Modeling, substrate docking and mutational analysis identify residues essential for the function and specificity of the purine-cytosine transporter FcyB.

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Introduction:

The Nucleobase Cation Symporter-1 (NCS1) family includes bacterial and fungal transmembrane transporters specific for the uptake of purines-pyrimidines as well as other structurally similar metabolites (e.g. allantoin, hydantoin, pyridoxine, thiamine). The recent elucidation of crystal structures of a bacterial member of the NCS1 family, the Mhp1 benzyl-hydantoin permease from *Microbacterium liquefaciens*, allowed us to construct and validate a three-dimensional model of the *Aspergillus nidulans* purine-cytosine/H^+ FcyB symporter. Based on primary sequence alignment, three-dimensional topology, and substrate docking, we identified residues as potentially essential for substrate binding in FcyB. To validate the role of these and other putatively critical for transport residues, we performed a systematic functional analysis of relevant mutants. The characterization of the mutated forms of FcyB was performed by growth tests, epifluorescence microscopic analysis, detailed kinetic studies and western blot analysis. Among the residues mutated we identified critical ones for the substrate binding affinity and/or the specificity, providing information concerning the molecular mechanism and evolution of the transporter specificity.

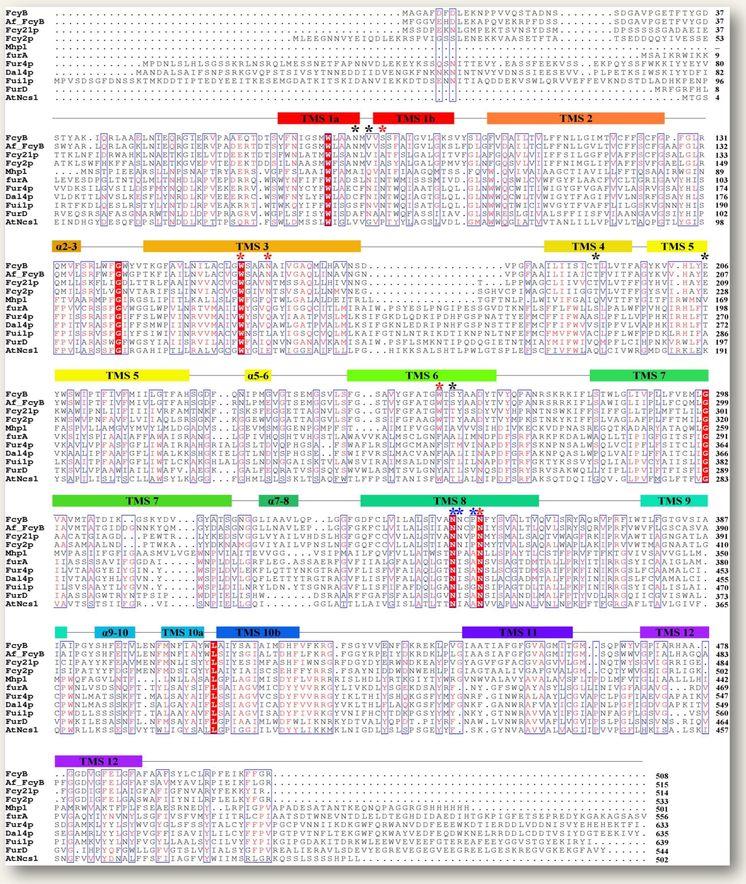
1. Multiple sequence alignment of FcyB and NCS1 homologues of known function.

NCS1 family members bind structurally different substrates, however the alignment of representatives of known function reveals that despite the low identity, there are highly conserved residues in the substrate docking area.

