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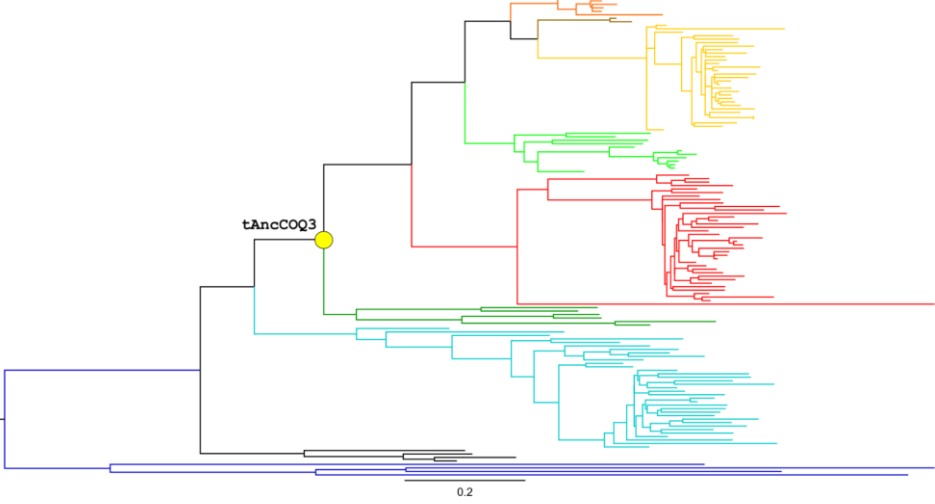
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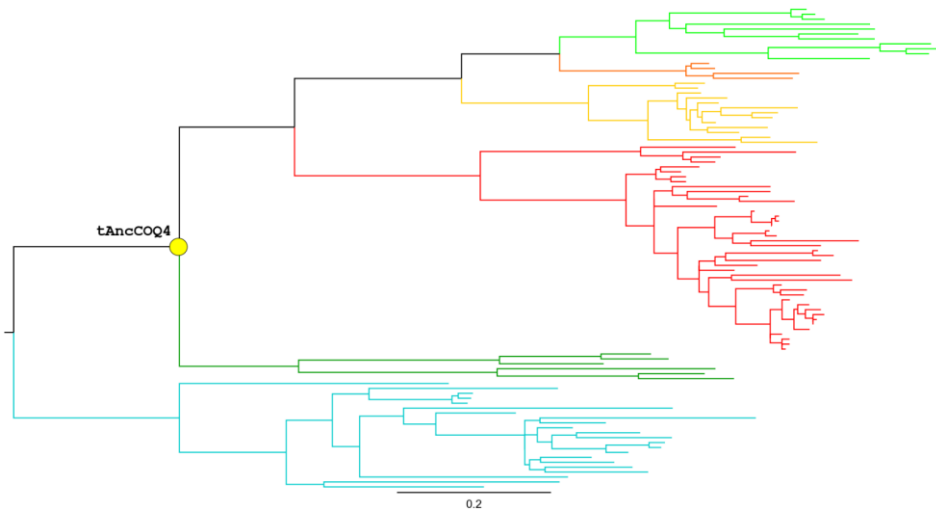
COQ6



