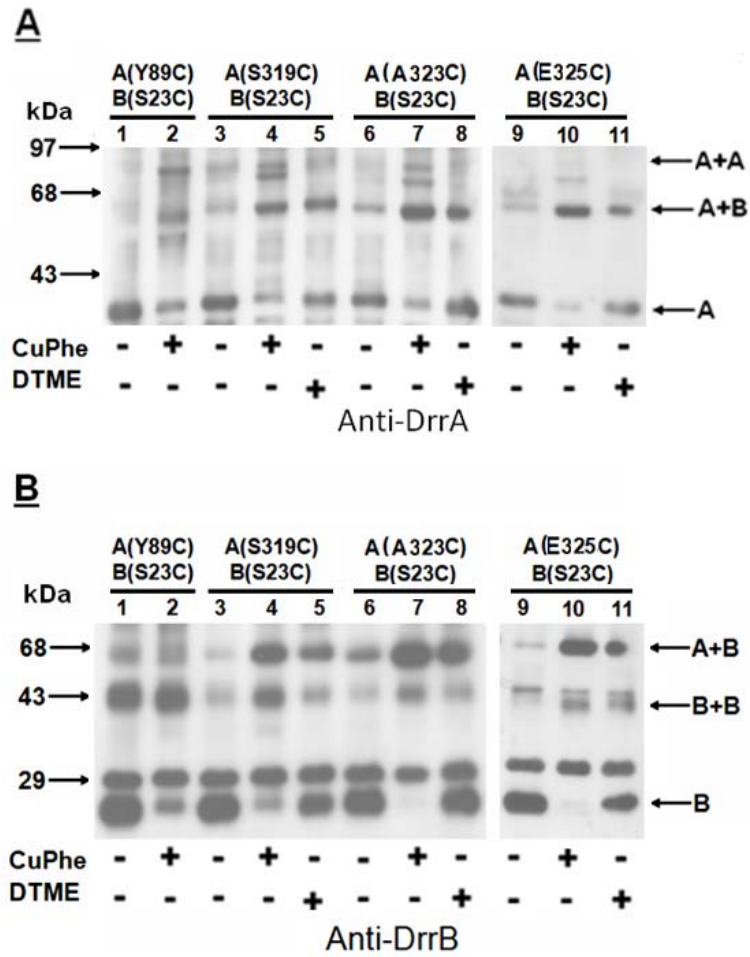


Supplementary Figures



**Fig. S1** Disulfide cross-linking between A323C or E325C in DrrA and S23C in DrrB. Two different cross linkers were used: CuPhe and DTME. *Panel A*, anti-DrrA. *Panel B*, anti-DrrB. *Panels A and B, lanes 1, 2:* A(Y89C)B(S23C). *Lanes 3-5:* A(S319C)B(S23C). *Lanes 6-8:* A(A323C)B(S23C); *Lanes 9-11:* A(E325C)B(S23C). The plus or minus indicates the presence or absence of the cross linker.

