the two structures is 1.2 Å for 299 Cα atoms compared). This shows that the main regions of contact between γD3 and domain I of Cdc123 are similar in the two structures (Supplementary Figure 2A) and conserved from yeast to human. When the Cdc123 molecules of the human and yeast complexes are superimposed, a small difference in the positioning of the γD3 domain with respect to Cdc123-D1 is

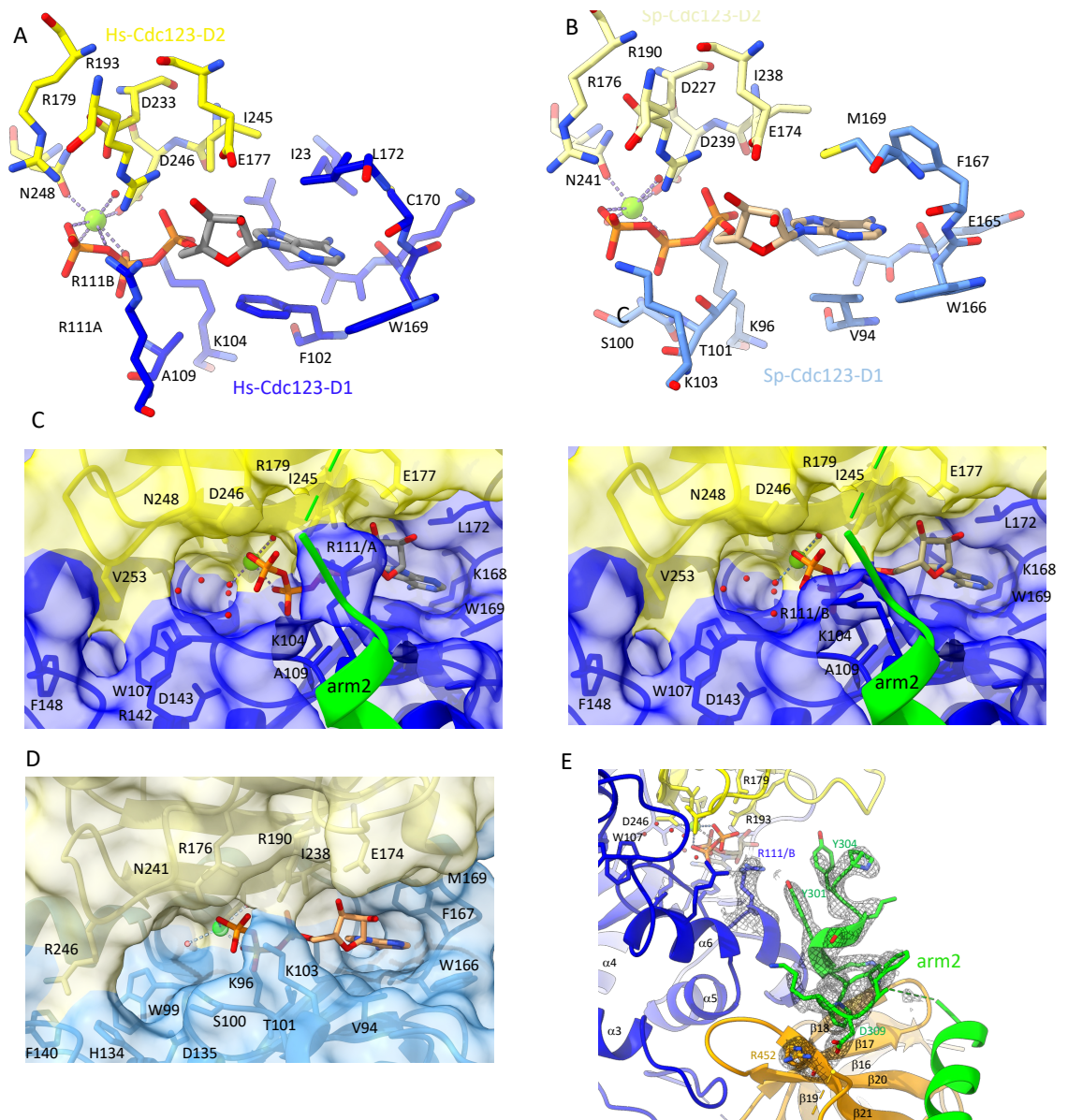


observed (3.06 Å translation and 9.6° rotation, Supplementary Figure 2A). This difference in the orientation of  $\gamma$ D3 is possibly related to a small movement of Cdc123  $\alpha$ 3 and the following loop as well as to the participation of C-terminal region of Hs-Cdc123 in the binding to  $\gamma$ D3 (Figure 2B). Indeed, in Hs-Cdc123 domain I, the long linker containing forty-four residues located between  $\alpha$ 3 and  $\alpha$ 4 (residues 40-83, Supplementary Figure 2) is partially ordered. Residues 40-50 and 69-83 were positioned in the electron density (Supplementary Figure 4). This region surrounds  $\gamma$ D3 and contacts it on the side of the  $\beta$ 18- $\beta$ 19 hairpin. This region is hereafter named arm 1 (Figure 2). On the other hand, the C-terminal region of Hs-Cdc123 could be partially built (Figure 2 and Supplementary Figure 4 A-C). This long C-terminal tail, from residue 290 to residue 336, starts from Hs-Cdc123 domain 2 and extends to Hs- $\gamma$ D3 with which it interacts via its C-terminal  $\alpha$ -helix. This region is hereafter named arm 2. These two regions contribute many interactions described in Supplementary Table 3. Arm1 and arm2 of Hs-Cdc123 