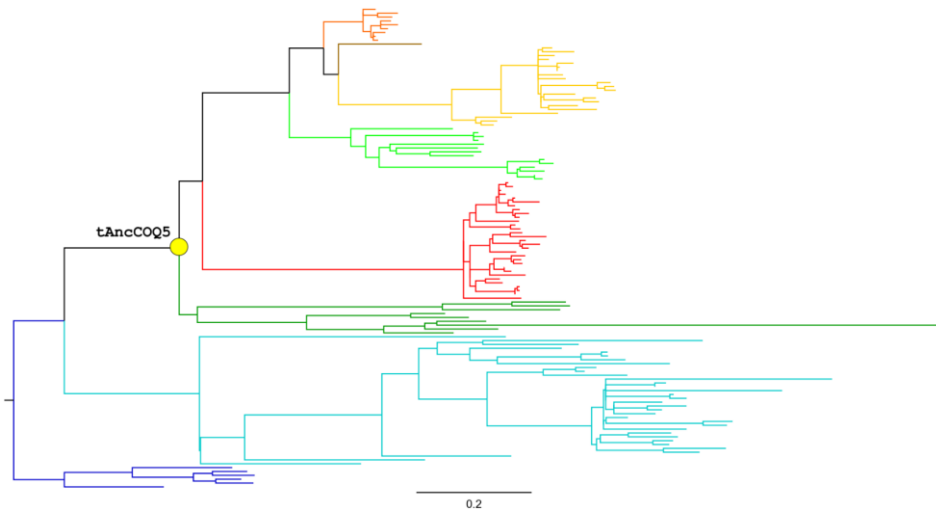
COQ3



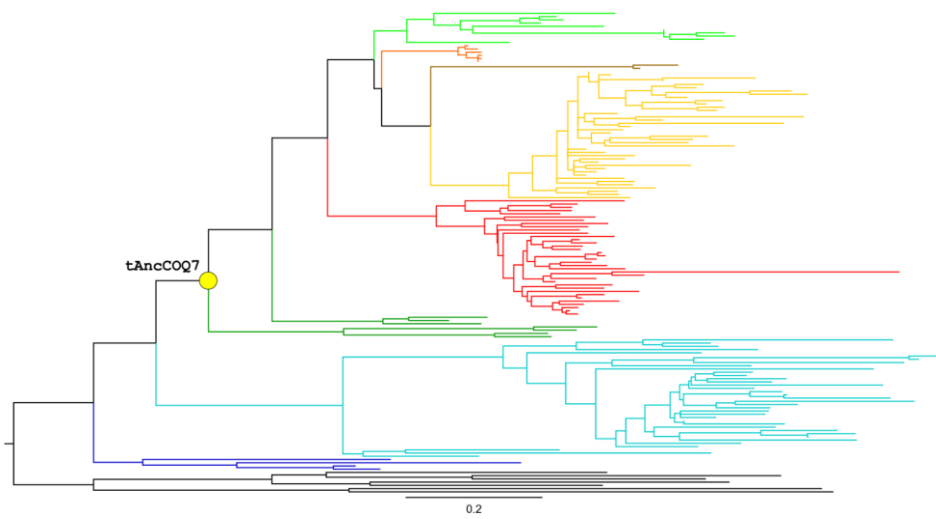
# COQ4



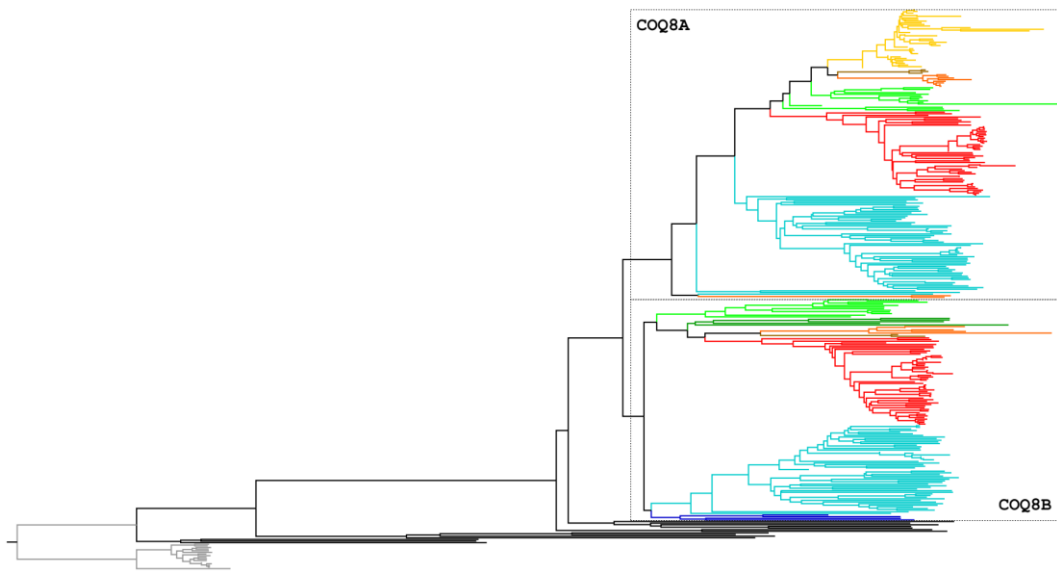
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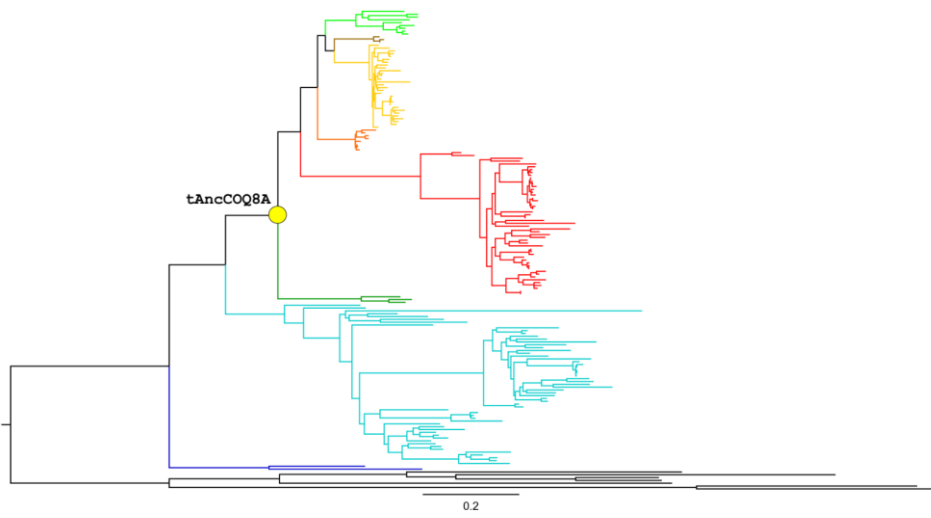
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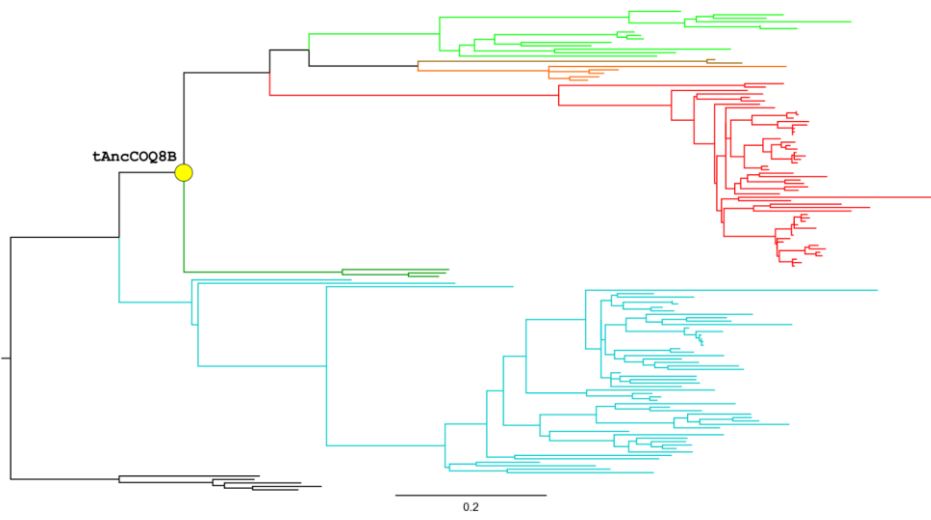
# COQ8



