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### ► **To cite this version:**

Jorgaq Pata, Alexis Moreno, Benjamin Wiseman, Sandrine Magnard, Idriss Lehlali, et al.. Purification and characterization of Cdr1, the drug-efflux pump conferring azole resistance in *Candida* species. *Biochimie*, 2024, 220, pp.167-178. <10.1016/j.biochi.2023.12.007>. <hal-04680964>

**HAL Id: hal-04680964**

**<https://hal.science/hal-04680964v1>**

Submitted on 30 Aug 2024

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# Purification and characterization of Cdr1, the drug-efflux pump conferring azole resistance in *Candida* species

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## Abstract

*Candida albicans* and *C. glabrata* express exporters of the ATP-binding cassette (ABC) superfamily and address them to their plasma membrane to expel azole antifungals, which cancels out their action and allows the yeast to become multidrug resistant (MDR). In a way to understand this mechanism of defense, we describe the purification and characterization of Cdr1, the membrane ABC exporter mainly responsible for such phenotype in both species. Cdr1 proteins were functionally expressed in the baker yeast, tagged at their C-terminal end with either a His-tag for the *glabrata* version, *cgCdr1*-His, or a green fluorescent protein (GFP) preceded by a proteolytic cleavage site for the *albicans* version, *caCdr1*-P-GFP. A membrane Cdr1-enriched fraction was then prepared to assay several detergents and stabilizers, probing their level of extraction and the ATPase activity of the proteins as a functional marker. Immobilized metal-affinity and size-exclusion chromatographies (IMAC, SEC) were then carried out to isolate homogenous samples. Overall, our data show that although topologically and phylogenetically close, both proteins display quite distinct behaviors during the extraction and purification steps, and qualify *cgCdr1* as a good candidate to characterize this type of proteins for developing future inhibitors of their azole antifungal efflux activity.

**Keywords** : ABC transporter, drug resistance, membrane proteins, purification, efflux pumps

## Introduction

Fungi are a constant health threat. More than 300 million humans are regularly infected by those microorganisms, and among them 3 million displays severe candidemia with a mortality rate up to 50% [1]. Plants and animals are not spared from this type of infection either, leading to significant reduction in agricultural crops [2]. A large part of human infections by fungi is due to the *Candida* species, a widespread and non-pathogenic yeast genus but aggressively opportunistic under

immunocompromised conditions of their host. The most severe form of infection is caused by invasion of blood tissue, which is particularly deadly for immunocompromised patients, particularly after surgery, HIV infection, or even more recently, COVID 19 infection [3]. Other *Candida* species involve *glabrata* [4], *tropicalis* [5], *krusei* [6], and more recently *auris* [7].

Five classes of fungicidal and fungistatic drugs have been developed since 70 years to treat fungal infections [8]. These compounds either disrupt membranes or target the biosynthetic pathways of ergosterol, sphingolipids, microtubules, nucleic acids and cell wall [9]. Among them, azole compounds constitute the most widely used class of antifungals. Early developed [8], azoles block CYP51 [10] which, by demethylating lanosterol generates the ergosterol. This cholesterol-like lipid is specific for the yeast and is required for membrane integrity, accounting for 20% of the lipid content [11]. Success of azoles is attributed to both their yeast-specific target as well as low cost [12]. They are also used in agriculture, accounting for about 25% of the fungicides market [13]. The other side of the coin, the massive usage of these molecules favors the development of resistance mechanisms. The appearance of strains resistant to their action rapidly followed their usage [14], a phenomenon that may have been accelerated by their combined use in humans, plants and animals [15].

As a long-lasting response to toxicity induced by the use of azoles, fungi alter the primary structure of the targeted enzyme CYP51 [16]. But as a fast and efficient response to drug administration, fungi detoxify their cytoplasm by upregulating the expression of gene encoding for membrane proteins which act as pumps to expel those drugs out of them, thereby reducing the azoles concentration below their effectiveness threshold [17, 18]. These pumps belong primarily to the ATP-binding Cassette (ABC) superfamily [19], early identified in yeast by André Goffeau and colleagues [20], and also to the Major Facilitator Superfamily (MFS) [21]. More worrying, certain representatives of these two families manifest a multi-specificity, a property by which they confer resistance not only to the antifungal agent which triggered their overexpression, but also to other azole or non-azole drugs, *i.e.*, with unrelated structures, that may be subsequently administered. This mechanism leads to a pleiotropic drug resistance (PDR) phenotype [22], which is also observed in cancer cells as a multidrug resistance (MDR) phenotype [23]. Combination of alanine scanning and molecular modeling approaches allowed us to shed light on the molecular basis of this poly-specificity in the case of Mdr1 and Cdr1, the two prominent MFS and ABC proteins of *C. albicans* responsible for the resistance to various antifungal agents, notably azoles [24, 25].

PDR exporters represent a significant part of ABC transporters in yeast, with e.g. 10 out of 30 proteins in *Saccharomyces cerevisiae* [20], 9 out of 26 in *C. albicans* [26] and 7 out of 25 in *C. glabrata* [27]. They share a structural organization made on one hand of 2 nucleotide-binding domains (NBDs) that generate 2 nucleotide-binding sites (NBS) when tightly associated during the transport cycle, and on the other hand of two trans-membrane domains (TMDs) to which drugs bind, are translocated from the inner to the outer membrane leaflet, and then expelled outside the cell. NBDs and TMDs are encoded as a single polypeptide in which one NBD precedes a TMD, a topology also found in the human ABCG sub-family according to the HUGO classification [28]. Progresses in 3D-structure knowledge of ABC transporters have more recently allowed to propose a new classification in which PDR exporters now belong to the type V sub-family [29] ; see Moreno *et al.* 2019 [19] for review. Recently, the first cryo-EM structures of the *S. cerevisiae* representative, Pdr5, has provided a lot of not only original but also critical information about structural shape and details of PDR exporters [30].

PDR exporters also share an intriguing and uncommon feature by which one of their NBS binds but cannot hydrolyses ATP. Mutagenesis approaches initially carried on Pdr5 [31, 32] and later on Cdr1 from *C. albicans* [33, 34] highlighted the functional role of this non-catalytic NBS in drug efflux, revealing its pivotal role in the long-distance cross-talk between NBSs and TMDs ; see Banerjee *et al.*

2021 [35] for review. Most of these studies were done *in vivo* or by characterizing the wild-type and mutated proteins in enriched membrane fractions. In order to take a step ahead for characterizing these features on the isolated protein, we have evaluated two different purification strategies based on polyHis and green fluorescent protein (GFP) affinity tagged versions of Cdr1 from two different species, *C. albicans* and *C. glabrata*.