**LDEADQLA**

S.p.DrrA	199	<b>LD RADQLAD RI AVIDHGRVIA ECT TGE LKSS</b> LGSNVLRLLRLHDAQSRAEAE RL LSAELG	257
S.a.ABV97910	199	<b>LD RADQMADDL TLIDHGRVVA RCT PAELK</b> TGRAD CVLEVLL TDS SRRAEAGRL LTRAI G	257
S.t.ABP53097	195	<b>LE RADRFAD RI AVI KHCVVLA GCT PD ELK</b> DQVGGHRLVTL SRPAD LSPARSVLAPVAA	253
M.e.CalT5	199	<b>LD RADHLAD EL TLIDHGRVIA GCT PP ELK</b> ASRAAGVLDVRL DPERRADAGAL LAKAVG	257
N.f.BAD59995	200	<b>LD RADQLAD RI AVIDRGRVIA DCT AD ELK</b> ASICGSS LHL TLADRAL LEQARRVIGELLG	258
C.k.BAD75195	206	<b>LQ RAEELAD RI AVIDHGRVVA ECT PD ELK</b> ASICQSS LQVKL QHP GEIETAKRI IERV LH	264
R.o.BAH55073	199	<b>LD RADQLAD RV AVIDHGRVIA ECT TGE LK</b> ASVCS GALHVRVTDIAT RPAAAAL LRDVLE	257
G.o.EE131000	199	<b>LE RADQLAD GI VVIDHGRVIA ECT PQQLK</b> ASVGT GS LHVRL LDP AQRAAA RVLEAAVG	257
A.s.ACC75227	195	<b>LD RADRLAE RMAVIDHGRVIA ECT SRELK</b> ASVCSNA LRL RLADACQRLAAQ QVITRVLG	253
B.c.BAD64015	200	<b>LE RADQLAD RI AVINHCRT IA ECT SS ELK</b> ASVGTNT LHL TLQHASD QERAI QL LAEDQN	258
M.s.ABG63493	209	<b>LE RADQLAA RI AVIDHGRVIA ECT SRELK</b> AAIGCS GF LHVAPADQSQLDEAAAL LEARLG	267
L.i.ABJ72056	195	<b>LD RADQLAD RI AITDHCSVIA QCT PS ELK</b> NMLGCTT FEL SLIKS SQIRQAKEMIEQKFN	253
J.s.EAP97255	196	<b>LD RADQLAD NI VVIDQ CRT IA QCT PL ELK</b> MQSGAAS LVVTVSRPHDEVHQAAL LRCVVG	254
A.e.ABI58055	195	<b>LE RADA LAD LI VVIDQ GRVIA ECT SAQLK</b> SRVCA RT LYVTVSDPA-LAP RTAE RVT AVT	252
P.s.EDS54065	206	<b>LD RADQLAD RI AVIDRGRVVA ECT VD ELK</b> QS VGTSS LQL RVLDQSD IRIARHTVHVHLQ	264
O.g.EAR51498	196	<b>LE RADQLAD RI AVIDHGRVIA ECT SRELK</b> AQ TCS CVLQVVPAAAAG---EAAALAV-LG	250
L.b.ABJ65324	197	<b>LD RADA LAD RI AVIDHGRVIA ECT PT ALK</b> QQICGAT LTL SLAKA TQGPLAQL LAQALH	255
S.t.EEP40806	201	<b>LD RADQLAE RL AITDRGRVIA ECT VD ELK</b> ASVCS CVLHI RL GDPARRPEAEAVLARS LD	259
S.s.EDX84899	202	<b>LE RADA LAD QI VVIDHGRVIA ECT VD ELK</b> DRVCGCK CEL RLANLADVPKVKLL LFD IGD	260
C.m.ACL16820	221	<b>LD RADQLAD NI VVIDKGRVVA ND PD CLK</b> RVCS SS LHL TVKADNAAMAAQ I IERILD	279
F.s.ABW09842	198	<b>LD RADQLAD RI CVLDCGRVVA ECT AA ELK</b> RRVPCGHVSVQF TDAAGLAAAAAHPEATT	256
M.g.ABP44416	197	<b>LE RADQLAD NI VVIDRGRVIA ECT SRELK</b> PLHLKQQACRAS LVVTVADAAD LESARGLLARTGA	255
M.v.ABML5569	216	<b>LE RADQLAD NI VVIDRGRVIA ECT SRELK</b> QQACRAS LVVTVGDAAD LEGARTLLCGTGA	274
T.b.EDY41301	195	<b>MD RA BQLAD RI AITDHGRVIA ECT SE ELK</b> KLUGNDV IYTI RIANGK--EEVKCLNAD FIR	251
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**LDEVFL**