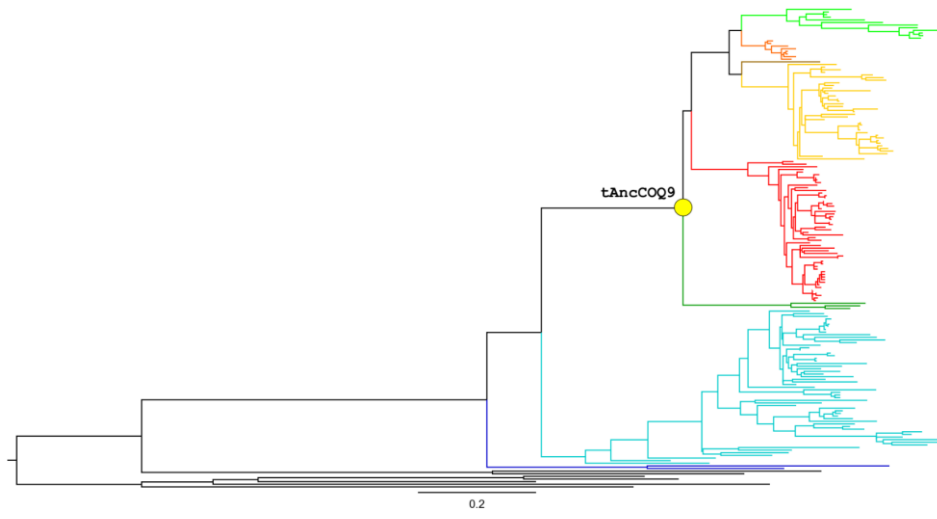
## COQ8A



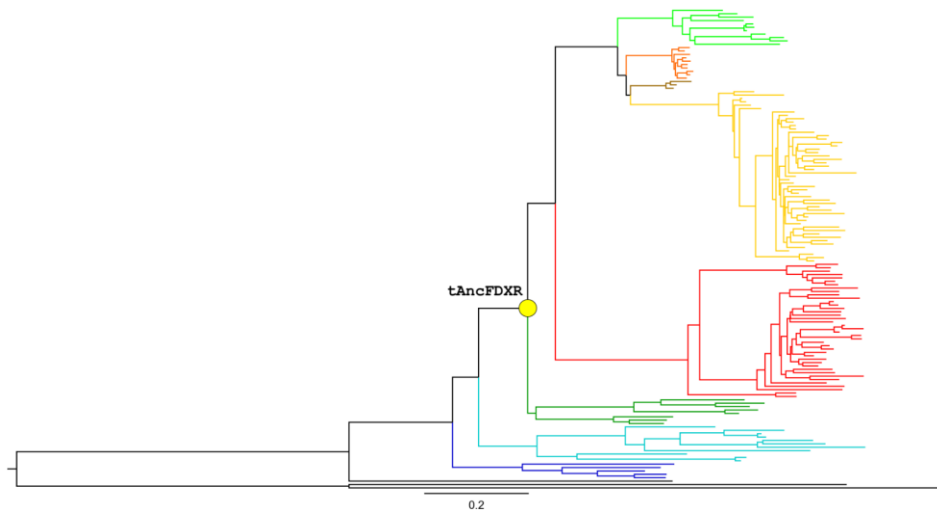
## COQ8B



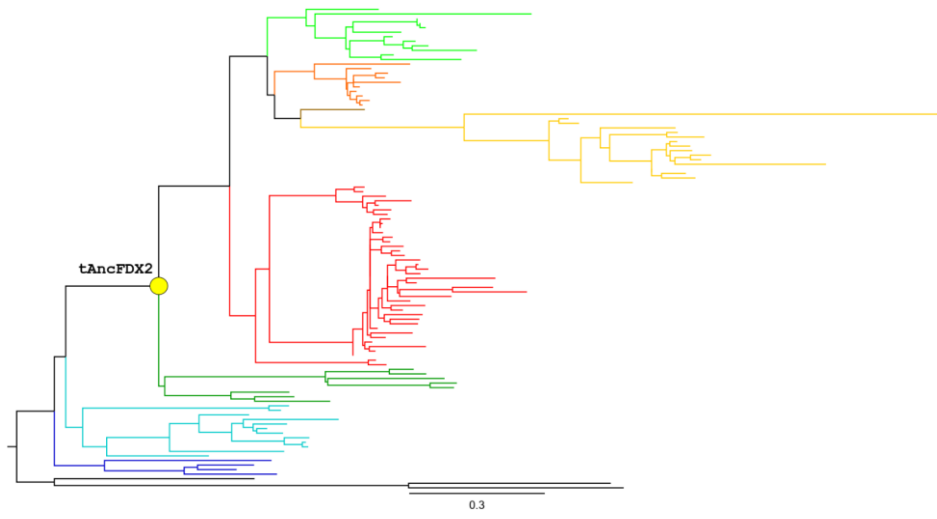
# COQ9



# FDXR



# FDX2



**Supplementary Figure 1. Maximum likelihood phylogenies from Chordata phylum obtained for each COQ, FDXR and FDX2.** Branches are colored according to the taxonomic groups: Mammalia (red), Aves (yellow), Testudines (orange), Crocodylia (brown), Lepidosauria (light green), Amphibia (dark green), Actinopterygii (cyan) and Chondrichthyes (blue). The root (black) is formed by sequences from hemichordata, echinodermata and cephalochordata groups. The resurrected tetrapod ancestors are marked with yellow circles. The scale bar indicates the substitutions *per site*. For COQ6 a multiple sequence alignment (MSA) including 138 sequences and 480 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.81$ ; for COQ3 a MSA including 181 sequences and 359 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.92$ ; for COQ4 a MSA including 98 sequences and 287 sites was employed, DUMMY2 was the best-fit substitution model and  $\alpha= 48$ ; for COQ5 a MSA including 125 sequences and 336 sites was employed, LG was the best-fit substitution model and  $\alpha= 0.53$ ; for COQ7 a MSA including 149 sequences and 217 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.91$ ; for COQ9 a MSA including 183 sequences and 323 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.93$ . For COQ8, first a NJ tree (MSA with 346 sequences) is presented where the paralog duplication (COQ8A and COQ8B) is evidenced at the Chordata emergence (note that COQ8B paralog has been lost in Aves group). Sequences from the related family ACDK1 (aarF domain containing kinase 1) were included in the analysis for rooting purposes (shown in grey branches). For COQ8A a MSA including 170 sequences and 643 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.84$ ; for COQ8B a MSA including 140 sequences and 527 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.87$ . For FDXR a multiple sequence alignment including 142 sequences and 500 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.83$ . For FDX2 a multiple sequence alignment including 174 sequences and 180 sites was employed, JTT was the best-fit substitution model and  $\alpha= 0.92$ .

# COQ6

	1	10	20	30	40	50
tAncCOQ6	MAA	...RLGLSGWGRRR	RLR	CGARLSLA	...RGLARCCRRR	SSGGPAVYDVVIS
XP_029454311_Rhinatrema	MAA	LLAGPRGL	...	CGFGL	...GASCLARR	SASSKPDLYDVVIS
NP_872282_Homo	MAA	...RGLVSR	CGAVRAAP	...HSGPLVSWRRW	SGASTDTVYDVVIS	
NWQ76339_Columbina	TSA	...	MAVVC	RGLLAPLAGRL	RAGPSAPLR	SVAAAAALYDVVIS

	60	70	80	90	100	110
tAncCOQ6	GGCMVGT	AMACALCYDP	PHQDKKII	LLEAGHKKV	FDTPE	SYSNRVSI
XP_029454311_Rhinatrema	GGCMVGT	AMACALCYDP	PHSNKRVI	LLEAGHKKV	IDQVAD	IYSNRVSI
NP_872282_Homo	GGCI	VGAAMACALCYD	IHHDKKII	LLEAGPKKVL	EKLS	ETYSNRVSI
NWQ76339_Columbina	GGCMVGS	AMAALVIGHNI	IHHDKKII	LLEAGPKKE	YDRMP	ESYSNRVSI

	120	130	140	150	160	170
tAncCOQ6	GAWDHI	CNMRFK	PFRRMQVWD	ACSDAMI	TFDKEN	LED
XP_029454311_Rhinatrema	GAWDHI	CNMRVKK	PFRRMQVWD	GCSDDAMI	TFDKEN	LED
NP_872282_Homo	GAWDHI	CNMRYRA	PFRRMQVWD	ACSEALIM	FDKDN	LED
NWQ76339_Columbina	GAWDHV	CSLRK	PFRRMQVWD	ACSEALIM	VEKDD	LED

	180	190	200	210	220	230
tAncCOQ6	RVQVLY	RSRAVGY	TWPLF	YHTAEAN	PWVQTE	ELADGR
XP_029454311_Rhinatrema	RVQVLY	KCAVGY	TWPLF	YHTAEXN	PWVQTE	ELADGR
NP_872282_Homo	RVQVLY	RSKAI	RYTWPC	PFPMADSS	PWVHT	TLGDG
NWQ76339_Columbina	RVQVLY	GSRAVGY	TWPLF	THNCDTS	PWVQTE	ELADGR

	240	250	260	270	280	290
tAncCOQ6	NVQWNY	DQSAVVA	TLLHSEAT	DNNVAWQ	RFLP	GP
XP_029454311_Rhinatrema	NVQWNY	DQSAVVA	TLLHSEAT	DNNVAWQ	RFLP	GP
NP_872282_Homo	NVQWNY	DQSAVVA	TLLHSEAT	DNNVAWQ	RFLP	GP
NWQ76339_Columbina	NVQWNY	DQSAVVA	TLLHSEAT	DNNVAWQ	RFLP	GP

	300	310	320	330	340	350
tAncCOQ6	LSMDEE	SFVDAT	NSAFWSN	ENHSEFF	IDTAG	S
XP_029454311_Rhinatrema	LSMDDER	FVDAT	NSAFWSN	ETHSEFF	IHSAG	S
NP_872282_Homo	VSMDEE	KFVDAT	NSAFWSN	ADHDID	IDTAG	AM
NWQ76339_Columbina	LAMDEE	SFVDAT	NSAFWSN	VNHID	IDTAG	AV

	360	370	380	390	400	410
tAncCOQ6	SRAMFPLC	MGHAEY	VRRR	RVALIG	DAHRVH	PLAGQGVN
XP_029454311_Rhinatrema	SRAMFPLC	MGHAEY	IRRR	RVALIG	DAHRVH	PLAGQGVN
NP_872282_Homo	SRVLFPLC	MGHAEY	VRRR	RVALIG	DAHRVH	PLAGQGVN
NWQ76339_Columbina	SRAMFPLC	MGHAEY	VHRR	RVALIG	DAHRVH	PLAGQGVN

	420	430	440	450	460	470
tAncCOQ6	DLGSTR	HLLE	EYETER	QRHNP	LMAAID	L
XP_029454311_Rhinatrema	DLGSIR	HLLE	EYETER	QRHNP	LMSAID	L
NP_872282_Homo	DLGSVSHL	TGYETER	QRHNP	TALLAAT	D	L
NWQ76339_Columbina	DLGSLKHL	LKFETER	QRHNP	SLIAAID	V	L

tAncCOQ6	IMAFASK
XP_029454311_Rhinatrema	IMNFASK
NP_872282_Homo	IMAFASK
NWQ76339_Columbina	MSLQWGP

# COQ3

```

1          10          20          30          40          50
tAncCOQ3      MWGGGR.GSRAGRLLV1ALRGRSRGR.GAGCRR2LSLA3AAGNHYGWTLQMAPRFKSS....
NWQ76719_Columbina
XP_029451244_Rhinatrema
NP_059117_Homo

60          70          80          90          100         110
tAncCOQ3      .NRTM4WLK5SNST6FA7SL8TK9MS10SRSAV11KRM12YST13SQT14TV15PK16EM17KK18FQA19LA20AK21WWD22EQ23GE24Y
NWQ76719_Columbina
XP_029451244_Rhinatrema
NP_059117_Homo

120         130         140         150         160         170
tAncCOQ3      AAL25HS26MND27IR28VP29F30IRD31TL32LN33MSGD34H35QL36GS37PL38SG39MKI40LDV41GCGGG42LL43SE44PL45GR46LGAS47VT48GI
NWQ76719_Columbina
XP_029451244_Rhinatrema
NP_059117_Homo

180         190         200         210         220         230
tAncCOQ3      DP49LED50NI51RTA52EL53HKS54FDP55VLD56KR57IQ58YK59AC60SLEE61I62VE63EAT64ET65FD66AV67VASE68VVE69HV70AD71VET72F
NWQ76719_Columbina
XP_029451244_Rhinatrema
NP_059117_Homo

240         250         260         270         280         290
tAncCOQ3      IK73CC74Y75QVL76KP77GG78SL79FIT80TI81NK82TQ83LSY84AL85GIV86VA87ER88IM89GI90V91PA92GH93HD94WE95KF96TS97PE98EL99ER100LL
NWQ76719_Columbina
XP_029451244_Rhinatrema
NP_059117_Homo

300         310         320         330         340         350
tAncCOQ3      ES101NG102F103SV104RT105VN106GML107YN108PL109SG110SW111SW112ENT113ST114INY115AL116HAV117KS118K119VQE120Q121SD122ST123EP124PS125EQ126EQ127EQ128H129Q
NWQ76719_Columbina
XP_029451244_Rhinatrema
NP_059117_Homo

360
tAncCOQ3      AETS130TSTIV.....
NWQ76719_Columbina
XP_029451244_Rhinatrema
NP_059117_Homo
ANACT131NP132AV133HE134LK135K

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# COQ4