## Methods

**Materials.** Bacto-yeast extract and bacto-peptone were purchased from Difco Laboratories, Detroit, MI. Luria Bertani (LB) medium and YPD medium were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). All detergents including diisobutylene/maleic acid copolymer [36] (DIBMA) were purchased from Anatrace Inc., Ohio, USA. Dicarboxylate oside 9b [37] (DCOD 9b) was from CALIXAR, Lyon, France. Trans-PCC- $\alpha$ -M [38] (PCC) was purchased from Glycon Biochemicals, Luckenwalde, Germany. Competent *Escherichia coli* strain BL21(DE3) Gold pLysS, were purchased from ThermoFisher Scientific, Illkirch, France. The pOPINE-GFP nanobody plasmid [39] was purchased from Addgene, Watertown, MA, USA. EDTA-free antiproteases mix was purchased from Roche SAS, Boulogne-Billancourt, France. The HiFloQ Nickel-NTA columns were purchased from Generon, Slough, UK. Superdex 200 column was purchased from CYTIVA Europe GmbH, Velizy-Villacoublay, France. Amicon® ultra-centrifugal filters were purchased from Merck KGaA, Darmstadt, Germany. Isopropyl -D-1-thiogalactopyranoside (IPTG), oligomycin and FK506 were purchased from Sigma, L'Isle-d'Abeau Chesnes, France. Peptidisc with the primary sequence FAEKFKEAVKDYFAKFWDPAAEKLKEAVKDYFAKLWD [40] was synthesized chemically by GenScript Biotech Corporation, Jiangsu, China.

**Strain.** The yeast strain used in this study was the  $\Delta$ ura3 version called AD $\Delta$  [41] of the *S. cerevisiae* AD1-8u<sup>-</sup> strain in which 7 genes coding for the ABC exporters Yor1 (“1”), Snq2 (“2”), Pdr5 (“3”), Pdr10 (“4”), Pdr11 (“5”), Ycf1 (“6”), Pdr15 (“7”), and one transcription factor, Pdr3 (“8”) were deleted, rendering the strain hypersensitive to drugs [42]. AD $\Delta$  was a gift of Richard Cannon and Edward Lamping.

**Proteins.** The pABC3 plasmids coding for *cgCdr1* and *caCdr1* studied here were a gift of Richard Cannon and Edward Lamping. *cgCdr1* corresponds to the sequence referenced as Q6FK23 in the Uniprot protein database, from the *C. glabrata* laboratory strain CBS 138, to which a hexa-histidine tag had been added at the C-terminal end, leading to the *cgCdr1*-His chimera [41]. *caCdr1* corresponds to the sequence referenced as P43071 in the Uniprot protein database, from the laboratory strain ATCC 32032, to which a GFP had been fused at the C-terminal end, leading to the *caCdr1*-P-GFP chimera [41]. Here, we inserted a PreScission Protease cleavage site (LEVL $\downarrow$ FQGP) coding sequence between Cdr1 and GFP encoding moieties to generate the *caCdr1*-P-GFP chimera. The proteolytic cleavage site was introduced by homologous recombination into the AD $\Delta$  strain, using the forward and reverse oligonucleotides 5'-tcgacctggagggtgctgttccagggacctg-3' and 5'-tcgacaggtccctggaacagcacctccagg-3'. Primary sequences of the *cgCdr1*-His and *caCdr1*-P-GFP constructs are displayed in Table 1.

**Microscopy.** Yeast strains were screened by growing them to the exponential phase followed by a wash with phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) and observation in a Zeiss LSM 800 confocal microscope equipped with a Plan-Apochromat 63x/1.40 Oil M27 lens, exiting at 493 and looking for fluorescence emission at 513 nm.

**Drug susceptibility assays.** Ten milliliters of YPD medium were inoculated with a single colony of control AD $\Delta$  yeast or AD $\Delta$ [Cdr1] and further incubated for 8 h at 30 °C, 200 rpm. Strains sensitivity to xenobiotics was then determined by inoculating yeasts in a 96-well plate at an initial optical density at

600 nm, OD<sub>600</sub>, of 0.03 using a SAFAS Xenius spectrophotometer (SAFAS, Monaco), in a total volume of 200 µL YPD containing 1% (w/v) bacto-yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose, in presence of a range of drug concentrations. Drugs being solubilized in DMSO, the final concentration of the later was set to 0.5%. Wells containing test samples were surrounded by wells filled with water to limit evaporation. The plate was then incubated for 14 h at 30 °C, 600 rpm in humidified atmosphere. Yeast growth was then measured at OD<sub>600</sub>.

**Large-scale yeast cultures.** Fifty milliliters of YPD medium were inoculated with a single colony of control ADΔ yeast or ADΔ[Cdr1] and further incubated for 14 h at 30 °C, 200 rpm. The culture was then used to inoculate a 17-L Chemap fermenter (Fermentec, Lyon, France) of YPD medium at an initial OD<sub>600</sub> of 0.005. Yeasts were grown 14 h at 30 °C, 200 rpm and 2 L air/min. Nitrogen source was refreshed when the OD<sub>600</sub> reached 1.5 by addition of 10% (v/v) 5xYP mix containing 100 g/L tryptone and 50 g/L yeast extract. Yeasts were harvested at OD<sub>600</sub> ~4 by pelleting them at 5,000 ×g for 15 min, 4 °C using a JLA-9.1000 rotor (Beckman Coulter). They were then washed twice with ice-cold pure H<sub>2</sub>O, weighted (~500 xg) and diluted 1.5x (w/v) in a Suspension Buffer (SB) containing 50 mM Tris-acetate pH 7.5, 1 mM EDTA, 20% glycerol, 1 mM PMSF, freshly complemented with an aliquot of EDTA-free antiprotease solution (chymostatin, pepstatin, leupeptin, antipain, aprotinin) at a count of one tablet for 50 mL buffer. Yeast samples were frozen in liquid nitrogen and stored at -80 °C.

**Membrane preparation.** Membranes were prepared using a protocol recently described for small-scale cultures [43], that we adapted to large-scale production as follows. Yeasts were thawed in cold water and then mechanically broken with a refrigerated CF cell disruptor (Constant systems Ltd.) by applying a pressure of 1.5 kbar and passing the solution through the disruptor one time, with an outlet lysate temperature remaining below 10 °C. Unbroken material were discarded by pelleting it by two centrifugation steps at 3000 ×g 5 min, 4 °C and a last one at 4200 ×g. The supernatant was 4x diluted in SB and then centrifuged at 20,000 ×g 1 h, 4 °C on a JLA 16.250 rotor (Beckmann Coulter). The resulting membrane fraction pellet was suspended in SB at about 10 g/L protein, estimated by BCA [44]. Membranes were frozen in liquid nitrogen and stored at -80 °C.

**anti-GFP nanobody production and purification.** A His-tagged anti-GFP nanobody (nanoantiGFP) was produced in the *E. coli* strain BL21(DE3) Gold pLysS and then purified as described by Kubala and colleagues [39]. Ten milliliters of LB medium (25 g/L) added of with 50 µg/mL ampicillin and chloramphenicol were inoculated with 1 colony of transformed bacteria and then incubated overnight at 37 °C 200 rpm. The culture was used to inoculate 1 L LB supplemented with antibiotics as above, and incubated at 37 °C, 160 rpm, until reaching an OD<sub>600</sub> of 0.5. The culture was cooled down to 20 °C for about 30 min under shaking and 0.5 mM IPTG was added, followed by 20 h incubation at 20 °C, 160 rpm. The bacteria were then harvested by centrifugation at 5000 ×g with a JLA-9.1000 rotor at 4 °C for 15 min. The pellet was suspended in 50 mM HEPES-HCl, pH 8.0, 1 mM EDTA, anti-protease tablet (one tablet/50 mL). Bacteria were mechanically lysed with a refrigerated CF Constant cell disruptor by applying a pressure of 1.5 kbar and passing the solution through the disruptor two times, with an outlet lysate temperature remaining below 10 °C. The lysate was centrifuged at 10,000 ×g for 10 min at 4 °C using a JLA 16.250 rotor to discard the unbroken material. The supernatant was loaded into a 5-mL HiFliQ Nickel-NTA resin (Generon) connected to an Aktä Prime™ (GE-healthcare®) purification unit, equilibrated with 50 mM HEPES-HCl buffer pH 8.0, 200 mM NaCl and 40 mM imidazole, at a 2 mL/min flow rate. The resin was washed with 10 volumes of the same buffer but 60 mM imidazole and the nanoantiGFP was then eluted by increasing again the imidazole concentration to 250 mM. The pool of nanoantiGFP was frozen in N<sub>2</sub> liquid and stored at -80 °C.