S.p.DrrA	258	VT IHFD-SDPTALSARIDD PQGMPALAE LSRTH-LEVRSFSLGQSSLD EVFLALTGHPA	315
S.a.ABV97910	258	AGVAAE-SDPSRLLVKIPDAD QAA RALGELARAG-ISVEDFTLGGP TLD TVFLALTGHST	315
S.t.ABP53097	254	GEAVSD EAEGR LAVTVA AADCVLV DGI RRLD AAG-I EVIDASLR RP TLDDVFLALTGHIA	312
M.e.CalT5	258	AAADLD-SDPARLSVRVTD PDRAALALGELARAG-IHVD DFTLGGP SLD TVFLALTGHST	315
N.f.BAD59995	259	VEAQIT-PEAGRLTAPLREAGLTTDL LIRLRHD-IAID EITSVKP SLD EVFLTITGHPA	316
C.k.BAD75195	265	VRPTVS-LETGNITAPMAD PDHVTDL LVALRQRC-IHLT ELSVQKP TLD EVFLTITGHCV	322
R.o.BAH55073	258	VPVTEE-ADPAALTARIDD PARVSRALPALDDAG-IAVSTFALGQP SLD EVFLSLTGRPT	315
G.o.EE131000	258	-TVVLE-PDPAALS AVCPDARLGA LGMCE LGRAG-LGIAEFSLGQP SLD EVFLGLTGHA	314
A.s.ACC75227	254	DCVMPG-SEPAEVAARLEKAAQACAVLTALS ECG-I EIAEVTVCMP SLD EVFLALTGRPA	311
B.c.BAD64015	259	TMVHPG-KDPTSLTAQTCNPD VAAA AVGR LASAN-IAVRTFSLGQP SLD EVFLTLTGEMV	316
M.s.ABG63493	268	NVVQRS-AEQAQLSVVACS AREANEALAA LISAG-I ELSDF SMCSP SLD EVFFALTGAPA	325
L.i.ABJ72056	254	IEVIVL-PEFATLS IKVTD TKIHT QILLLLE TEH-IAINEF ETRKP TLD EVFL ELTGK--	309
J.s.EAP97255	255	EVHVD--ADARKLTAPGCDVPALT RIAAQ LD EAG-IEVD DLGMQ RP SLDDVFLALTGHKA	311
A.e.ABI58055	253	GAQAQ--VDGEQISAPVQD PGLLP AVLRALD QAG-IEVAELGLRGA SLDDVFLALTGHSG	309
P.s.EDS54065	265	VQATLT-SEAAKITAPMANAD LITDL LIALRREK-I TLS EVSVQRP TLD EVFLTITGHV	322
O.g.EAR51498	251	EGAQYS-AECCRIS LPVTGAAE ARARLAALEAAG-IEVAEFSLGSP SLD EVFFALTGKPL	308
L.b.ABJ65324	256	AQPTLH-CAQ--LTAHLENPNQITDL LVQLQAAD-ISLANI AVQ EP SLDDVFFALT-TGK	310
S.t.EEP40806	260	LT TYSE-CDPARLSARVSEGE EVA RALAVLS SHG-IPVTTF SFGQP SLD EVFLALTGHPT	317
S.s.EDX84899	261	-MVTGEMENGLAIAA PRGANTLT EIVLRLE EAG-IAIADI SLR RP SLDDVFFKLTGHTT	318
C.m.ACL16820	280	ARKVHLA-KAT-ELIAPIDNIDKLT DVLT LTKDAG-IALS GVNILQP TLD EVFMALTGEGS	336
F.s.ABW09842	257	-----DARALT LRVPDCG STACLRRVLD GLDDDDVA CLTVR SPDLDDVFLALTGHAT	308
M.g.ABP44416	256	EVYVD--AGARRLTATADGLDDMVKRVAGWRDSC-ITVDDI GLS RP SLDDVFLMLTGHRT	312
M.v.ABML5569	275	EVFVD--AGARRLTASADGLDDMI RVAEWRDSC-IAVDDI GLS RP SLDDVFLTLTGHRT	331
T.b.EDY41301	252	GCKVLP---DGRVRLEVANAAEAL PKLFE LAQQMDIKIL EVTYHRP TLNDVFLYLTGREL	308
		* : : : : * : * : * :	

**Fig. S2** ClustalW alignment of the C terminal sequence (residues 199-315) of DrrA with the C-terminal sequences of bacterial homologs identified by BLAST search. Sequences in LDEADQLA and LDEVFL motifs are highlighted.