```

1           10           20           30
tAncCOQ4      MA...TLRRARAGLLP LRRPVLGSP...GLTGRALCMR...QAPAA...
Q9Y3A0_Homo   MA...TLRR...PVLRRLCGLP...GL...QRPAA...
NXE46430_Casuarius HP..GAGGSRSPG...PR...ALSAGAVRPRHAGRRAAA...
XP_029467832_Rhinatrema MAMVTA LRRLEVRGCSL LRRSLGRVPRQSVIAAATGKGQRWY...QTNKASYYDMHEDAF

           40           50           60           70           80           90
tAncCOQ4      ...DSPLRRAEFEGYGLYPGHPTSPYOKALLAAGSACMALLYNRYRHMVAVLGETTGH
Q9Y3A0_Homo   ...EMPLRARSDGAGPLYSHHPTSPYOKGLLAAGSARMALLYNRYRHMVAVLGETTGH
NXE46430_Casuarius ...EDGAAEEQEGCCQLYPGHPTSPYOKALLAAGSARMALLYNRYRHMVAVLGETTGC
XP_029467832_Rhinatrema HMEEDNTEAEAGKYHLYPNHPTNAFOKALLTVGSAMMSLYDPRYRHMVAVLGETTGP

           100          110          120          130          140          150
tAncCOQ4      LALQNLDRDRMRNDPEGVQILQERPRIITSTLDLAKLRSLPDDGSGREYVRFLLDNRVSPD
Q9Y3A0_Homo   RTLKVLDRDQMRDDEPGAQILQERPRISTSTLDLQKLSLPEGSDGREYVRFLLDNRVSPD
NXE46430_Casuarius LVLPLNRDKMKHDPEGYRILRERPRIITSTLDVNRRLRGLPDGTGREYVRFLELDNKNVSPD
XP_029467832_Rhinatrema LVLQNLDRDRMRNDPEGNQILQERPRIITSTLEMHRRLRPLDPTGREYVRFLLDNKNVSPD

           160          170          180          190          200          210
tAncCOQ4      TRAPVKFVDEELAYVIQRYREVHDLHTLLGMPTNMLGEEVVVKWFEEQTGLPMLILGA
Q9Y3A0_Homo   TRAPT RFVDEELAYVIQRYREVHDMHTLLGMPTNMLGEEVVVKWFEEQTGLPMLILGA
NXE46430_Casuarius TRMPAKFVDEELAYVIQRYREVHDLHTLLGMPTNMLGEEVVVKWFEEQTGLPMLVILGA
XP_029467832_Rhinatrema TRMPVKFVDNEELAYVIQRYREVHDLHTLLGMPTNMLGEEVVVKWFEEQTGLPMLILGA

           220          230          240          250          260          270
tAncCOQ4      AFGPIRLSARKLQVLVTELPWAVONGRNARCVLNIIYERRWEOSLESREELIGTTPPP
Q9Y3A0_Homo   FFGPIRLGAQSLQVLVSELPWAVONGRRAPCVLNIIYERRWEOSLRAREELIGTTPAPP
NXE46430_Casuarius AFGPVRLENARKLRVLTTELPWAIQSGRNANCIINIIYERRWEOTVESREELIGTFSP
XP_029467832_Rhinatrema TFGPLRLNARKRLQVLMMLLPWVIOCGRNSQFVMNVYERRWEOTMESREELIGTTPPP

tAncCOQ4      RVTGLA
Q9Y3A0_Homo   HVQGLA
NXE46430_Casuarius .....
XP_029467832_Rhinatrema IK...V

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# COQ5

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1           10           20           30           40
tAncCOQ5      ..MAASMRCA...C...SRAICGCR...S GARVCCRAHS TEAAEKETHFGFQT
NWQ77319_Columbina .....RGL...AAGPEMHFGFQT
Q5HYK3_Homo     ..MAAPGSCALWSYCGRGW SRAMRGCQLLGLRSSWPGLLSARLLS QEKRAAETHFGFET
XP_029474956_Rhinatrema MAVLAAGRC.LGGLC...SRLIGS...PKLWFLRQHTDQYKKAASFGFQS

           50           60           70           80           90           100
tAncCOQ5      VSEEEKCFKVVYQVFENVAKKYDVMNDAMS LGIHRVWKDMLHQMNPYPGTQLLDVAGGTG
NWQ77319_Columbina VTEAERREKIIYQVFESVAKKYDVMNDSMT LGIHRVWKDMLVHKMNPSPCTQLLDVAGGTG
Q5HYK3_Homo     VSEEEKGGKVVYQVFESVAKKYDVMNDSMT LGIHRVWKDMLLWKMHPLPCTQLLDVAGGTG
XP_029474956_Rhinatrema VTEAERNEKVHELFDRVSLKYDVMNDML LGIHRVWKDMLLRMNPYPGTQLLDVAGGTG

           110          120          130          140          150          160
tAncCOQ5      DIAFRFINYVRSQRERQVRQELRSHQNLVSWQETS KSYQEEEQDSLGGSQAVICDINKEMML
NWQ77319_Columbina DIAFRFINYVRSVRERQLQRKLRHHQNLVSWQETAESYQEDKSKSLGGSQVVCIDINKEMML
Q5HYK3_Homo     DIAFRFLNYVRSQHRKQKRLRAQNLVSWQETAKEYQNEE.DSLGGSRVVVCDINKEMML
XP_029474956_Rhinatrema DIAFLFLDYIHSQREVQLRRDLRSYQNLVSWLEVS KSYQKTRQDLGGSHVVICDINKEMML

           170          180          190          200          210          220
tAncCOQ5      KVGKQKAQQLGYSEGLSVVVGNAABELPFD DDKFDVYITAFGIRNVTHIDQALQEAHRVLRK
NWQ77319_Columbina KVGKQKALAQGYRAGLAVVLDGAEELPFD DDKFDVYITAFGIRNVTRIDQALQEAHRVLRK
Q5HYK3_Homo     KVGKQKALAQGYRAGLAVVLDGAEELPFD DDKFDIYITAFGIRNVTHIDQALQEAHRVLRK
XP_029474956_Rhinatrema AIGKQKAQH LGYTEGLSVVVGNAABELPFTADKFDVYSVAFGIRNVTHIDQALQEAHRVLRK

           230          240          250          260          270          280
tAncCOQ5      PGGFRFLCLEFSQVNNPLSRLYDLYSFQVIPVLGEVIAGD WKSQYLVESIRRFPSOEEF
NWQ77319_Columbina PGGFRFLCLEFSHVSNNPLSRLYDLYSFQVIPVLGEVIAGD WKSQYLVESIRRFPSOEEF
Q5HYK3_Homo     PGGFRFLCLEFSQVNNPLSRLYDLYSFQVIPVLGEVIAGD WKSQYLVESIRRFPSOEEF
XP_029474956_Rhinatrema PGGFRFLCLEFSHASNNPLSRLYDLYSFQVIPVLGEVIAGD WKSQYLVESIRRFPSOEEF

           290          300          310
tAncCOQ5      KAMIEDAGFFKVFYHNLTSGLVAIHSGFKL
NWQ77319_Columbina KAMIEDAGFLKVFYQNLNLGIVAIHSGFKL
Q5HYK3_Homo     KDMIEDAGFHKVFYESTLTSGLVAIHSGFKL
XP_029474956_Rhinatrema KAMIEDAGFMKVFYHNLTSGLVVAIHSGFKL

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# COQ7

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1          10          20          30          40
tAncCOQ7      M.....ERAAAVRRGWRHCRRLR LGAGP RRPCC AQA RRTSVRF CSTG
Q99807_Homo  M.....SCAGAAAPRLW.....RLRPGARRSLSA YGRRTSVRF RRS SG
XP_029432753_Rhinatrema M.....ARALALRRASCACSRRLCLIGA.PFGCCAPADRTSVRFSISG
PKK24439_Columba MPRRGRANRRDRAGWRREAGABAARRDDALAVRRGGGGVGP.....LAPGVSRRLCGLT

50          60          70          80          90          100
tAncCOQ7      MTLDNVDKAVIDRIIRVDHAGEYGANRIYAGQMAVLGRFVS VGIQMWDEQKPHLKKFN
Q99807_Homo  MTLDNISRAAVDIRIIRVDHAGEYGANRIYAGQMAVLGRFVS VGIQMWDEQKPHLKKFN
XP_029432753_Rhinatrema MTLDNVDKAVVHPIIRVDHAGEYGANRIYAGQMAVLGRFVS VGIQMWDEQKPHLKKFN
PKK24439_Columba RVFGDIKPVIEHPIIRVDHAGEYGANRIYAGQMAVLGRFVS VGIQMWDEQKPHLKKFN