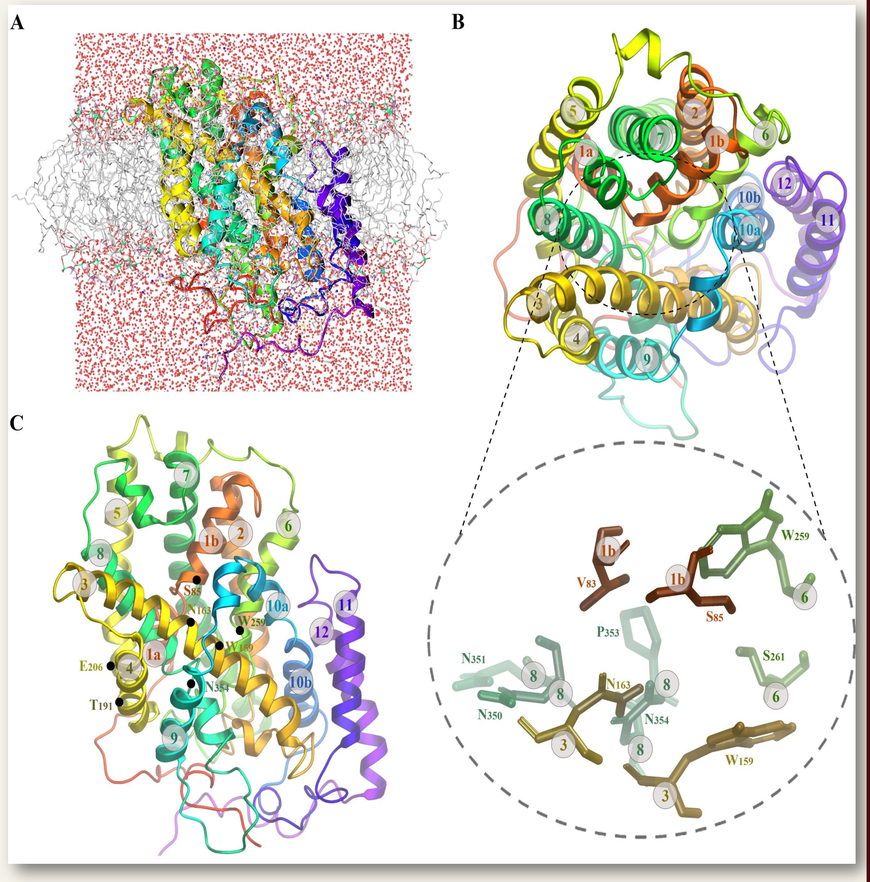
The proteins used for the allignment are: FcyB: *A. nidulans* purine-cytosine transporter; Af_FcyB: *A. fumigatus* purine-cytosine transporter; Fcy21p and Fcy2p: *Saccharomyces cerevisiea* purine-cytosine transporter; Mhp1: *M. liquefaciens* hydantoin transporter; FurA: *A. nidulans* allantoin transporter; Fur4p: *S. cerevisiae* uracil transporter; Dal4p: *S. cerevisiae* allantoin transporter; Fui1p: *S. cerevisiae* uridine transporter; FurD: *A. nidulans* uracil transporter; AtNcs1: *Arabidopsis thaliana* adenine, guanine and uracil transporter.

In order to construct the 3D FcyB model we used the alignment shown with Mhp1. The software used for the construction was Modeller 9v8. Putative TMSs of FcyB are denoted in colored cylinders. Invariant and highly conserved amino acids are shaded in red and blue-lined boxes, respectively.

Amino acids selected and mutated are those predicted critical for function and specificity and are divided in 3 categories highlighted with asterisks: red for residues interacting with substrates, blue for those critical for substrate binding and transport, and black for other residues mutated.