**LDEADQLA**

```

S.p.DrrA      199  LDEADQLADRIAVIDHGRVIAEGTTGELFSSSLGS-NVLRRLRHDA----- 242
ABCA1_human  1081 MDEADVLDRIAIIISHGKLCVCGSSLFLEWQLGTGYLTLVKRDVESLSSCRNSSSTV 1139
ABCA2_human  1182 MDEADLLGDRIAIIISHGKLRCCGSPFLFRGTYGDGYRLTLVKRPAEPGGP----- 1231
ABCA3_human  723  MDEADLLGDRIAIMARGELQCCGSSLFLEKRYGAGYHMTLVKKEPH----- 767
ABCA8_human  635  MDEADILADRRVFLSQKLRKACGSSLFLEKRWGIGYHLSLQLNEI----- 679
Ced-7        737  MDEARLGDWVFMISHGKLVASCTNQYLRQKFCYGLLTVVLDHN----- 781
          :***: *.*  .: :*.: . *:  **  *  : :

S.p.DrrA      243  -----QSPRAEAERLLSAELGVTIHRDSDPTAL 269
ABCA1_human  1140 SYLKKEDSVSQQSSDAGLGSDEHSDTLTIDVSAISNLIRKHWSEARLVEDIGHELTYVLP 1199
ABCA2_human  1232 ---QEPGLASSPPGRAPLSSCSLQ-----VSQFIRKHWASCLLVSDTSTELSYILP 1280
ABCA3_human  768  -----CNPED-----ISQLVHHHPNATLSSAGAEISFILP 799
ABCA8_human  680  -----CVEEN-----ITSLVKQHIIDAKLSAKSEKGLITYILP 711
Ced-7        782  -----GDKRK----MAVILTDVCTHYVREARERGEMHCQQIETILP 817
          : . . : . .

          LDEVFL

S.p.DrrA      270  SARIDDP-----QGMRALAELESRTHLEVRSFSLGQSSLDDEVFLAL 310
ABCA1_human  1200 YEAAKEGA-----FVELFHEIDRLSDLGISSYGISFTTLEEIFLKV 1241
ABCA2_human  1281 SEAAKKA-----FELFQHLERSLDAHLSSFCGLMDTTLEEVFLKV 1322
ABCA3_human  800  RESTHR-----FECLFAKLEKQKELGCIASFCASITTEEVFLRV 839
ABCA8_human  712  LERTNK-----FPELYKDLDSYPLGCIENYGVSMTTLNEVFLKL 750
Ced-7        818  EARFKKFPVPLFQALEAIQDRNYRSMVFDNMPNLTLSQLATLEMPFGLSLMTLEQVFTTI 877
          . . . . . * : . . . . . :* :

S.p.DrrA      311  TGHPADDPSTEEAAEERKVA----- 330
ABCA1_human  1242 AEE-----SCVDAE----- 1250
ABCA2_human  1323 SEEDQSLENSADVKESPKD----- 1342
ABCA3_human  840  GKLVD-----SMDIQAIQLPALQYQ 860
ABCA8_human  751  ECK-----STINESD----- 760
Ced-7        878  GDKVDKALASRQNSRISHNSRNA-- 900

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**Fig. S3** ClustalW alignment of the last 132 amino acids of DrrA with the C-terminal sequences of NBD1 of eukaryotic homologs identified by TC-Blast search. Their homology in LDEADQLA and LDEVFL motifs are highlighted.

**LDEADQLA**

```

S.p.DrrA      199  LDEADQLADRLAVIDHGRVIAEGTTGELKSSLGSSN-VLRLRLHD---AQSARAERLLS  253
ABCA2_human  2247  MEECEALCTRLAIMVNGRLRCLGSIQHLKRNRFDCGYMITVTRK---SSQSVKDVVRF  2301
ABCA1_human  2104  MEECEALCTRMAIMVNGRFRCLGSSVQHLKRNRFDCGYTIVVRIAG--SNPD LKPVQDF  2160
ABCA3_human  1574  MEECEALCTRLAIMVQQQFRCLGSPQHLKSKFGSGYS LRAKVQSEGGQEALEEFKAF  1630
ABCA8_human  1438  MARAEAVCDRVAIMVSGRLRCTGSIQHLKSKFGKDY LEMKVKN---LAQVPLHAEIL  1493
Ced-7        1563  MDECEALCSRIVAVLNRCGLIATGSSQELKSLYGNNYTM TLSLYE---PNQEDMVVQLV  1618
: *.: .: *:*.: * . . *: .** .*.. :