110         120         130         140         150         160
tAncCOQ7      ELMVAHVRVPTL LMPFNNVAGFVLGAGTALLGKEGAMACTVAVEESIS SHYNNQIRTLME
Q99807_Homo  ELMVTFVRVPTV LMPLWNLGFGALGAGTALLGKEGAMACTVAVEESIS AHYNNQIRTLME
XP_029432753_Rhinatrema ELMIAHVRVPTL LMPFNNVVGFGALGAGTALLGKEGAMACTVAVEESIS SHYNNQIRTLME
PKK24439_Columba ELMVAHVRVPTV LMPFNNVAGFVLGAGSALLGKEGAMACTVAVEESIS SHYNSQIRTLME

170         180         190         200         210         220
tAncCOQ7      EDPEKYRELLQITKFRDDELEHDDTGLEHDAEIPAPYSLKKNVIOIGCKAAIYLSERL
Q99807_Homo  EDPEKYRELLQITKFRDDELEHDDTGLEHDAEIPAPYAVLKSITQAGCRVAIYLSERL
XP_029432753_Rhinatrema EDPEKYRELLQITKFRDDELEHDDTGLEHDAEIPAPYSLKKNVIOIGCKRAAVLSQRIT
PKK24439_Columba EDPEKYRELLQITKFRDDELEHDDTGLEHDAKCAPAYSVLKTAITQIGCKAAITLSERL

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# COQ9

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1          10          20          30          40          50          60
tAncCOQ9      MMAAAVAGLIRRAGWRLLQLRCRVVVRQQLSPVQRAFHASAVLRRVSDDEKQPPFPSSSO
O75208_Homo  MAAAASGALGRAGWRLLQLRCLPVARQALVPRAFHASAVGLRSSDDEKQPPFPSSSO
NWQ80522_Columbina_picui KMAAAAGSIRRAGWRLLA..SLSVLRGQLSVPORALQASAVLRRVSDDEKQVPLASSSO
XP_029464793_Rhinatrema_bivittatum .MGAVV..ILSRVGRLLQRCPEVLRQQLIPKRRPFCRSSVLRASDENKRR..PLSSVK

70          80          90          100         110
tAncCOQ9      QHSRSOPT.EEPDPSORSHPSTDOGGEESEDYESEEQLOHRLITAALEFVPAHGWTAE
O75208_Homo  QHSETQGA.EKPPDPSGSHSPRYTDOGGEESEDYESEEQLOHRLITAALEFVPAHGWTAE
NWQ80522_Columbina_picui QHFDSPPTDQPPDQEPQSPRPSSTGGQSESEDYESEEQLOHRLITAALEFVPAHGWTAE
XP_029464793_Rhinatrema_bivittatum QHPEEQPDDEQPASSWHPRPSYTDOSGNSESEDYESEEQLOHRLITAALEFVPAHGWTAE

120         130         140         150         160         170
tAncCOQ9      AIAEAKSLGLSAAAGMFGNDGSDLTHFVSCNLSLSELEEEHKLVLQIGAEKRRKTD
O75208_Homo  AIAEAKSLGLSAAASMFGRDGSGLTHFVSCNTRLFVLEEEHKLVLQIGAEKRRKTD
NWQ80522_Columbina_picui AIAEAKTLGLSAAAGMFRNDGSDLTHFVSCNTRLFLELEEEHKLVLQIGAEKRRPLD
XP_029464793_Rhinatrema_bivittatum AIAEAKSLNLSAAMGMFSSDGSGLTHFVSCNTRLFLELEEEHKLVLQIGAEKRRKTD

180         190         200         210         220         230
tAncCOQ9      QPLRDAVEIRLRMLIPYIEKWPQALSLLIPHNIPASLNLITSMVDDMWHYAGDQSTFIN
O75208_Homo  QPLRDAVEIRLRMLIPYIEKWPQALSLLIPHNIPASLNLITSMVDDMWHYAGDQSTFIN
NWQ80522_Columbina_picui QPLRDAVEIRLRMLIPYIEKWPQALSLLIPHNIPASLNLITSMVDDMWHYAGDQSTFIN
XP_029464793_Rhinatrema_bivittatum QPLRDAVEIRLRMLIPYIEKWPQALSLLIPHNIPASLNLITSMVDDMWHYAGDQSTFIN

240         250         260         270         280         290
tAncCOQ9      WYTRRAVLAGIYNTTELVMQDSSPD EDTWRFLNRRINDAMNMGHTAKQVKS TGEALVQ
O75208_Homo  WYTRRAVLAAIYNTTELVMQDSSPD EDTWRFLNRRVNDAMNMGHTAKQVKS TGEALVQ
NWQ80522_Columbina_picui WYTRRAVLTVGYNTTELVMQDSSPD EDTWRFLNRRVTDAMNMGHTANKVQS TGEALVQ
XP_029464793_Rhinatrema_bivittatum WYTRRAVLVLAGIYNTTELVMQDSSPD EDTWRFLNRRLDNAMMGHAAKQVKS TGEALVQ