**Cdr1 extraction tests.** Assays were carried out at a protein concentration of 5g/L using 1% (w/v) extractant and 0.1% additive (DCOD or cholate) when used, both prepared as 10% solution in SB, for 1

h, 4 °C under gentle agitation. DIBMA and SMA solutions were used at 2.5%. Solutions were then centrifuged at 100,000 xg for 1 h, 4 °C, in a MLA80 rotor (Beckman Coulter) to remove the insoluble material, and loaded on a 10% acrylamide-bis-acrylamide SDSPAGE [45]. Cdr1 proteins were revealed on SDS-PAGE stained with Coomassie blue stain or illuminated at 473 nm to reveal GFP fluorescence at 510 nm using a Typhoon FLA 9500 Fluorescence Imager (GE Healthcare, Illinois, USA). Proteins on gel were quantified using the Fiji software [46].

### ***Cdr1 purifications.***

***Extraction.*** Membrane proteins were extracted as above using 1% (w/v) detergent. The solution was then centrifuged at 100,000 xg for 1 h, 4 °C, to remove insoluble material and used immediately further chromatographic steps.

***Ion-metal affinity chromatography (IMAC).*** Both proteins were purified by using a 5-mL HiFliQ Nickel-NTA column connected to an Aktä Prime™ purification unit. This was done either directly for *cgCdr1*-His or *via* the GFP moiety for *caCdr1*-P-GFP, by preloading 10 mol of nanoantiGFP, per mol of extracted *caCdr1*-P-GFP, estimated by using the GFP fluorescence. The extracted material was loaded to each resin at a flow rate of 1.0 mL/min and then washed with 50 mM HEPES-HCl, pH 7.5, 150 mM NaCl, 70 μM (2 CMC) PCC ± 4 μM DCOD (as indicated in the text), 20 mM imidazole. Cdr1 proteins were then eluted in 2.0 mL fractions by increasing the imidazole concentration in the buffer up to 150 mM.

***Size exclusion chromatography (SEC).*** Each pool (~10 mL) was concentrated to about 500 μL using Amicon® ultra-centrifugal filters with a cutoff of 100 kDa, at 1500 xg, a speed reduced for preventing detergent over-accumulation as recently set up [47]. Concentrated solutions were then loaded on a Superdex 200 Increase 10/300 GL column with a running buffer containing 50 mM HEPES-HCl, pH 7.5, 100 mM NaCl, and detergent, either 70 μM (2 CMC) PCC ± 4 μM DCOD (as indicated in the text), 1 mM (6 CMC) DDM or 50 μM (5 CMC) LMNG. Fractions of 1 mL were collected, and the proteins were used immediately or frozen in liquid nitrogen and stored at -80 °C.

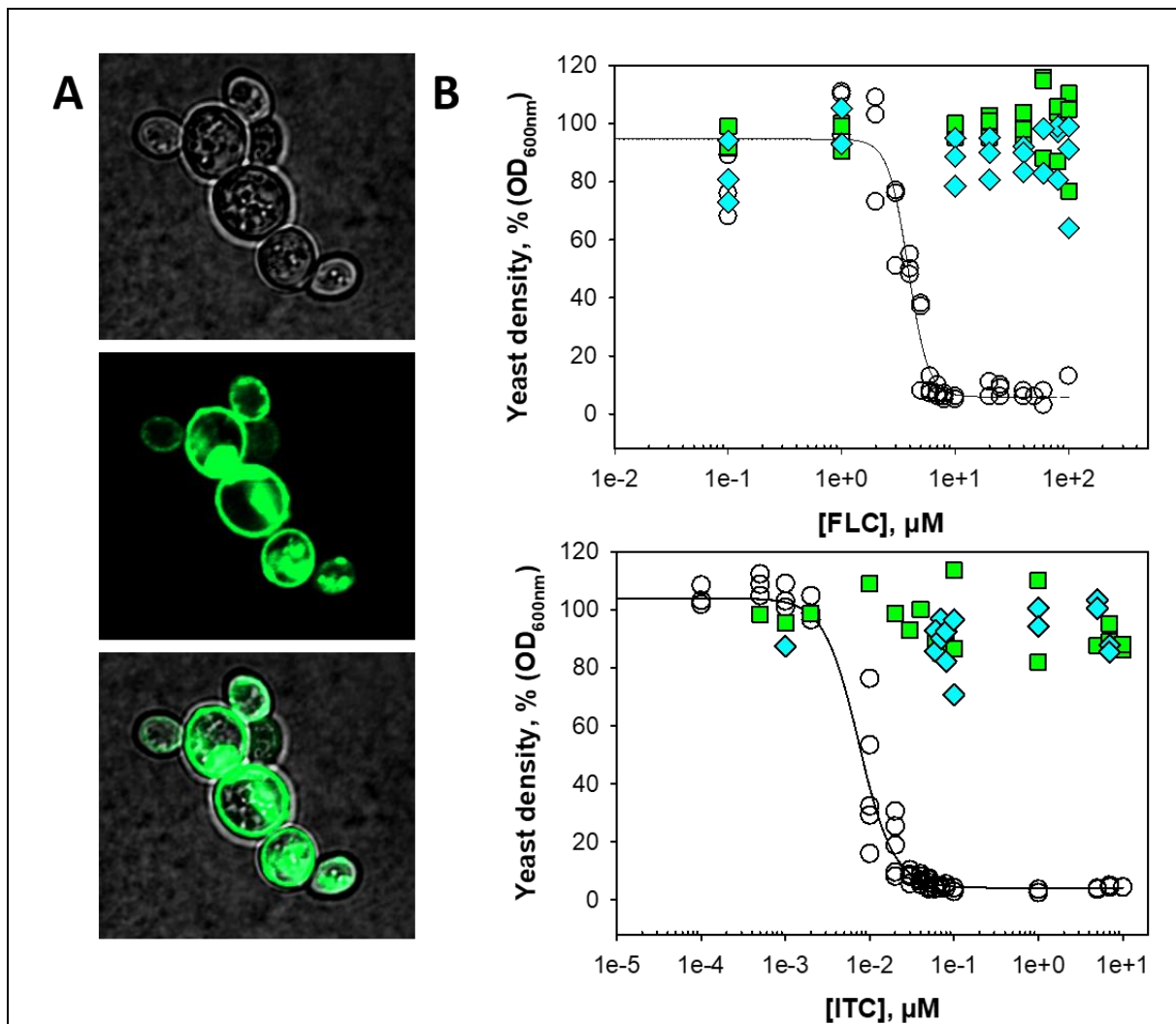
***Substrate, inhibitors and ligands binding assays.*** Substrate-binding assays were performed using intrinsic fluorescence, probed on a SAFAS Xenius spectrophotofluorimeter. Tryptophan residues, and N-acetyl tryptophan amide (NATA) used as negative control, were excited at 290 nm with a slot of 5 nm and their fluorescence emission spectra were recorded between 310 and 380 nm with a slot of 5 nm. Experiments were done in a final volume of 200 μL in a quartz cuvette, in which increasing amounts of compounds were added. Resulting emission curves were integrated and deduced from the same experiments carried out with NATA. The results were fitted using GraphPad Prism 9 or SigmaPlot V12.5.