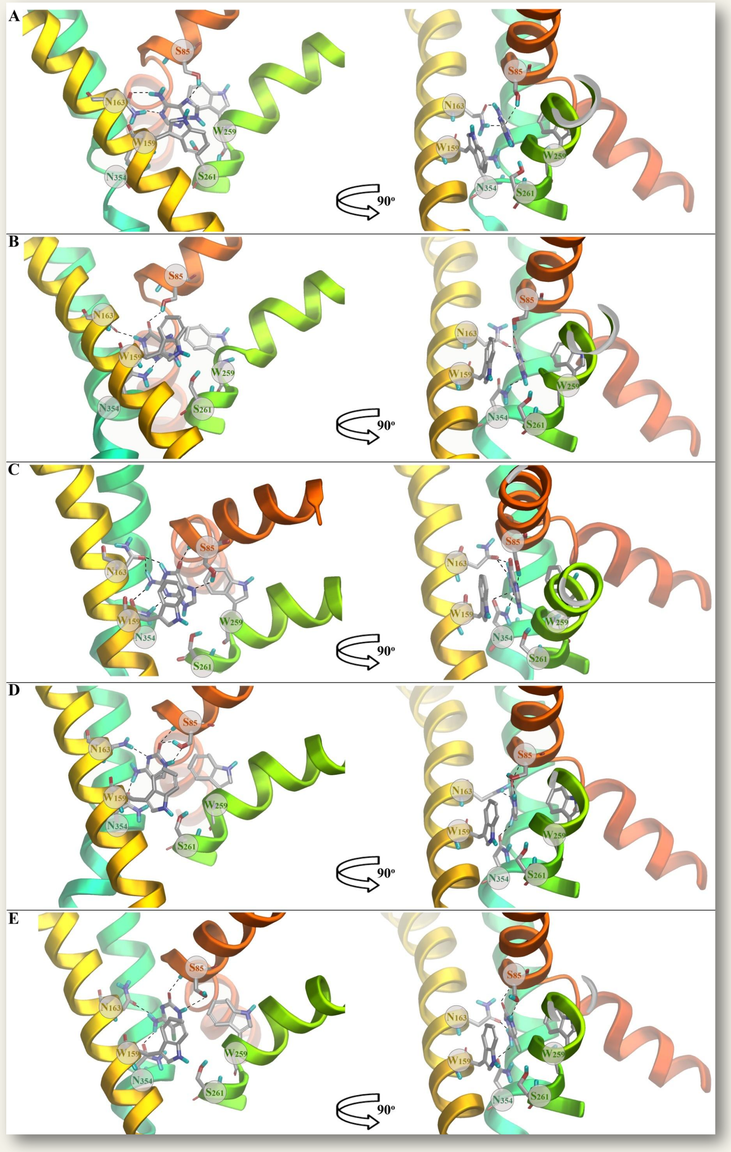
2. FcyB structural model

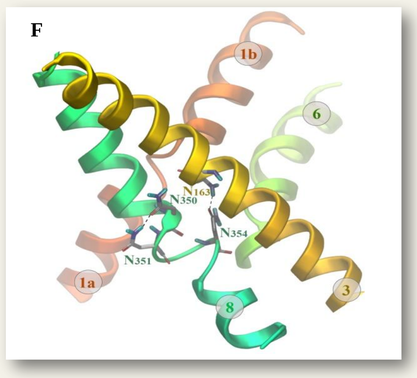


A. Modeled 3D structure of the FcyB validated with molecular dynamics using Desmond software **B.** Top view of FcyB model where the core of the first 10 TMSs is clearly distinguished from the last two TMSs 11 and 12, which probably do not affect transport catalysis per se. The core can also be seen as two subdomains, the first made of TMSs 1, 2, 6 and 7 and the second from TMSs 3, 4, 8 and 9, linked with flexible helices TMS5 and TMS10. The substrate-binding site is located in the space between the two subdomains of the core. In the lower panel, the topology of residues critical for the function of the substrate binding site, S85, W159, N163, W259, N350, N351, P353 and N354, is shown in zoom-out. **C.** Side view of FcyB structure showing the topology of residues S85, W159, N163, W259 and N354, involved in direct interactions with substrates.

3. Substrate docking in FcyB

Docking assays were held, the more consistent poses are presented here for each substrate. Hydrogen bonds are depicted with dashed lines. In all cases the two Trps (W159 and W259) participate in substrate binding by π-π stacking interactions between the purine/pyrimidine ring and the indolic ring. **A.** Adenine binds with 3 hydrogen bonds in total that are formed with S85 and N163. **B.** Hypoxanthine forms 3 hydrogen bonds with S85, N163 and N354. **C.** Guanine forms 6 hydrogen bonds with S85, N163 and N354. **D**. Cytosine forms 5 hydroden bonds with S85, N163 and N354. **E.** 5-Flurocytosine forms 4 hydrogen bonds with S85, N163 and N354.