300         310
tAncCOQ9      GLMGAIVTLKKNLTGLNQR
O75208_Homo  GLMGAIVTLKKNLTGLNQR
NWQ80522_Columbina_picui GLMGAIVTVSNA...ETRR
XP_029464793_Rhinatrema_bivittatum GLMGAIVTLKKNMTGLNQR

```

# COQ8A

	1                    10                    20                    30                    40                    50
tAncCOQ8A	...MAGDAIMLMFGLAKLSKAVLETQAGQLR...LGGEAVAIARTWQATAEEGFSAAMGK
NWX02076_Caloenas	.SVMAGDAIMVVRGLTKLSKAVLETQAGQLRQVLMGGDAVTIAKTLQATAEEQFSSAALGK
XP_029448900_Rhinatrema	...MASDATMLLRGLKLSRAVLETQSGQLRIGLAGKAAGLTRKWOVTAEEQGFSAAMER
Q8NI60_Homo	MAA...ILGDTIMVAKGLVKTQAAVET...LHLQHLGIGGELIMAAARALOSTAVEQIGMFLGK
	60                    70                    80                    90                    100
tAncCOQ8A	MQELGKQENLSDIGEDFG...SEYDFSGPESSANKDFSSPSGQPHH...HSGAEG
NWX02076_Caloenas	MQELGKQENLSDLSDFDG...KDYDFSAAREPSDASMDFASTAPGKPHH...HSSSEEG
XP_029448900_Rhinatrema	MQELGKQENVSD...PFG...TEYDFSEPVLESIGKNPPPPDSWPEH...SHG
Q8NI60_Homo	VGGQDKHEEYFA...ENFGPEGEFFHFSVPHAAAGASTDFSSASAPDQSAPPSLGHASHSG
	110                    120                    130                    140                    150                    160
tAncCOQ8A	PAYSATNGPFR...NTGDSRRADSPVSAKNGKLFGGFRD...PAGNPF...FAAAGQTRAFHQDH
NWX02076_Caloenas	PAHSYTTNGPFRSVGETGDSMGQKPFPPKVDARLFGGFRDFGNPF...FAATFGONRAFHQDH
XP_029448900_Rhinatrema	...STDGHVESSSKEMGSSGRADTPT...PEGNRERVVVERKDSRDLF...AAAGQTRAFHQDH
Q8NI60_Homo	PAPAYVASGPF...EAGFPGAS...LGRANGLFANFRD...FSAMGFORRFHQDQ
	170                    180                    190                    200                    210                    220
tAncCOQ8A	SSVGGTLAEDIEKARAKANPEENKPHKOMLSEARARERKVPVTRIGRLANFGGLAVGLGEG
NWX02076_Caloenas	SSVGGTLAEDIEKARAKATGSEOKPYKOMLSEARARERKVPVTRIGRLANFGGLAVGLGEG
XP_029448900_Rhinatrema	SSVSRLLAEDIEKARAKADTENKPYKOTLSEARARERKVPVTRIGRLANFGGLAVGLGEG
Q8NI60_Homo	SPVGGTLAEDIEKARAKARPEENKQHKOTLSEARARERKVPVTRIGRLANFGGLAVGLGEG
	230                    240                    250                    260                    270                    280
tAncCOQ8A	ALAEVAKKSLRPEERNCKKAVLDSPPFLSEANAERIVRTLCKVRGAALKLGMLSIQDDA
NWX02076_Caloenas	ALAEVAKKSLRPEERNCKKAVLDSPPFLSEANAERIVRTLCKVRGAALKLGMLSIQDDA
XP_029448900_Rhinatrema	ALAEVAKKSLRSEHTGKAVLDSPPFLSEANAERIVRTLCKVRGAALKLGMLSIQDDA
Q8NI60_Homo	ALAEVAKKSLRSEDPGKAVLGSPPFLSEANAERIVRTLCKVRGAALKLGMLSIQDDA
	290                    300                    310                    320                    330                    340
tAncCOQ8A	FINPQLQKIFRVRQSADFMPKQMMKTLNNDLGNWRKLEEFEEERFFAAASIGQVHLA
NWX02076_Caloenas	FINPHLQKIFRVRQSADFMPKQMMKTLNNDLGNWRKLEEFEEERFFAAASIGQVHLA
XP_029448900_Rhinatrema	FINPQLQKIFRVRQSADFMPKQMMKTLNNDLGNWRKLEEFEEERFFAAASIGQVHLA
Q8NI60_Homo	FINPHLQKIFRVRQSADFMPKQMMKTLNNDLGNWRKLEEFEEERFFAAASIGQVHLA
	350                    360                    370                    380                    390                    400
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NWX02076_Caloenas	RFKNGREVAMKIQYPGVAQSINSDVNNLMTVLSMSNALLEPGLFPEHLIVLSRELALECD
XP_029448900_Rhinatrema	RFKDGRVAMKIQYPGVAQSINSDVNNLMTVLSMSNALLEPGLFPEHLIVLSRELALECD