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**LDEVFL**

```

S.p.DrrA      254  AELCVTIHDDSDPT-----ALSARIDDP RQGMRALAELSRTHLEVRSFSLGQSSLDEVFL  308
ABCA2_human  2302  RNFPEAMLKE RHHT---KVQYQLKSEHIS-LAQVF SKMEQVSGVLGIEDYSVSTTLDNV  2359
ABCA1_human  2161  LAFPGSVLKERHPN---MLQYQLPSLSS-LARIF SILSQSKKRLHIEDYSVSTTLDQV  2218
ABCA3_human  1631  LTFPGSVLED EHQG---MVHYHLPGEDLS-WAKVFGILEKAKKYGDDYSVSTISLEQV  1691
ABCA8_human  1494  RLFPQAAARQEFYSS---LMVYKLPVEDVQPLAQAF EKLEKVKQSFDEEYSLSQSTLE  1552
Ced-7        1619  TRLPNSVLKTS TNKTLNMLKQIQPKKEDCWSAKF EMVQALARDLGVKDFILAQSSLE  1679
: : . : . : . : . : . : . : . : . : . : . : . : . : . : . : . : . :

```

```

S.p.DrrA      309  ALTGHPADDRSTEE-----AAEEKVA-----  330
ABCA2_human  2360  NFAKQSDNLEQQET EPPSALQSPLCCLLSLLRPRSAPTELRLALVAD EPEDLDT EDEG  2418
ABCA1_human  2219  NFAKQSDDDHLKD-----LSLHKNTV-VDVAVLTS-----  2251
ABCA3_human  1692  SFAHLQPTAEEGR-----  1706
ABCA8_human  1553  ELSKEQELGDFEED-----FDP SVKWKLLPQ-----  1580
Ced-7        1680  RLAGLDEDQLDTHS-----TVEISHSTHV-----  1704
::

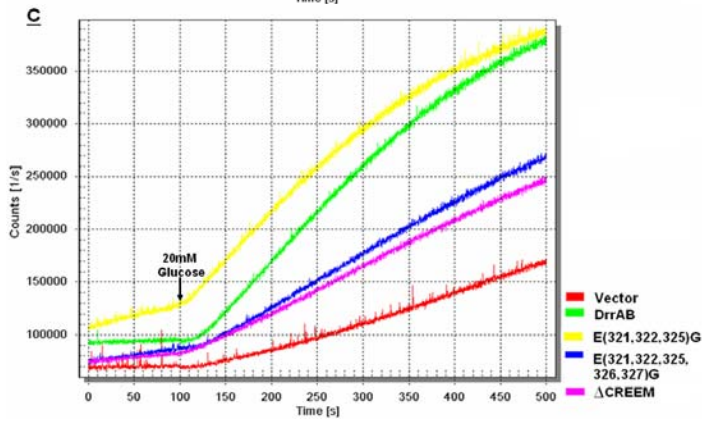
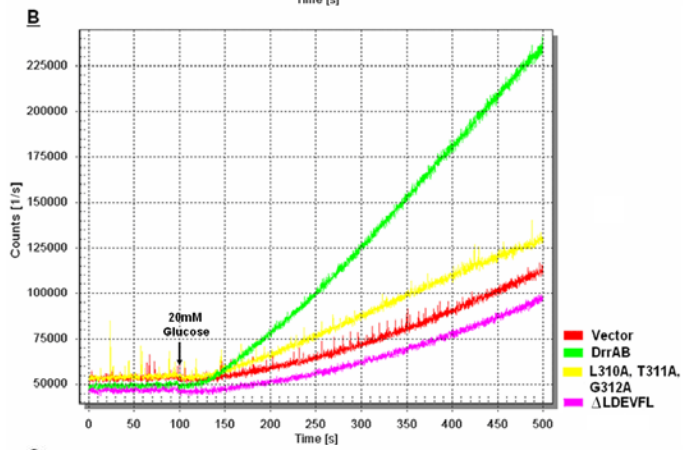
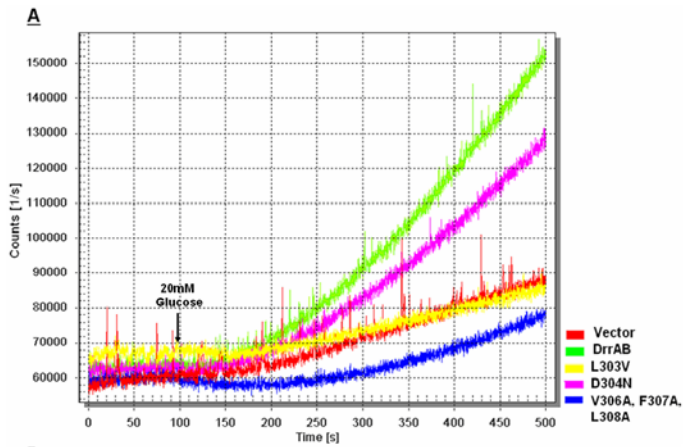
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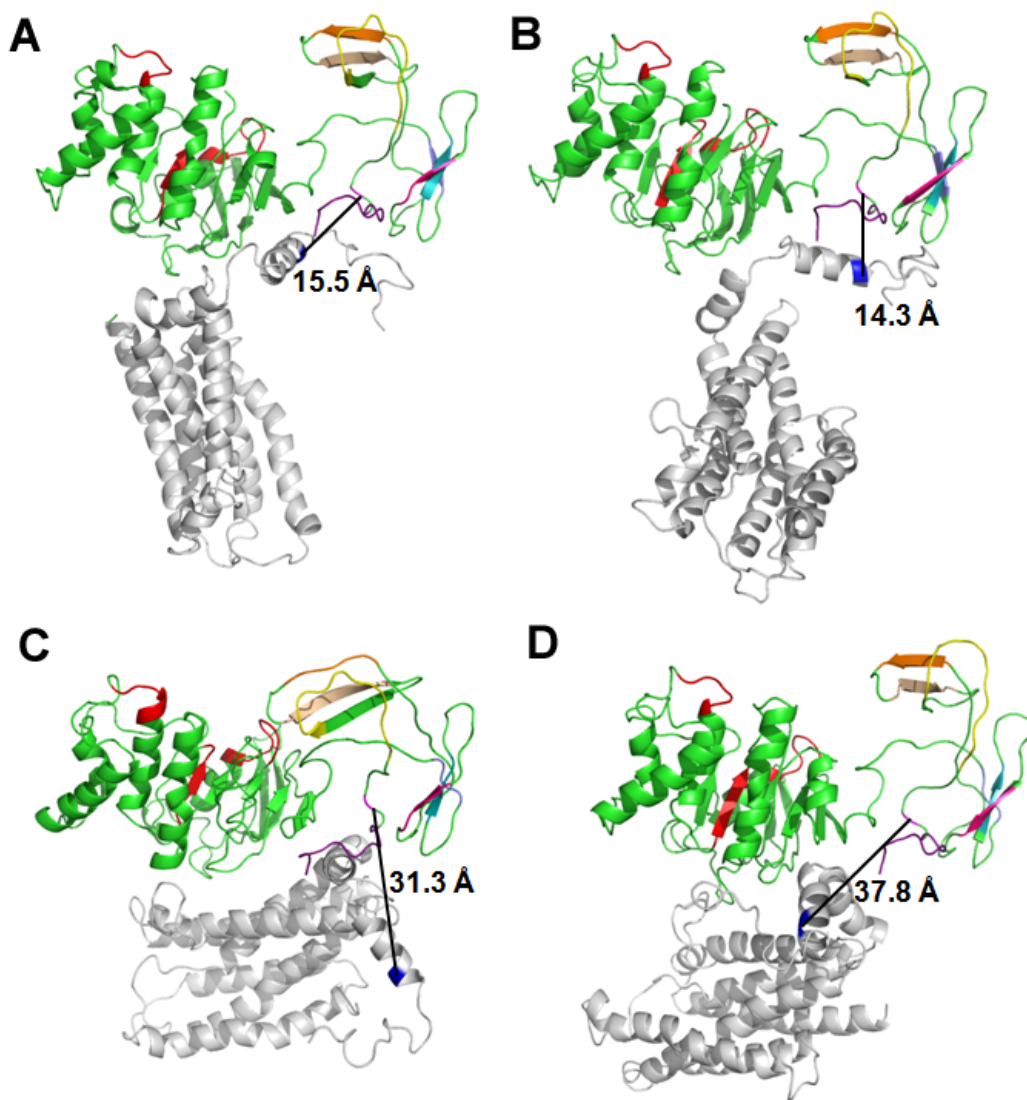
S.p.DrrA      -----
ABCA2_human  2419  LISFEERQAQLSFNTDTLC  2434
ABCA1_human  2252  --FLQDEKVKESYV-----  2261
ABCA3_human  -----
ABCA8_human  1581  -----EEP-----  1584
Ced-7        -----