act as tweezers that wrap the Hs- $\gamma$ D3 domain and increase the surface of interaction between Cdc123 and  $\gamma$ D3 to 4530 Å<sup>2</sup>, as compared to 1307 Å<sup>2</sup> for the Sp-Cdc123:Sc- $\gamma$ D3 complex or 1742 Å<sup>2</sup> for the present complex where regions 69-80 and 291-336 have been deleted from the model (Supplementary Figure 2B). These two regions have B-values above the mean B-values for the model (Supplementary Figure 2C) indicative of their flexibility. Packing analysis shows that region from residue 290 to 319 of Hs-Cdc123 is not involved in packing interactions in the crystals. However, the C-terminal  $\alpha$ 10 helix of one Hs-Cdc123 also contacts a neighbor molecule. Therefore, its positioning could have been stabilized by crystal packing interactions. In the Sp-Cdc123:Sc- $\gamma$ D3 structure, arm 2 was mostly disordered and only the C-terminal helix could be built. However, the positioning of this C-terminal helix with respect to the main part of Cdc123 could not be established. Overall, the present data suggests that these two arms are mobile regions of Cdc123 that are involved in the binding of the  $\gamma$ D3 domain.

### **ATP-Mg<sup>2+</sup> binding site of Hs-Cdc123**

ATP-Mg<sup>2+</sup> is bound to Hs-Cdc123 at the interface between D1 and D2. Residues involved in ATP-Mg<sup>2+</sup> binding were analyzed with Ligplot (Wallace et al., 1996) and shown in Figure 3A. For

comparison, the Sp-Cdc123 structure bound to ATP-Mg<sup>2+</sup> is shown in Figure 3B. Most residues involved in ATP binding are conserved in the two proteins (Supplementary Figure 3).