Q8NI60_Homo	RFKGGRVAMKIQYPGVAQSINSDVNNLMAVLSMSNALLEPGLFPEHLIVLSRELALECD
	410                    420                    430                    440                    450                    460
tAncCOQ8A	YKREAAACAKKFKELLLKDHPPFFVYPAVVDLCSQHVLTTELVSQFPDQAEGLSQEIRNEI
NWX02076_Caloenas	YBREAAACAKKFKELLLKDHPPFFVYPRVVDLCSQHVLTTELVSQFPDQGVGLSQEIRNEI
XP_029448900_Rhinatrema	YKREAAACAKKFKELLLKDHPPFFVYPAVVDLCSQHVLTTELVSQFPDQAEGLSQEIRNEI
Q8NI60_Homo	YOREAAACAKKFRDLLKSHPPFFVYPEIVDELCSQHVLTTELVSQFPDQAEGLSQEIRNEI
	470                    480                    490                    500                    510                    520
tAncCOQ8A	CHNILVLCLELFEFRFMQTDPNWSNFFYDPOHKVALLDFGATRGFDEDFDVIYEVIK
NWX02076_Caloenas	CHNILVLCLELFEFRFMQTDPNWSNFFYDPOHKVALLDFGATRGFDEKFTDVIYEVIK
XP_029448900_Rhinatrema	CHNILVLCLELFEFRFMQTDPNWSNFFYDPOHKVALLDFGATRGFDEEFTDVIYEVIK
Q8NI60_Homo	CYNILVLCLELFEFRFMQTDPNWSNFFYDPOHKVALLDFGATREYDRSFTDVIYQITR
	530                    540                    550                    560                    570                    580
tAncCOQ8A	AAADQDRERVLKKSIEMKFLTGYESKAMENAHLDVAVLILGEAFASDEPPDFGQSTTEKI
NWX02076_Caloenas	AAADMDRERVLKKSIEMKFLTGYEVKEMEDAHLDVAVLILGEAFASDEPPDFGQSTTEKI
XP_029448900_Rhinatrema	AAAEKDRERVLKKSIEMKFLTGYESKTMENAHLDVAVLILGEAFASDEPPDFGQSTTEKI
Q8NI60_Homo	AAADRDRERTVRAKSIEMKFLTGYEVKVMEDAHLDVAVLILGEAFASDEPPDFGQSTTEKI
	590                    600                    610                    620                    630                    640
tAncCOQ8A	HGLIPVMLKRRLLVPPPEETYSLHRKMGGSFLLCSKLKAKIPCKNMFQEAYSKYNSRRRAKK
NWX02076_Caloenas	HGLIPVMLKRRLLVPPPEETYSLHRKMGGSFLLCSKLKAKIPCKNMFQEAYSKYNSRRRAKK
XP_029448900_Rhinatrema	HGLIPVMLKRRLLVPPPEETYSLHRKMGGSFLLCSKLKAKISCSNMFQEAYSKYNSRRRAKK
Q8NI60_Homo	HNLPVMLRHRLLVPPPEETYSLHRKMGGSFLLCSKLKARFPCAMFQEAYSKYNSRRRAKK
tAncCOQ8A	QEQ
NWX02076_Caloenas	PED
XP_029448900_Rhinatrema	RRK
Q8NI60_Homo	...



# FDXR

tAncFDXR  
 XP\_029456456\_Rhinatrema  
 XP\_013222637\_Columba  
 NP\_077728\_Homo

1 10 20 30 40 50  
 MGAPRGAVCWLVG.....RSLARSLPRAGSPVRRLLSTASFTPTQTCIVGSGPAGFYT  
 MGPCR...WTNSVSSRCVLRSLARLQTAGALGLRKRLLSTAPSSPLVCIIVGSGPAGFYT  
 MEPVR.....EAVRRWLSAAPLPRLCVIVGSGPAGFYT  
 M.ASRCWRWGS.....WPTRRLPPAGSTPSFCHHESTQEKTPQTCIVGSGPAGFYT

60 70 80 90 100 110  
 AQHLLKHHKQAQVDIYEKIPVPPGLVRFVGVADHPEVKNVINFTOTASSDRCSFYGNVT  
 AQHLLKHHKQVQVDIYEKIPVPPGLVRFVGVADHPEVKNVINSETOTASSNRCAFYGNIT  
 AQHLLKHGGAHVDIYEKIPVPPGLVRFVGVADHPEVKNVINSETOTASSERCAYYGNVT  
 AQHLLK.HPQAHVDIYEKIPVPPGLVRFVGVADHPEVKNVINFTOTASSGRCAFVGNVE

120 130 140 150 160 170  
 VGRDVTVEELOYAYHAVVLSYGAEDNRRLGIPGENLFCVYSARAFVGYWNGLPENRDLNLP  
 VGRDISVEELOYAYHAVVLSYGAEDNRQLGIPGENLFCVYSARAFVGYWNGLPENQNLDP  
 VGRDVTVAELOYAYHAVVLSYGAEDNRVLGIPGENLSVYSARAFVGYWNGLPENRDLNLP  
 VGRDVTVEELOYAYHAVVLSYGAEDHRALEIPGELEFCVYSARAFVGYWNGLPENQELDP

180 190 200 210 220 230  
 DLSSETAVILGQGNVALDVARILLSPDLLLKKTDITQHSLLEALAQSKVKRVLWVGRGRL  
 DLSSETAVILGQGNVALDVARILLAPDLLLKKTDITASSLKATAHRSKVKRVLWVGRGRL  
 DLSSETAVILGHGNVALDVARILLSPHLHLRQTDITEGSLAALACSKVKRVLWVGRGRL  
 DLSCDTAVILGQGNVALDVARILLTPPEHLERTDITKAAALGLVLRQSRVTRVLWVGRGRL