```

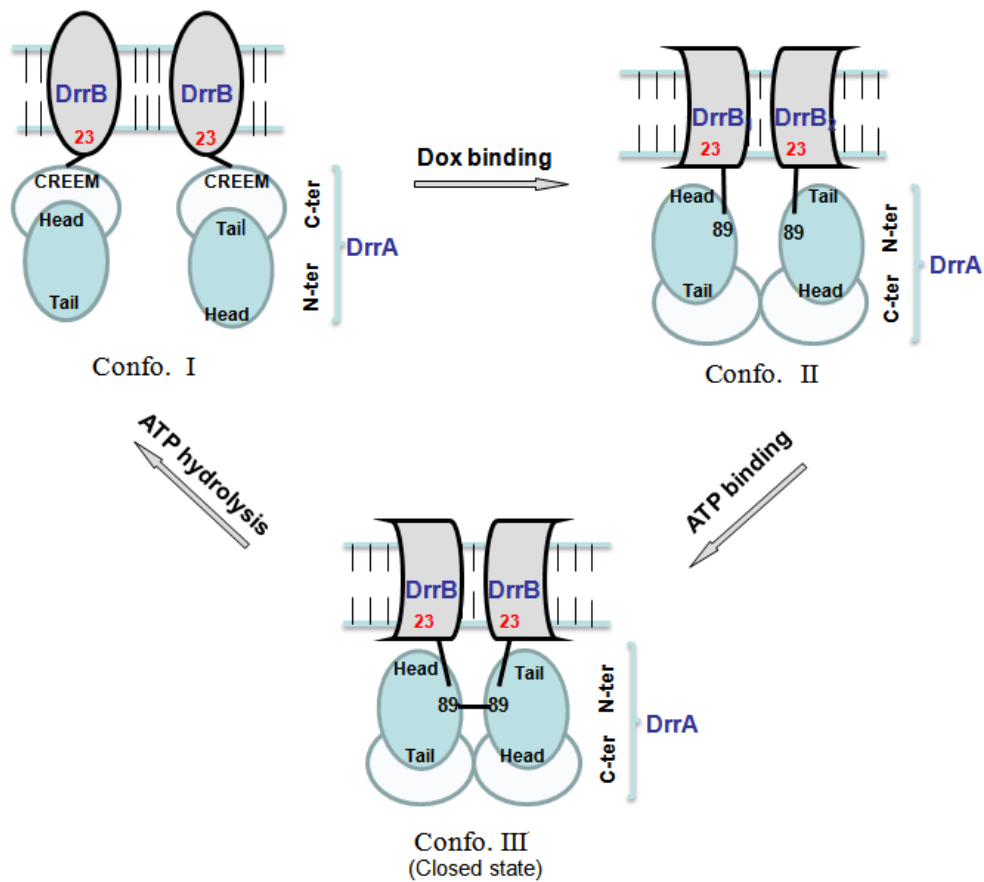
**Fig. S4** ClustalW alignment of the last 132 amino acids of DrrA with the C-terminal sequences of NBDII of eukaryotic homologs identified by TC-BLAST search. Their homology in LDEADQLA and LDEVFL motifs are highlighted.