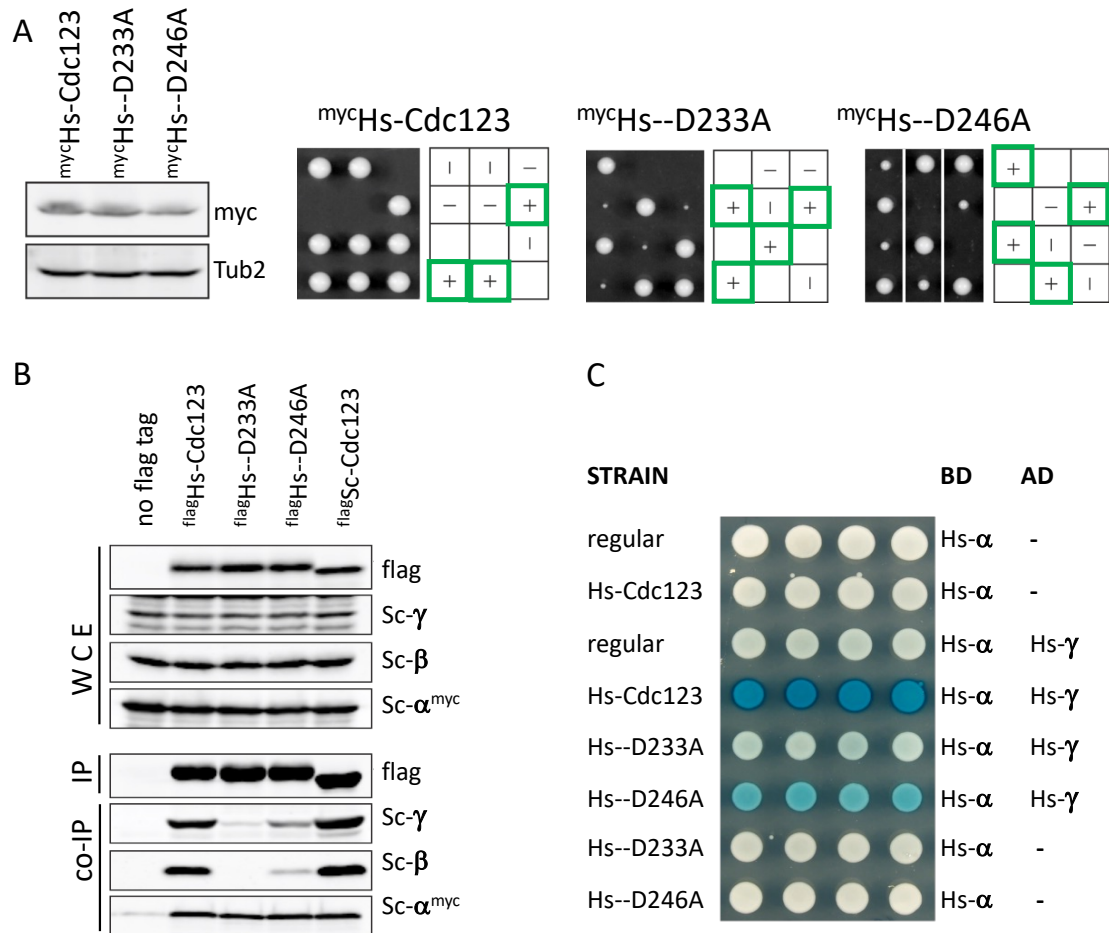
**Figure 3: Binding of ATP-Mg<sup>2+</sup>**

- (A) The Hs-Cdc123 residues involved in ATP binding are shown in sticks. D233 (D227 in Sp-Cdc123) is also represented as it tightly interacts with R179, N248, and a water molecule (see also Supplementary Figure 4D). Residues belonging to domain 1 are colored in blue and those belonging to domain II are colored in yellow. Water molecules are shown with red spheres and the magnesium ion is shown as a green sphere.
- (B) Same as view A but for Sp-Cdc123 (Panvert et al., 2015).
- (C) Molecular surface representation of Hs-Cdc123:ATP-Mg<sup>2+</sup>. The color code is the same as in view A. The view shows the cavity facing the ATP binding pocket with the side chain of R111 in two alternative conformations (A, right and B, left).
- (D) Same as C but for Sp-Cdc123:ATP-Mg<sup>2+</sup>.
- (E) Close-up view showing the contacts of Hs-Cdc123 arm 2 with the ATP binding site and with  $\gamma$ D3.

To address the relevance of ATP binding to the human Cdc123 ortholog, we generated two variants altered in the ATP-binding site and studied their properties. To this end, we replaced two conserved aspartate residues (D233 and D246 in Hs-Cdc123 corresponding to D227 and D239 in Sp-Cdc123, Panvert et al., 2015); Figure 3A and B, Supplementary Figure 3) by alanine residues through site-directed mutagenesis of the Hs-Cdc123 gene. In a first step, we tested for biological function by complementation analysis in budding yeast. For this, we expressed wild type Hs-Cdc123 and the mutant versions D233A and D246A in a diploid yeast strain heterozygote for a *CDC123* gene deletion. Western analysis confirmed that the mutant Hs-Cdc123 versions were expressed at levels comparable to the wild type protein (Figure 4A). By tetrad dissection following meiosis and sporulation, we recovered haploid progeny carrying a Hs-Cdc123 expression construct but lacking the yeast *CDC123* gene. In case of wild type Cdc123, these cells had normal growth properties as indicated by the formation of regular sized colonies (Figure 4A). Apparently, Hs-Cdc123 can take over the function of its yeast ortholog consistent with a previous systematic gene replacement study (Kachroo et al., 2015). In contrast, yeast *cdc123-Δ* cells expressing the Hs-Cdc123-D233A or -D246A mutant version formed only tiny or small colonies, respectively (Figure 4A). These data suggest that the biological function of Hs-Cdc123 depends on the integrity of its ATP binding site.