***Nano Differential Scanning Fluorimetry (nanoDSF).*** Melting temperature ( $T_m$ ) of each purified protein with and without ligands was determined using a Prometheus NT.48 (NanoTemper Technologies GmbH, München, Germany). Measures were carried out using Cdr1 proteins at 0.2 g/L and a temperature gradient ranging from 20 to 95 °C with a slope of 1 °C/min. Ligands were added at 5 μM for substrates, dyes and inhibitors, 2 mM for ATP-Mg<sup>2+</sup>-VO<sub>4</sub> and 0.1 μg for yeast lipids (10% ergosterol content) per μg of Cdr1. Solutions were incubated either for 30 min at room temperature or overnight at 4 °C when containing lipids before measure.

***ATPase activity.*** ATP hydrolysis in presence of inhibitors and substrates was assayed by measuring the Pi produced for purified protein, in presence and absence of orthovanadate (VO<sub>4</sub>) [48]. Alternatively, an ATP regenerating system [49] was also used as follows: reactions were performed at 30 °C in a final volume of 200 μL consisting of 0.1 g/L lactate dehydrogenase, 1 mM phosphoenolpyruvate, 0.1 g/L pyruvate kinase, 0.6 mM NADH, 2 mM MgCl<sub>2</sub> and 10 mM KCl. The solution was added of 5 mM NaN<sub>3</sub>, 75 mM KNO<sub>3</sub> and 0.3 mM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> as inhibitors of F1 ATPase, pyrophosphatases and



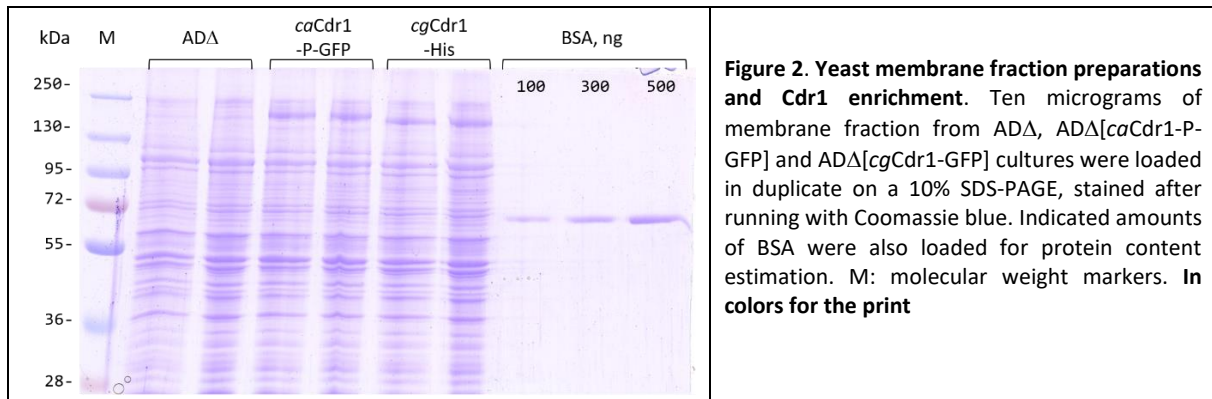




**Figure 1. Functionality of the *caCdr1*-P-GFP construct.** **A.** Fluorescence of GFP fused to *caCdr1* localization by confocal microscopy. **B.** Resistance and sensitivity pattern to fluconazole (FLC, upper panel) and itraconazole (ITC, lower panel) of *ADΔ[caCdr1-P-GFP]* (green squares) compared to the host *ADΔ* (circles) and *ADΔ[cgCdr1-GFP]* (blue diamonds) strains. Maximal OD<sub>600</sub> values obtained in absence of antifungal ranged between 0.9 and 1.2. Half-maximal cytotoxic concentrations, EC<sub>50</sub>, for the *ADΔ* strain are of  $3.8 \pm 0.2 \mu\text{M}$  and  $7.4 \pm 0.6 \text{ nM}$  ( $p < 0.0001$  each) for fluconazole and itraconazole, respectively. **In colors for the print**

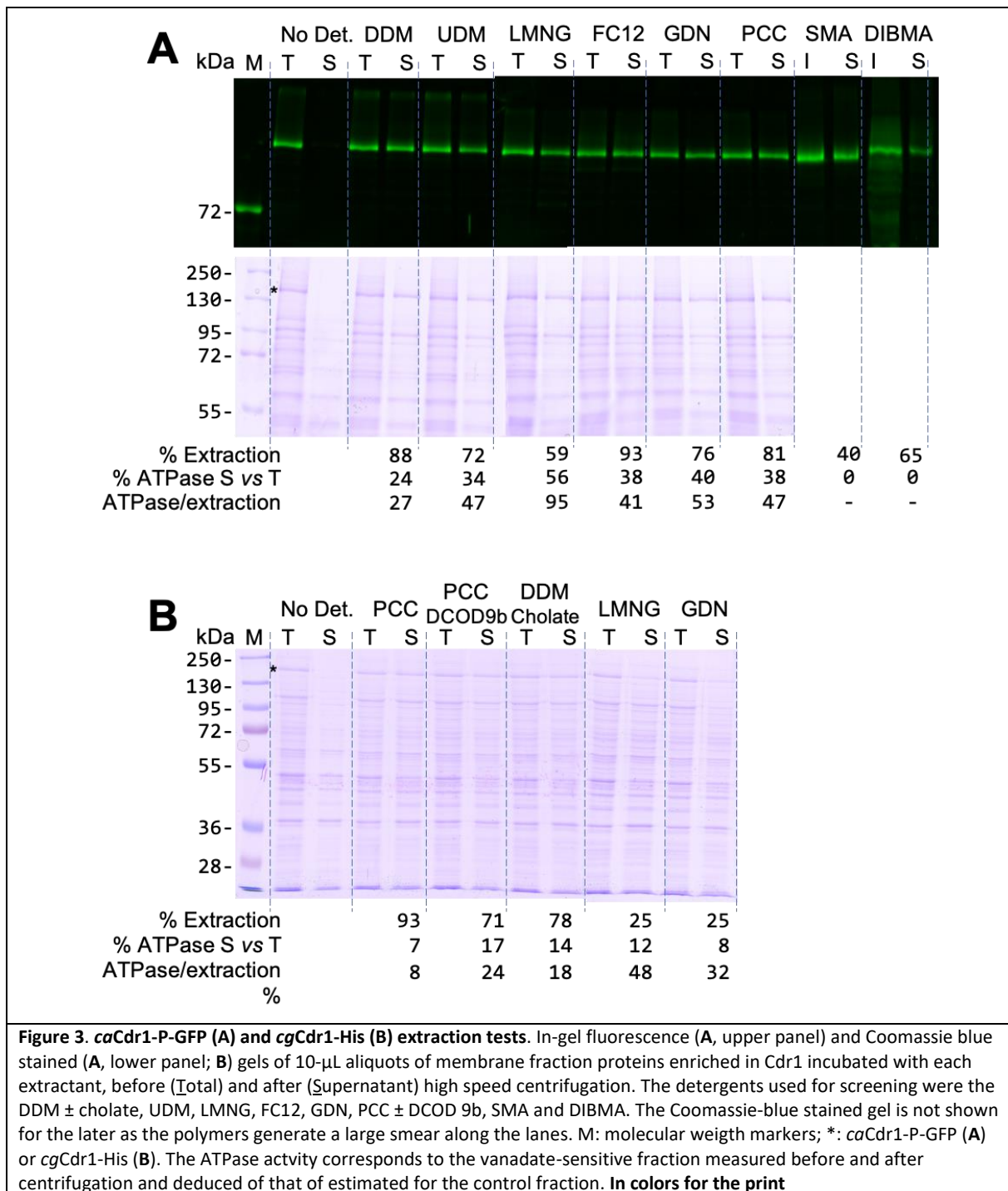
### Membrane preparations.