**Fig. S5** Effect of LDEVFL or CREEM mutations on doxorubicin efflux. *E.coli* LE392 $\Delta$ uncIC cells containing the indicated plasmids were grown in TEA medium and induced with IPTG at OD=0.6, as described under Methods. Washed cells were de-energized with 5mM DNP and loaded with 10  $\mu$ M doxorubicin for 11 hours. Loaded cells were washed twice, and doxorubicin fluorescence was measured for 100 seconds. Doxorubicin efflux by the cell suspension was then initiated by providing 20 mM glucose, shown with an arrow. The fluorescence was monitored for additional 400 seconds. The linear region of each curve was used for calculation of the slope of the curve. *Panel A*, effect of L303V, D304N or V306A/F307A/L308A mutation on doxorubicin efflux by DrrAB. The slopes obtained were: vector, 82; wild type DrrAB, 331; DrrA(L303V)DrrB, 63; DrrA(D304N)DrrB, 229; DrrA(V306A/F307A/L308A)DrrB, 84. *Panel B*, effect of L310A/T311A/G312A mutation or  $\Delta$ LDEVFL in DrrA on doxorubicin efflux by DrrAB. The slopes were: vector, 172; wild type DrrAB, 558; DrrA(L310A/T311A/G312A)DrrB, 220; DrrA( $\Delta$ LDEVFL)DrrB, 143. *Panel C*, effect of E(321, 322, 325)G, E(321, 322, 325, 326, 327)G mutation or  $\Delta$ CREEM on doxorubicin efflux by DrrAB. The slopes were: vector, 233; wild type DrrAB, 930; DrrA(E(321, 322, 325)G)DrrB, 832; DrrA(E(321, 322, 325, 326, 327)G)DrrB, 507; DrrA( $\Delta$ CREEM)DrrB, 429.



**Fig. S6** Docking analysis of the predicted structures of DrrA and DrrB. DrrA protein was modeled using AMMP modeling software and the known structure of MalK as a template. DrrB protein was modeled using the Phyre modeling software (27), as described under Methods. The coordinates obtained for the predicted structures of DrrA and DrrB were then used for docking analysis of DrrA and DrrB by Rosetta Docking server (28). *Panel A*, wild type DrrA and DrrB; *Panel B*, D304N and DrrB; *Panel C*, L303V and DrrB; *Panel D*, X306-308A and DrrB. In all panels, DrrA is shown in green and DrrB is shown in grey. The Walker A, Signature, and Walker B motifs of DrrA are shown in red. The LDEVFL and CREEM motifs in DrrA are shown in yellow and purple, respectively. The  $\beta$ -strands in the C-terminal domain of DrrA are shown in different colors following the same color scheme as seen in Fig. 9. The distances between S319 (shown in magenta) in DrrA and S23 (shown in blue) in DrrB are shown in angstroms.



**Fig. S7** A model showing various interactions between DrrA-DrrB and DrrA-DrrA during different stages of the catalytic cycle. Both DrrA and DrrB proteins are shown as dimers. DrrA protein contains two domains: an N-terminal nucleotide binding domain (abbreviated as N-ter, filled with blue) in the front, and a C-terminal domain (showing the CREEM motif) (abbreviated as C-ter) in the front. In Conformation I, the two nucleotide binding domains in the N terminus of the DrrA protein are in the open state, while the extreme C terminus of DrrA forms an interface with the N-terminal tail of DrrB. It is proposed that this interaction between the extreme C terminus of DrrA and the N-terminal cytoplasmic tail of DrrB plays a role in assembly and biogenesis of the DrrAB complex. Doxorubicin binding to DrrB produces a conformational change: the extreme C terminus of DrrA disengages from the N-terminal tail of DrrB, which is now involved in communicating conformational changes to the Q-loop region (represented by residue 89) in the N-terminal domain of DrrA (Confo. II). Simultaneously, the extreme C terminus of DrrA undergoes homodimerization. This is followed by the ‘closed’ state, produced by the head-to-tail dimerization of the NBDs (Confo. III). Hydrolysis of ATP returns the complex to the resting state.