To define further the defect caused by a compromised ATP binding site, we studied the interaction of the Hs-Cdc123 mutant proteins with eIF2 subunits. For this, flag-tagged Hs-Cdc123 constructs were expressed in yeast and a strain expressing flag-tagged yeast Cdc123 served as a positive control. Flag-tagged proteins were immunoprecipitated from cell extracts and analyzed for co-precipitation of eIF2 subunits by Western analysis. Precipitates of Hs-Cdc123 contained eIF2 subunits in amounts similar to precipitates of Sc-Cdc123 (Figure 4B). In contrast, the mutant versions of Hs-Cdc123 co-precipitated hardly any (D233A) or only very little (D246A) of the  $\beta$  and  $\gamma$  subunits of eIF2 consistent with the view that integrity of the ATP binding site of Cdc123 is essential for the interaction of Cdc123 with eIF2 $\gamma$  and the eIF2 $\gamma$ -eIF2 $\beta$  dimer (Panvert et al., 2015; Vanselow et al., 2022). To facilitate detection of Sc-eIF2 $\alpha$ , the yeast strains used in this analysis carried a myc-epitope fusion of the *SUI2* gene, which encodes eIF2 $\alpha$  in budding yeast. The myc-tagged Sc-eIF2 $\alpha$  subunit was present

in similar amounts in the precipitates of Sc-Cdc123 and both the wild type and mutant versions of Hs-Cdc123. This confirms a previously observed direct contact of Sc-Cdc123 with Sc-eIF2 $\alpha$  (Vanselow et al., 2022) and suggests that the Cdc123-eIF2 $\alpha$  contact does not require intactness of the ATP binding site of Cdc123.



**Figure 4: ATP binding site variants of Hs-Cdc123.**

- (A) Complementation analysis in yeast. Expression of myc-tagged wild type or mutant versions of Hs-Cdc123 in CDC123/cdc123- $\Delta$  diploid strains (Hs-Cdc123: W15001; Hs-Cdc123-D233A: W15002; Hs-Cdc123-D246A: W15003) was compared by Western analysis. Tub2 served as a protein loading control. The four haploid spores generated by a single diploid cell were recovered by tetrad dissection and placed in a vertical line on solid growth medium to monitor colony formation. Green squares indicate the positions of haploid progeny carrying both the cdc123- $\Delta$  allele (horizontal line) and the Hs-Cdc123 expression construct (vertical line).
- (B) Hs-Cdc123 protein interaction with Sc-eIF2 subunits. Expression of flag-tagged wild type or mutant versions of Hs-Cdc123 in a SUI2-myc strain (Hs-Cdc123: W14968; Hs-Cdc123-D233A: W14969; Hs-Cdc123-D246A: W14970) was inspected by Western analysis. Negative and positive controls were the SUI2-myc strain lacking a flag expression construct (no flag tag: W9877) or expressing flag-tagged Sc-Cdc123 (W14974), respectively. Whole cell extracts (WCE) of these strains were also probed for Sc-eIF2 $\gamma$ , Sc-eIF2 $\beta$  and Sc-eIF2 $\alpha$ -myc. From the whole cell extracts, flag-tagged proteins were immunoprecipitated by an antibody against the flag

epitope (IP). These precipitates were probed by Western analysis for the presence of flag-tagged proteins, Sc-eIF2 $\gamma$ , Sc-eIF2 $\beta$  and Sc-eIF2 $\alpha$ -myc (co-IP).

- (C) Yeast-two-hybrid analysis of Hs-eIF2 $\gamma$  association with Hs-eIF2 $\alpha$ . Y2H-reporter strains that lack any Hs-Cdc123 construct (regular: W276) or express flag-tagged wild type (Hs-Cdc123: W15327) or mutant versions (Hs--D233A: W15328; Hs--D246A: W15328) of Hs-Cdc123 were used to express the *lexA* DNA binding domain (BD) fused human eIF2 $\alpha$  subunit (Hs- $\alpha$ ) either alone to test for autoactivation or in combination with a B42 transcriptional activation domain (AD) fused human eIF2 $\gamma$  subunit (Hs- $\gamma$ ) to test for association of Hs-eIF2 $\alpha$  with Hs-eIF2 $\gamma$ . Yeast colonies were overlaid with a X-gal containing top agar to visualize  $\beta$ -galactosidase activity resulting from transcriptional activation of the *lexAop-lacZ* reporter gene.