Membranes were prepared using a protocol recently set up [43], that we adapted for a large-scale production. We obtained the best yields of Cdr1 enrichment by passing the yeast suspensions through the cell disruptor only one time set to 1.5 kbar. Such pressure was rather low for breaking yeast but yields always reduced when increasing the pressure or repeating one or two times the breaking step. Membrane preparations from *ADΔ*, *ADΔ[caCdr1-P-GFP]* and *ADΔ[cgCdr1-GFP]* cultures (Figure 2) gave comparable yields, and for the latter about the same enrichment in Cdr1 of  $3.6 \pm 0.1\%$  (w/w, referring to bovine serum albumin, BSA as BCA and Coomassie blue staining standard).



### ***Cdr1* extraction tests.**

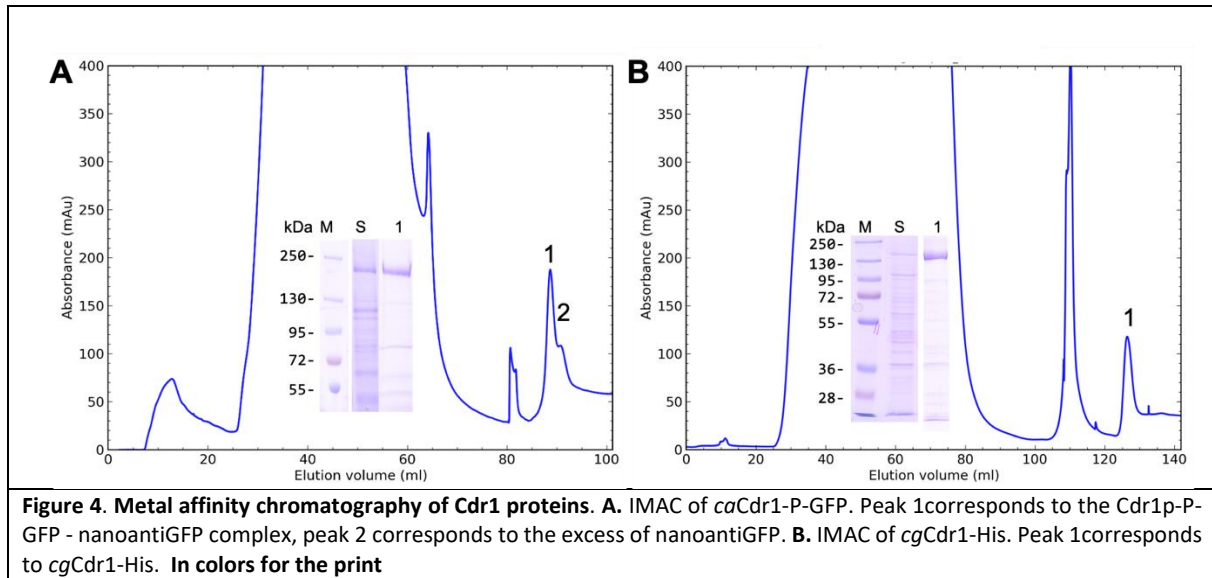
A series of extractants were assayed to explore *Cdr1* solubilization from host membranes. Compounds varied from classical to most recent, used either alone, combined or added to stabilizers. Extractants and stabilizers shown here comprise the dodecyl  $\beta$ -D maltoside (DDM)  $\pm$  cholate (20 mM DDM, 4.5 mM cholate as described [56]), the undecyl  $\beta$ -D maltoside (UDM), the lauryl maltose neopentyl glycol [57] (LMNG), the foscholine 12 (FC12), the glyco-diosgenin [58] (GDN), the cyclohexyl  $\alpha$ -maltoside [38] (PCC)  $\pm$  dicarboxylate oside 9b [37] (DCOD-9b, 1 mol/20 mol detergent), the polystyrene-co-maleic acid [59] (SMA) and the diisobutylene/maleic acid copolymer [36] (DIBMA). Extractions tests were mainly assayed with the *caCdr1*-P-GFP protein, taking advantage of the GFP moiety easily detectable on SDS-PAGE using a Typhoon imager. Some of them are displayed in Figure 3A. The protein was extracted more or less in all conditions, averaging from 40% with SMA to 93% with FC12. The extraction step had a variable impact on the VO<sub>4</sub>-sensitive ATPase activity of the fraction, presumably attributed to *Cdr1* by comparing that to the control fraction. No ATPase activity was detected when using SMA and DIBMA co-polymers. In the case of detergents, the ATPase activity was partially lost to different extents, pointing to LMNG, GDN and PCC for bringing the best results. The *cgCdr1*-His protein (panel B) did not display the same extraction patterns with LMNG or GDN which extracted rather poor amounts of protein although with a good level of ATPase activity, but lower than with the *C. albicans* version. The use of cholate with DDM, which was shown to give a high ATPase activity of the prokaryotic type IV ABC pump BmrA [56], was also of not much use in our case. The PCC extracted pretty well the protein but with a very low level of ATPase activity, which was improved when the DCOD-9b was added as a stabilizer.



### **Metal affinity chromatography step.**

As introduced above, we used a Nickel-affinity resin to enrich each Cdr1, either indirectly *via* the GFP moiety fused to *caCdr1* bound to a preloaded His-tagged nanoantiGFP or directly *via* the His-tag fused to *cgCdr1*. Results displayed in Figure 4 show that both proteins were well enriched and to comparable levels. We expected from the use of the nanoantiGFP to get a better enrichment level as it gives the possibility to remove His-rich contaminants by passing the extracted solution through the resin before loading the nanobody. It turned out that it is not the case, as comparable levels of purity were obtained with both Cdr1 proteins. This is probably due to the imidazole concentration stringency of the conditions used to equilibrate the resin (40 mM imidazole) and wash it after nanobody binding (60 mM

imidazole), which strongly limited the contamination. In any case, it remains an excellent strategy to limit the presence of contaminants having a high affinity for the metal.