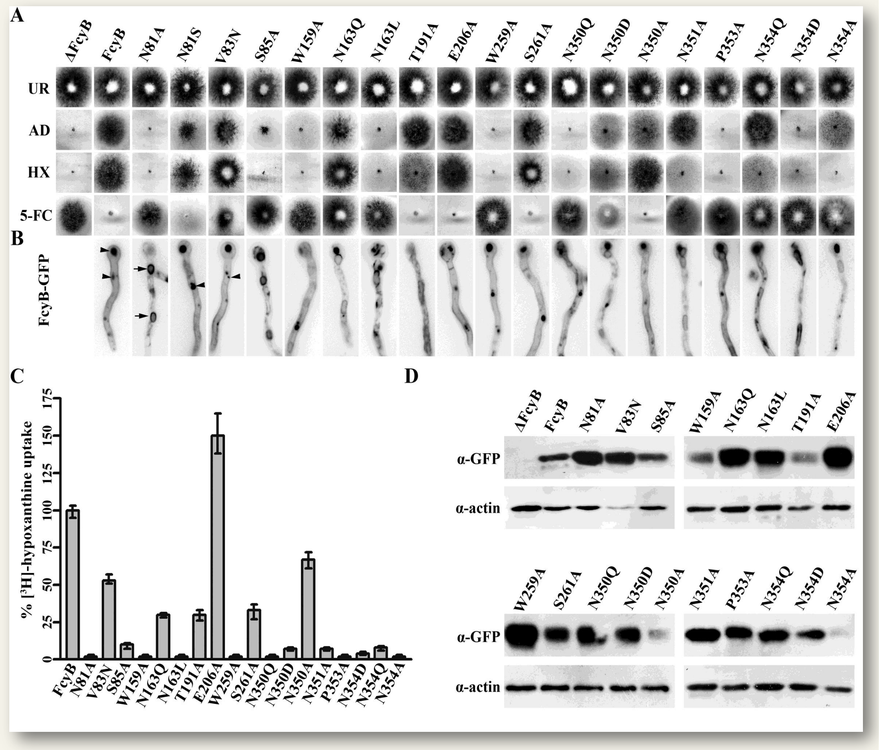


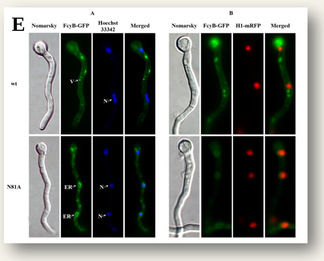


F. Topology of the four functionally critical Asn residues in TMSs 3 and 8 and their possible H bond interactions.

4. Functional analysis of FcyB mutations

A. Growth tests on HX (hypoxanthine) and AD (adenine) as sole nitrogen sources and resistance/sensitivity test on 5-FC (5-fluorocytosine) at 25° C of mutant alleles of FcyB. Growth on urea is also shown (UR) as a control. Positive (FcyB) and negative (*Δ*FcyB) isogenic control strains are shown. **B.** Epifluorescence microscopy showing *in vivo* subcellular expression of FcyB-GFP mutant alleles and a wild-type control (FcyB) presented as dark structures in a grayscale inverted mode. In selected samples, arrows and arrow heads depict perinuclear ER membrane rings and vacuoles, respectively. **C.** Comparative initial uptake rates of ^{3}H-radiolabeled hypoxanthine in FcyB mutant alleles and a wt control. 100% is the transport rate in the wt (FcyB). **D.** Western blot analysis of total proteins from FcyB-GFP mutants detected with anti-GFP antibody. Antibody against actin was used as an internal marker for equal loading.





E. *In vivo* subcellular localization of wild-type FcyB-GFP (upper panel) and mutant FcyB-N81A (lower panel) in relation to positioning of nuclei in order to verify that the rings formed in the case of FcyB-N81A are endoplasmic reticulum rings. (A) Nuclei labeled with Hoechst 33342, (B) Nuclei labeled with HhoA-mRFP (histone H1). V, indicates vacuoles, ER, indicates endoplasmic reticulum, N, indicates nucleus.

Kinetic and specificity profile of mutant versions of FcyB. $K_{m/i}$ values (μM) determined were determined as described in Experimental procedures. >500 or >1000 stand for inhibition values close to 10-20 % at 0.5 and 1 mM respectively. Results are averages of at least three independent experiments in triplicates for each concentration point. Standard deviation was <20 %.

	Hypoxanthine	Adenine	Guanine	Cytosine
wt	11	7	17	20
V83N	16	2	7	18
S85A	78	26	16	45
N163Q	47	2	> 500	73
T191A	9	11	4	3
E206A	6	7	10	14
S261A	4	6	50	17
N351A	3	1	4	16
N350D	5	8	31	6
N350A	6	> 1000	25	5
N354D	3	1	> 500	4

Discussion:

Our results fully support the involvement of specific residues in TMS1, TMS3, TMS6 and TMS8, in substrate binding and/or transport. In particular:

- ❖S85, W159, N163, W259, N350, N351, P353 and N354 were shown to be irreplaceable for FcyB-mediated transport, among these: S85, N163, N350, N351 and N354 seem critical for determining the substrate binding affinity and/or specificity of FcyB.
- ❖N81, V83, S261, T191 and E206 seem to have a less critical role on protein turnover or FcyB transport activity, however only T191A moderately affects the affinity and substrate specificity of FcyB.
- ❖S85, N163 and N354 make direct H-bonds with substrates,.
- ❖E206A, concerning a residue within TMS5 (putative inward-facing gate), leads to a reduction in FcyB turnover and an increase in apparent transport capacity
- ❖W159 and W259 stabilise binding through *pi-pi* stacking interactions with purine or pyrimidine-ring.
- ❖N350, N351 and P353 have an indirect role in substrate binding through local interactions between themselves and the residues binding substrates.

Some of the chosen residues were rationally mutated in order to mimic the binding sited of homologous transporters of different specificity, however none of the single mutations constructed and analysed in this work, including N163L, N163Q or P353A which introduce residues present in uracil, allantoin, hydantoin or uridine transporters, conferred to FcyB the ability to bind or/and transport substrates. This observation strongly suggests that substrate specificity has a more complex molecular basis and might not even be solely determined by the architecture of the binding site, but also by elements acting as gates or selectivity filters, or by elements contributing to protein stability.

Our work is the first systematic approach to address structure-function specificity relationships in a eukaryotic member of NCS1 family by combining genetic and computational approaches. Knowledge from this work is the basis for further research in order to understand in more detail not only FcyB's transport mechanism, but also how substrate specificity has evolved within the NCS1 family, and probably a first step in the design of novel, targeted antifungals taken-up specifically by FcyB-like transporters of pathogenic fungi.

References:

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