As a third test, we analyzed the ability of Hs-Cdc123 to promote eIF2 assembly. For this, we used a Y2H-assay to monitor the association of Hs-eIF2 $\alpha$  fused to a DNA-binding domain with Hs-eIF2 $\gamma$  fused to a transcriptional activation domain. Co-expression of the human  $\alpha$  and  $\gamma$  subunits activated the *lacZ* reporter gene only weakly (Figure 4C). Interestingly, the additional expression of wild type Hs-Cdc123 in the Y2H strain increased the reporter signal substantially. This suggests that Hs-Cdc123 can promote assembly of human eIF2 subunits in yeast. In contrast, the ATP binding site mutants of Hs-Cdc123 had either marginal (D233A) or significantly reduced (D246A) activity in this assembly assay. Together the data point to a critical role of the ATP binding site for the biological function of Hs-Cdc123 and its biochemical activity in eIF2 subunit binding and assembly.

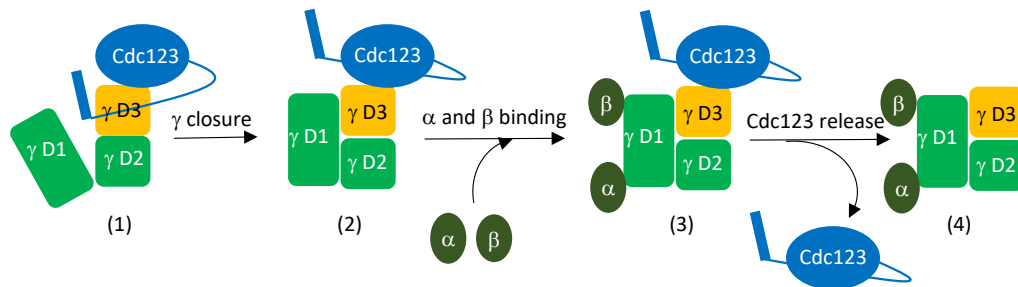
Interestingly, R111 in Hs-Cdc123 replaces K103 of Sp-Cdc123 (Figure 3A and B). R111 shows two alternative side chain conformations that make several interactions with the  $\gamma$  and  $\beta$  phosphate of ATP-Mg<sup>2+</sup> (Figure 3C and D). These additional contacts could participate in the high binding affinity of Hs-Cdc123 for ATP-Mg<sup>2+</sup>. An R residue is also found at this position in Sc-Cdc123 which also displays high affinity for ATP (Table 1, Supplementary Figure 3B). As already observed in Sp-Cdc123 (Panvert et al., 2015), a cavity borders the gamma phosphate group of ATP (Figure 3C and D). This cavity, filled with water molecules, may be the binding site of another substrate. The shape of the cavity in Hs-Cdc123 is different from that of Sp-Cdc123 due to some variations in the side chains of residues that border it. Notably, the 300-306 region in the C-terminal of Hs-Cdc123 is located close to R111 and the ATP binding site (Figure 3E). In particular, Y304 contacts the side chain of R111 in its B conformation. Moreover, as mentioned above, the “300” region of Hs-Cdc123 also contacts  $\gamma$ D3 and the  $\alpha$ 10 helix is

packed onto the  $\gamma$ D3 barrel. Therefore, the present structure shows a link between the ATP binding site and the binding of the  $\gamma$ D3 domain (Figure 3E).

## DISCUSSION

Previous results showed that the C-terminal tail of yeast eIF2 $\gamma$  was required for the interaction with yeast Cdc123. Removal of the last 13 amino acids of Sc-eIF2 $\gamma$  rendered eIF2 $\gamma$  unable to co-precipitate Cdc123 or  $\alpha$  and  $\beta$  subunits (PerzImaier et al., 2013). On the other hand, the C-terminal region of yeast Cdc123 is required for its function in vivo (Panvert et al., 2015). Moreover, similarly to the situation in yeast, it was shown using a yeast strain expressing Hs-eIF2 subunits, that the C-terminus of Hs-eIF2 $\gamma$  was also required for interaction with Hs-Cdc123 or Hs-eIF2 $\alpha$  or Hs-eIF2 $\beta$  (Vanselow et al., 2022). The present structure shows for the first time the tracing for the C-terminal region of Cdc123 that goes from Cdc123 domain 2 to eIF2 $\gamma$ D3 binding site.  $\gamma$ D3 interacts with residues 305 to 309 and the C-terminal helix of Cdc123. These two regions contact the face  $\beta$ 17-18-20-21 (Figure 2B) of the  $\gamma$ D3  $\beta$ -barrel which overlaps the binding site of domain I of eIF2 $\gamma$ . Therefore, the binding of the C-terminal domain of Cdc123 at this position is not compatible with the binding of the active conformation of eIF2 $\gamma$  as it is observed in the heterotrimeric eIF2 (Brito Querido et al., 2020; Llacer et al., 2015; Schmitt et al., 2012). This observation agrees well with recent conclusions obtained with the yeast proteins that Cdc123 can interrupt the intramolecular association of the G-domain (D1) of eIF2 $\gamma$  with domains 2 and 3 (Vanselow et al., 2022). One could imagine that in a first step, Cdc123 binds an immature form of eIF2 $\gamma$  that prevents its interaction with  $\alpha$  and  $\beta$  subunits. This immature conformation may resemble the open form of elongation factor 1A -a protein homologous to eIF2 $\gamma$  (Schmitt et al., 2002)- observed in the presence of GDP (Andersen et al., 2001; Berchtold et al., 1993; Crepin et al., 2014; Kjeldgaard and Nyborg, 1992). Such an open conformation of eIF2 $\gamma$  would be compatible with the binding of Cdc123 as observed here. In a second step, during Cdc123-guided maturation of eIF2 $\gamma$ , the C-terminal part of Cdc123 would be released from its binding site on the  $\gamma$ D3 domain allowing eIF2 $\gamma$  to reach its active conformation capable of binding eIF2 $\alpha$  and eIF2 $\beta$  (Figure 4). Importantly, the C-terminal part of Cdc123 contacts both the ATP binding pocket and the eIF2 $\gamma$ D3 domain. These contacts