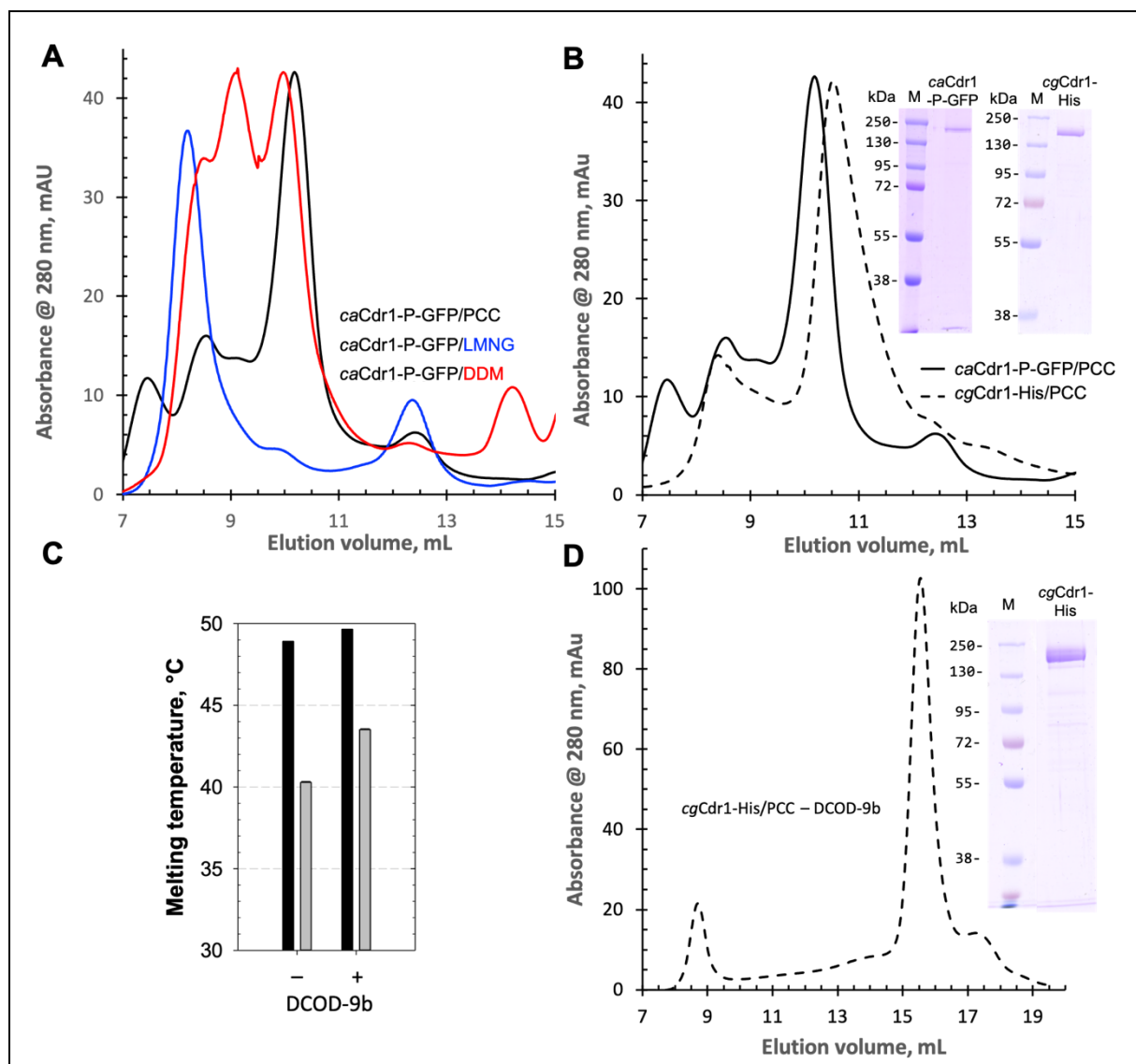


#### **Size-exclusion chromatography of Cdr1 proteins.**

We selected LMNG and PCC as detergents from the extraction tests, compared to the DDM as standard, to evaluate the SEC behavior of *caCdr1*-P-GFP. The protein was first enriched by IMAC as described above using either 70  $\mu$ M PCC, 1 mM DDM or 50  $\mu$ M LMNG. Each pool of the protein in complex with the nanoantiGFP was then  $\sim$ 10x concentrated and then loaded on a Superdex 200 10/300 column (Figure 5). Considering the size of such type of protein-detergent complex that we characterized in detail before [60], we expected its elution between 10 and 12 mL, with aggregates eluting in the void volume at about 8 mL. We observed such pattern when using the PCC, with a main peak eluting at 10.2 mL (black trace in panel A). DDM also produced such peak but two additional peaks almost equivalent in size and eluting at 8.5 and 9.2 mL were also present, thus presumably corresponding to oligomeric aggregate of the protein (red trace in panel A). Unexpectedly, LMNG mostly generated only aggregates eluting at the dead volume, suggesting a poor capacity of the detergent to maintain a monomeric state of the complex (blue trace in panel A). PCC was thus selected and then assayed with *cgCdr1*-His. A similar profile to that of the *caCdr1*-P-GFP-nanoantiGFP complex was obtained, with a main peak eluting slightly later at 10.5 mL due to the difference of molecular mass (dotted trace in panel B). These data confirm that the PCCs prevents, or at least limits, the aggregation tendency of membrane proteins, as also reported previously [61], and seems particularly well adapted to extract and prevent aggregation of yeast ABC pumps, as initially observed with Pdr5 from *S. cerevisiae* [62]. The cyclohexyl tandem forming the alkyl chain of the PCC compared to the aliphatic one of the DDM or the LMNG, and more precisely its higher rigidity as emphasized by its designers [38], seems determining for preserving the membrane region of Cdr1 from exposure to the surrounding water from which originates the aggregation pattern.

Finally, we evaluated the addition of DCOD-9b in the purification process. We previously designed this additive to improve the stability of detergent-extracted membrane proteins by favoring salt-bridge interactions between the 2 carboxylates born by the molecule and the bulk of basic residues enriched at the cytoplasm-membrane interface of the protein. Assayed on BmrA, a prokaryotic ABC pump and A2A, the human adenosine G-protein receptor, both extracted with DDM, the DCOD-9b was found to increase their melting temperature ( $T_m$ ) by 20 and 10  $^{\circ}$ C, respectively [37]. Herein, the Cdr1 proteins

were firstly purified as above and their  $T_m$  was then measured by nano-differential scanning fluorimetry (NanoDSF), which probes the increase of unfolded protein level in respect of the temperature from the intrinsic fluorescence observed at 330 and 350 nm (Figure 5, panel C). The *caCdr1*-P-GFP-nanoantiGFP complex displayed a rather high  $T_m$  of  $\sim 49^\circ\text{C}$ , to our surprise much higher than the  $\sim 40^\circ\text{C}$  observed with *cgCdr1*-His. Addition of DCOD-9b at a 1:20 PCC ratio increased the  $T_m$  of *cgCdr1*-His by  $\sim 4^\circ\text{C}$ , confirming its stabilizing effect. The  $T_m$  of the *caCdr1*-P-GFP-nanoantiGFP complex was modestly increased by only  $\sim 1^\circ\text{C}$ , maybe due to the high  $T_m$  that the protein already displays without additive. In both cases the level of increase in  $T_m$  remained lower than that observed initially with BmrA and A2A, which may plausibly be attributed to the higher rigidity of the PCC compared with that of the DDM, as discussed above. We therefore introduced the additive in the purification process at the extraction step and along the chromatographic steps. This is exemplified with the *cgCdr1*-His protein for which a purification at a preparative scale was then assayed with the additive and which gave a nice SEC profile of a protein eluting quite homogeneously at  $\sim 16\text{ mL}$  and lower amount of aggregates eluting at  $\sim 8\text{ mL}$ , carried out using a Superose 200 10/300 column (Figure 5, panel D).



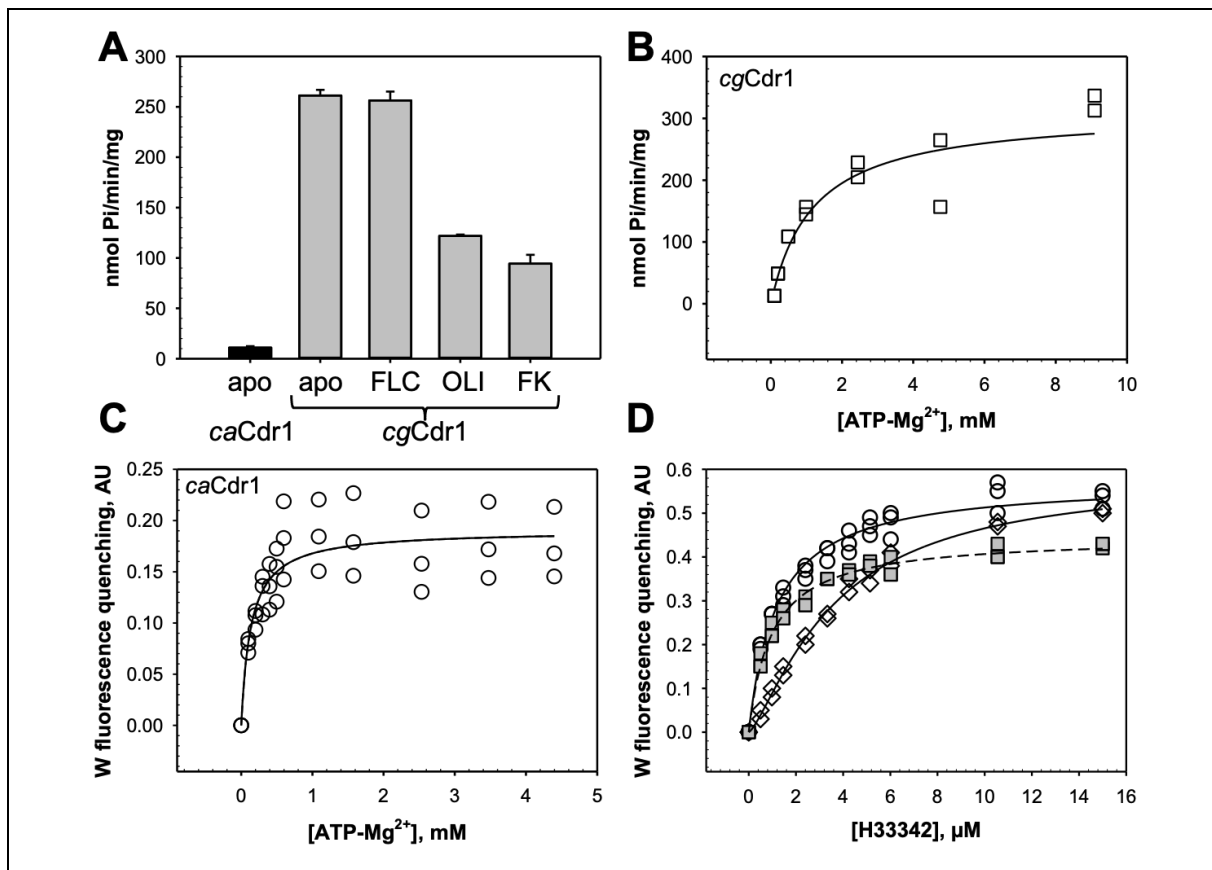
**Figure 5. Size-exclusion chromatography of Cdr1-detergent complexes.** **A.** Superdex 200 10/300 profiles of the *caCdr1*-P-GFP-nanoantiGFP complex in solution with either  $70\ \mu\text{M}$  PCC (black),  $1\ \text{mM}$  DDM (red) or  $50\ \mu\text{M}$  LMNG (blue). **B.** Superdex 200 10/300 profiles of the *caCdr1*-P-GFP-nanoantiGFP complex (solid) and *cgCdr1*-His (dots) in solution with  $70\ \mu\text{M}$  PCC. SDS-PAGE of the head of each main peak. **C.** Melting temperature of the *caCdr1*-P-GFP-nanoantiGFP complex (black) and

of *cgCdr1*-His (grey) from panel B with and without 4  $\mu\text{M}$  DCOD-9b, measured by nano-differential scanning fluorimetry. D. Superose 200 10/300 profile of *cgCdr1*-His in solution with 70  $\mu\text{M}$  PCC and 4  $\mu\text{M}$  DCOD-9b. SDS-PAGE of the head of main peak. In colors for the print

### Biochemical characterization of detergent-purified Cdr1 proteins.

As purified, *caCdr1* did not display any ATPase activity (Figure 6A). Assaying DDM, LMNG, adding or removing DCOD-9b did not change this behavior, nor the addition of lipids, reconstitution of the protein in a detergent-free peptidiscs environment or removing the GFP tag using the PreScission site (data not shown). On the contrary, *cgCdr1* prepared as described displayed an ATPase activity with a  $V_M$  of  $0.31 \pm 0.06 \mu\text{mol}/\text{min}/\text{mg}$ , comparable to that of recently reported for Pdr5 ( $0.2 \mu\text{mol}/\text{min}/\text{mg}$ ) [62], but a 3 times poorer  $K_M$  of  $1.2 \pm 0.7 \text{ mM}$ , which is surprisingly rather close to the cytoplasmic ATP concentration ( $\sim 1.5 \text{ mM}$  [63]) (Figure 6B). Addition of 5  $\mu\text{M}$  fluconazole did neither stimulate or inhibit this activity, hinting at an uncoupling of ATP hydrolysis and substrate transport, and addition of two well-established inhibitors, oligomycin and FK506, inhibited the hydrolysis by 53% and 64% respectively (Figure 6A).

We then evaluated the extent of inactivation of *caCdr1*. We took advantage of its ATPase-inactive state to estimate the binding constant of the ATP-Mg<sup>2+</sup> complex by recording the intrinsic fluorescence of the protein. The fluorescence quenching remained rather low, capping at 15-20%, but from which we could estimate a single  $K_d$  of  $134 \pm 32 \mu\text{M}$  ( $p = 0.0002$ ), thus displaying a rather good affinity for the catalytic and non-catalytic ATP-binding sites which looked indistinguishable (Figure 6C). We then assayed the affinity for transported substrate using the same approach. Adding increasing concentrations of the dye H $\ddot{o}$ chst 33342 led to a larger fluorescence quenching than observed with nucleotides, capping this time at 40-60% which is coherent with the localization of most of the tryptophan residues in the membrane region. Fitting of the data set allowed to estimate a binding-affinity constant of  $1.18 \pm 0.09 \mu\text{M}$  ( $p < 0.0001$ ), close to that of *cgCdr1*,  $0.97 \pm 0.05 \mu\text{M}$  ( $p < 0.0001$ ) assayed in the same conditions, and both rather good considering the detergent environment (Figure 6D). Interestingly, when we carried out the same experiment in the presence of a saturating concentration of ATP-Mg<sup>2+</sup>, the nucleotide decreased the affinity of the dye to  $3.6 \pm 0.2 \mu\text{M}$  ( $p < 0.0001$ ) and triggered an allosteric binding mechanism ( $h = 1.33 \pm 0.24$ ,  $p < 0.0001$ ). This behavior reveals the presence of 2 binding sites for the dye that become distinguishable in a tight ATP-bound state. This observation is in line with the allosteric behavior we previously observed with the human P-glycoprotein transporting the same dye in the presence of the QZSSS inhibitor, which also revealed the presence of 2 H $\ddot{o}$ chst 33342-binding sites [64]. Overall, these data show that although unable to hydrolyze ATP for a reason remaining to discover, *caCdr1* displays functional substrate binding and inter-domain communication.