may allow Cdc123 to couple eIF2 $\gamma$  binding to ATP consumption. Recent results show that effector-triggered immunity in plants is enhanced by Cdc123 through elevated ATP levels and that impairment of ATP hydrolysis disfavors Cdc123-mediated eIF2 assembly (Chen et al., 2023). This supports the idea that Cdc123 activity might be regulated by ATP concentration.



**Figure 5: Model of Cdc123-assisted eIF2 assembly.**

Cdc123 would bind an open conformation of eIF2 $\gamma$  unable to bind  $\alpha$  and  $\beta$  subunits (1). In a second step, during Cdc123-guided maturation of eIF2 $\gamma$ , the C-terminal part of Cdc123 would be released from its binding site on  $\gamma$ D3 allowing eIF2 $\gamma$  to reach its active conformation (2). eIF2 $\gamma$  is now capable of binding to  $\alpha$  and  $\beta$  (3) triggering Cdc123 release (4). During the process, ATP hydrolysis would occur on Cdc123, which would be released in an ADP-bound form. Because of the lower affinity for ADP (Table 1), Cdc123 would be spontaneously recycled to its ATP-bound form, under the control of intracellular concentration of ATP.

The present work also gives a structural support for interpreting the effects of mutations in eIF2 $\gamma$  gene that are responsible for MEHMO diseases. A P432S mutation in the gene coding for eIF2 $\gamma$  was isolated in patients affected by X-linked hypopituitarism and glucose dysregulation (Gregory et al., 2019). The Hs-Cdc123: $\gamma$ D3 structure shows hydrophobic contacts between P432 of eIF2 $\gamma$ D3 and Hs-Cdc123 domain 1 (Figure 2C). Moreover, many interactions between the 430-432 region of eIF2 $\gamma$ D3 and  $\eta$ 2, $\eta$ 3 Cdc123 helices are observed (Supplementary Table 3). Therefore, it is possible that mutation P432S destabilizes the contacts between eIF2 $\gamma$  and Cdc123 and results in deficiency of Cdc123-assisted eIF2 assembly. The frameshifting mutation, known to impair Cdc123 promotion of eIF2 complex formation, changes the C-terminal sequence of eIF2 $\gamma$  from G<sub>462</sub>VTIKPTVDD to G<sub>462</sub>VTSQQ. The last residue visible in the present structure is eIF2 $\gamma$ -I465. Interestingly, I465 side chain is close to P432 (4.5 Å). Thus, the frameshifting mutation may affect contacts between the C-terminal extremity of eIF2 $\gamma$  and Cdc123. To conclude, the present study provides structural basis for the design of new molecules that

could reinforce the interaction of Cdc123 with eIF2 $\gamma$  to compensate for the effect of MEHMO associated mutations.



## **Data availability**

X-ray data and models have been deposited to the Protein Data Bank under the accession numbers 8PHD (Hs-Cdc123:Hs-eIF2 $\gamma$ D3:ATP-Mg<sup>2+</sup>), 8PHV (Hs-Cdc123:Hs-eIF2 $\gamma$ D3 selenomethione).

## **Supplementary Data**

Supplementary Data are available.

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