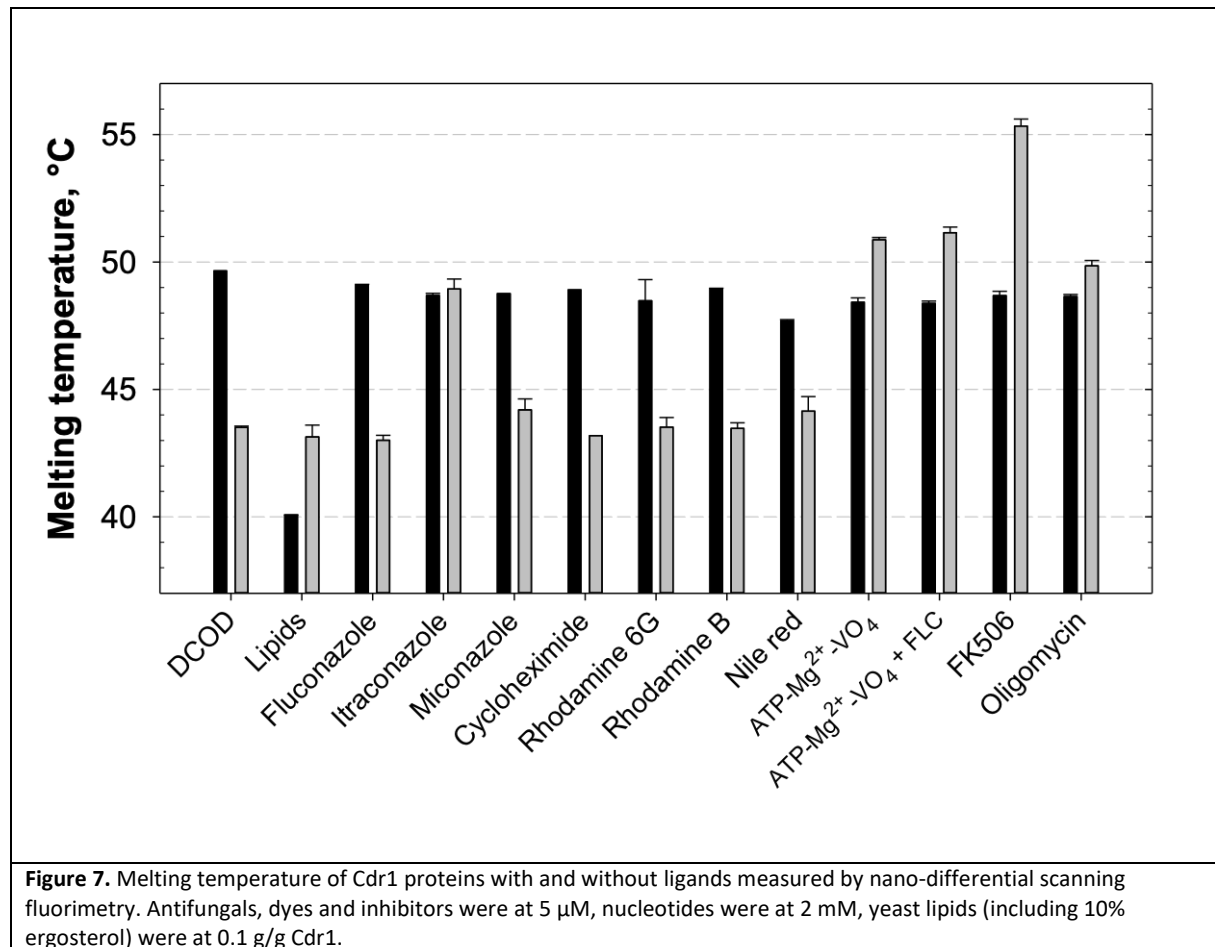
### Interaction of Cdr1 proteins with ligands

We then probed the behavior of Cdr1 proteins with various ligands including lipids,azole and non-azole antifungals, dyes (excluding H $\ddot{a}$ echst 33342 that cannot be used due to its fluorescence parameters), nucleotides and substrate-transport inhibitors by nanoDSF (Figure 7). Unexpectedly, except for the addition of lipids which reduced the  $T_m$  of *caCdr1* by almost 10  $^{\circ}\text{C}$ , none of the other ligands assayed increased or decreased it.

Assayed in the same conditions, *cgCdr1* displayed a rather differential levels of stabilization, depending on the ligands. Lipids had no effect, contrarily to what we observed with *caCdr1*. Transported substrates such as fluconazole, miconazole, cycloheximide and dyes had either no or poor effect on the  $T_m$ . On the contrary, itraconazole triggered an increase of 5  $^{\circ}\text{C}$ . This stabilizing effect may be attributed to the structure of the latter, which is by far the largest one and the most hydrophobic compound of the series assayed here, displaying a Log p of 7.31 vs e.g., 0.5 for fluconazole, which probably favors contacts inside the drug-binding pocket. Addition of ATP-Mg<sup>2+</sup> and VO<sub>4</sub> which, once hydrolyzed generates an ADP-Mg<sup>2+</sup>-Pi-VO<sub>4</sub> complex stably bound to the catalytic nucleotide binding site and switches the protein to its outward-facing conformation, had also a significant stabilizing effect on *cgCdr1* with a  $T_m$  increase of about 7.5  $^{\circ}\text{C}$ . Such an increase was not modulated by the addition of fluconazole. Finally, addition of inhibitors of Cdr1,

FK506 and oligomycin, also induced a large increase of the  $T_m$ , up to 55 °C in case of FK506, again in contrast to *caCdr1* which did not display any change in the presence of those compounds or with nucleotides.

These data highlighted once again the differences between two proteins. It is likely that *caCdr1* is in a very stable conformation, only modulable by lipids which lower its stability to the same level as *cgCdr1*. As for *cgCdr1*, these measures proved that binding of substrates except itraconazole has a small stabilizing effect on the protein, whereas inhibitors are far more efficient. This may be interesting when screening for new inhibitory molecules and may be implemented in a high-throughput screening pipeline.



## Author Contributions

## Funding

This work was supported by the Centre National de la Recherche Scientifique (CNRS), Lyon-1 University, Grenoble-Alpes University, the French Research Agency (CLAMP2 - 18-CE11-0002-01 to P.F., A.B. V.C. and S.M.) and the Auvergne-Rhône-Alpes (ARA) region (R&D BOOSTER 2021 – IMABGEN to P.F., V.C., S.M. and M.D.). A.M.'s Ph. D. was supported by grant n° 16 013104 01 ARC SANTE 2016; J.P.'s Ph. D and Master were supported by the doctoral school EDISS and R&D BOOSTER – IMABGEN grants.

## Acknowledgments

We thank Drs Richard Canon and Edward Lamping for the gift of the strain and Cdr1 expression plasmids.

## Conflicts of Interest/Disclaimer

Authors declare no conflict of interest.

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