240 250 260 270 280 290  
 QVAFTIKELREMINLPGTRPILLPDPSDFBGLGDAIKDLPRPRKRLTELMIKTALPKPGKE  
 QVAFTIKELREMISSLPGTRPILLPDPSDFBGLGDVTKDLPRPRKRLTELMVKSALPKPSEKD  
 QVAFTIKELREMINLPGTRPVLNPADEFTGLENAVKDVPFRPRKRLTELMIKTALPKPGEKA  
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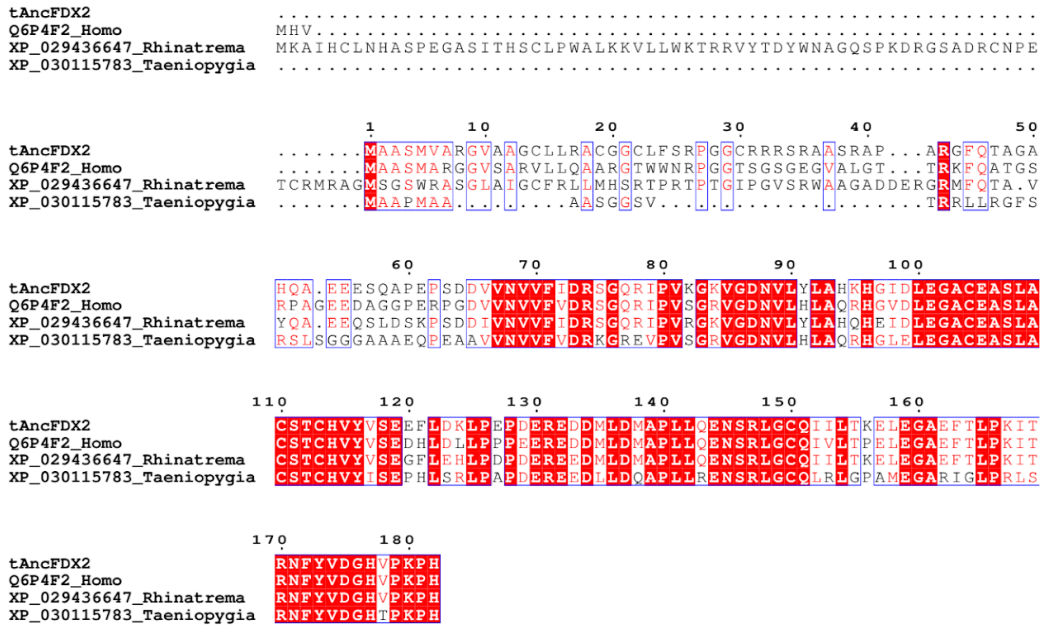
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 VEVQAAAPREWGLKFRSPQAVLPTADGRRRARGVRLSLTHLECSGDSAKAVPTGDVBELE  
 AARQASASRAWGLRFRSPQVLPSPDGRRAAGVRLAVTRLEGVDEATRAVPTGDMEDLE

360 370 380 390 400 410  
 CGLTLLSSITGYKSRPIIDPSVFPDPKQGITPNSSMRVQGPGLYCSGWVKRGPTGVIIITMNN  
 CGLTLLSSITGYKSRPIATSVFPDAKQGITPNTMGRVHNPGLYCSGWVKRGPTGVIIITMNN  
 CGLVLLSSITGYKSRPLDPAVFPDTRQGITPNSSSRVEGVPGLYCSGWVKRGPTGVIIITMNN  
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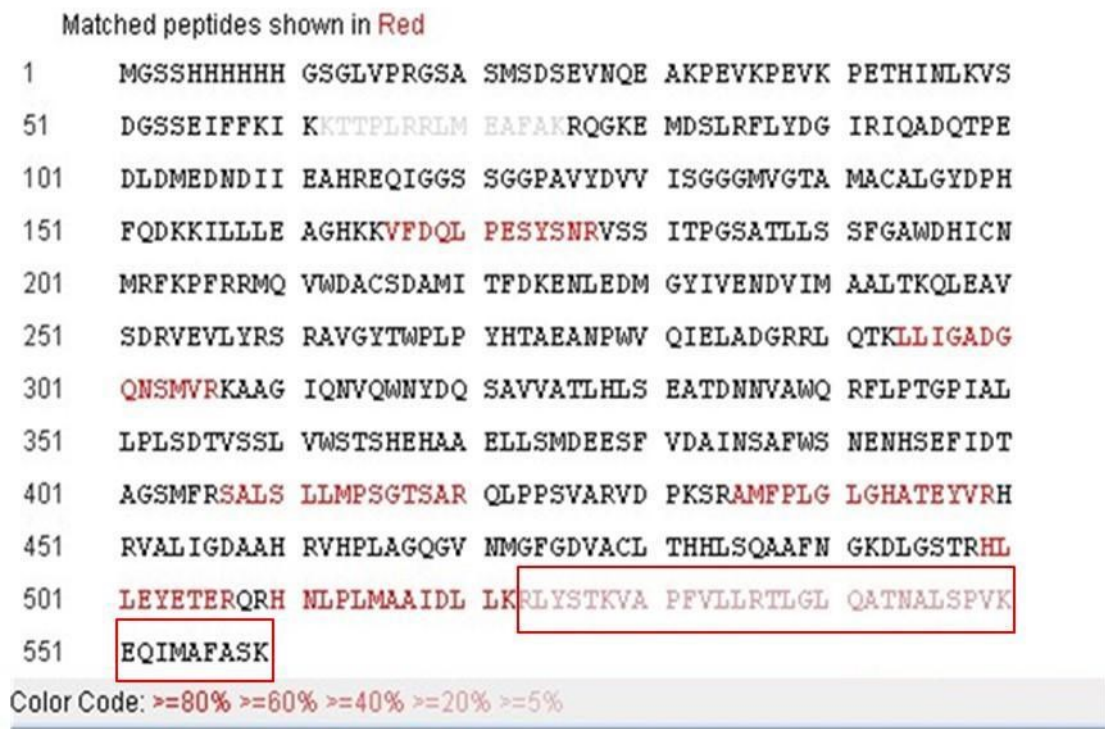
420 430 440 450 460 470  
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 DSFDTAQSVLEDDIQAGVLDVATSREGEFQAVESILCSRGRVPSVPSFDWEKIDAAEVARGKA  
 DSFLTGQMLLQDLKAGLLP.SGFRPGVAIQALLLSRGRVPSVPSFDWEKIDAEVARGQG

480 490  
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 VGKPREKILDLEMLRLASQ  
 AGKPREKIVDPQEMLRLLGH  
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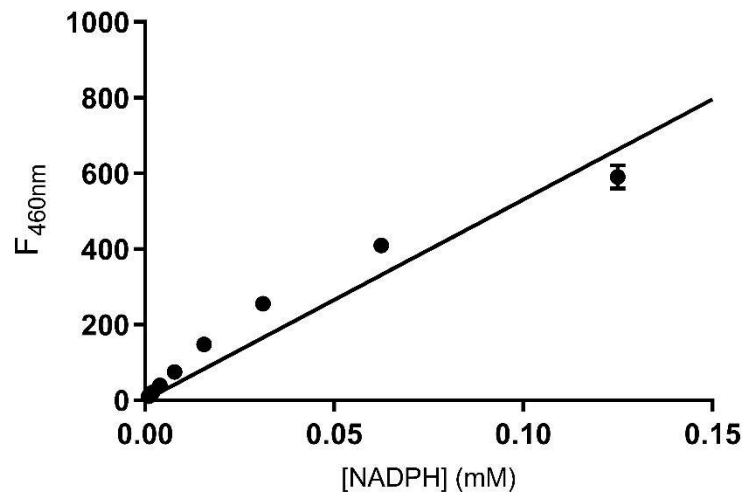
# FDX2



**Supplementary Figure 2. Representative Multiple Sequence Alignments of each COQ.** For each COQ a multiple-sequence alignment was constructed including the ancestral sequence reconstructed (full-length) from the tetrapoda superclass plus representative extant sequences from each of the three classes: amphibia (*Rhinatrema bivittatum* in all cases), reptilia (*Columba livia*, *Columbina picui*, *Casuaris casuaris*, *Caloenas nicobarica*, *Lacerta agilis* or *Taeniopygia guttata*) and mammalia (*Homo sapiens* in all cases). For each selected sequence the GenBank accession code is given in the name. The multiple-sequence alignments are colored according to the percentage of equivalence among residues calculated from their physical-chemical properties.

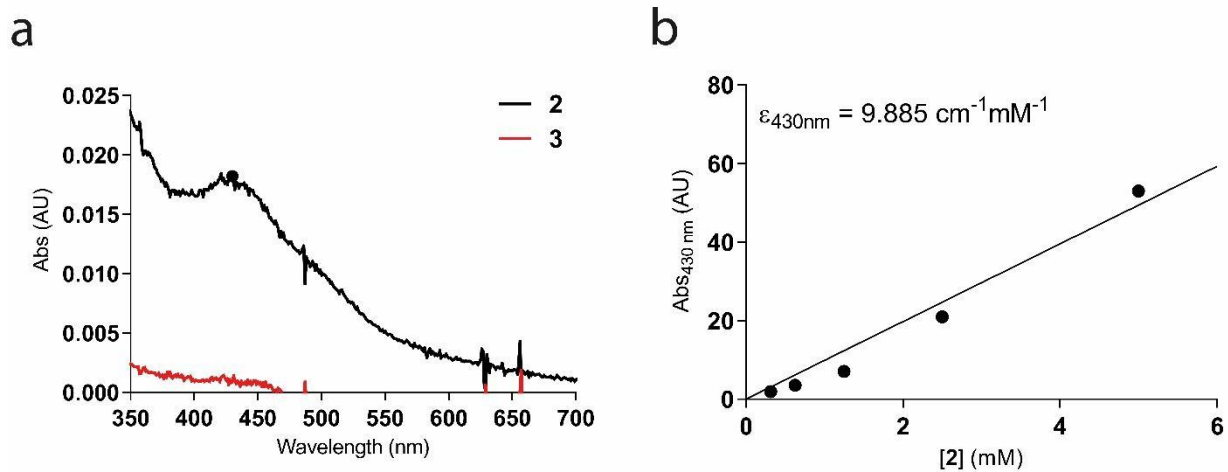


**Supplementary Figure 3. Limited proteolysis of COQ6 analysed by UHPLC-HRMS and peptide mapping.** The peptide in the red box was removed in the COQ6 construct employed for most of the experimental work.

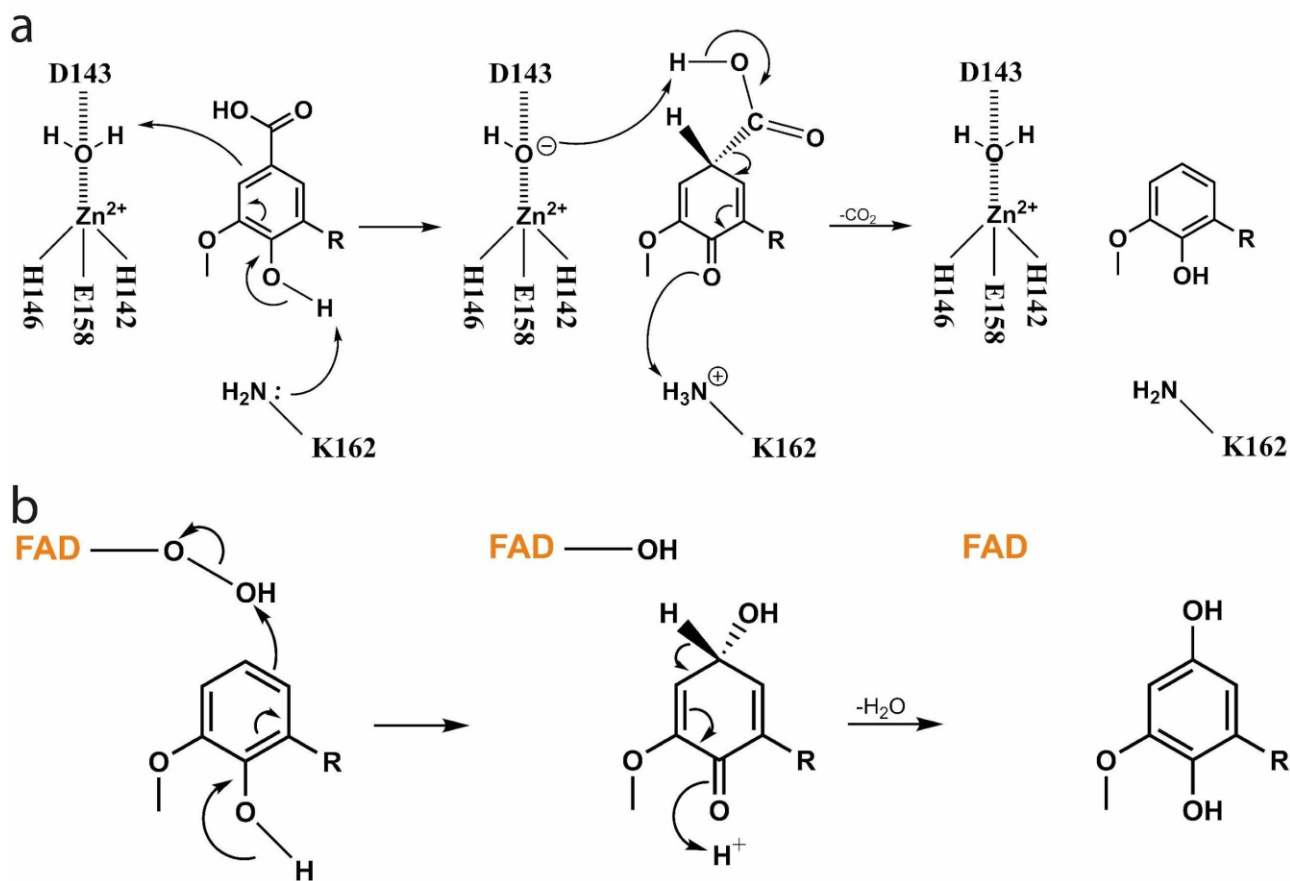


**Supplementary Figure 4. Fluorescence NAD(P)H calibration line** built by measuring  $F_{460\text{nm}}$  of known concentrations of NAD(P)H. All data are presented as mean values  $\pm$  s.d. The error bars correspond to the standard deviations in  $n = 2$  independent measurements for each datum.

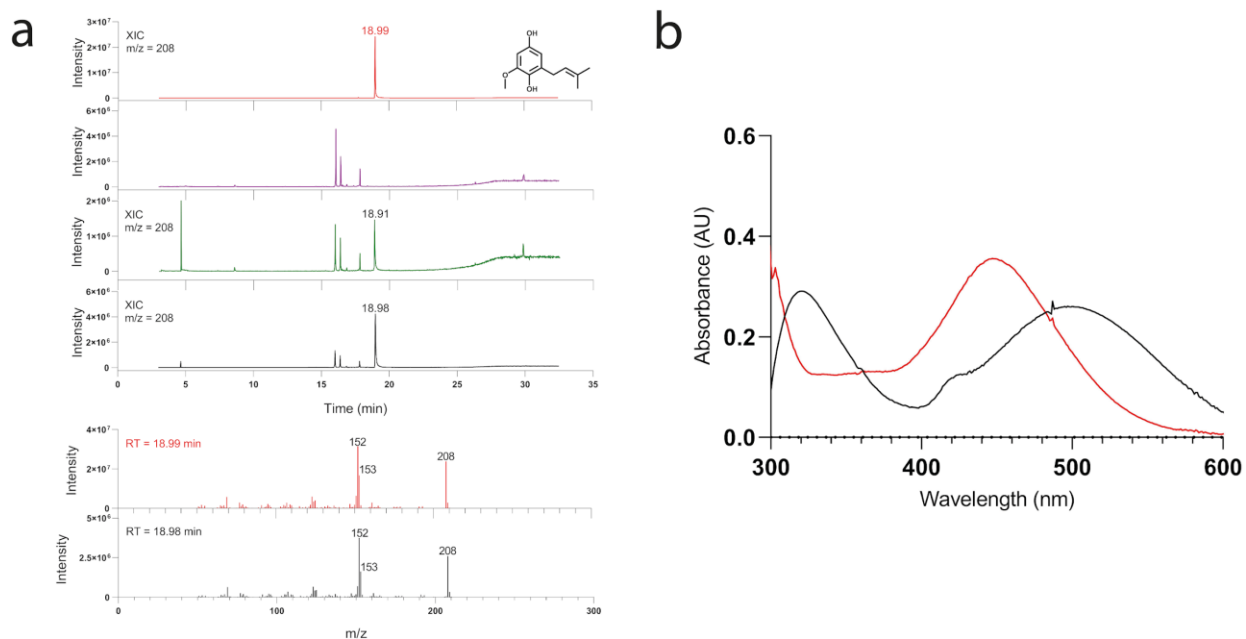




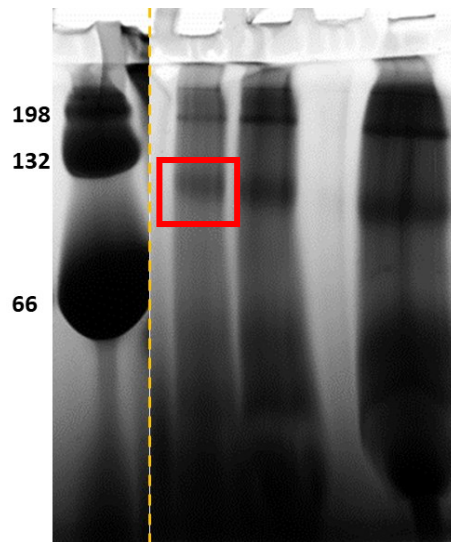
**Supplementary Figure 5. UV/Vis spectra of 2 and 3. a.** Visible spectra of **2** and **3**; the absorption peak at 430 nm disappears after O-methylation in position 5. **b.** Calibration line for the absorbance of **2** at 430 nm at known concentrations determined an extinction coefficient of  $9.924 \text{ cm}^{-1}\text{mM}^{-1}$ .



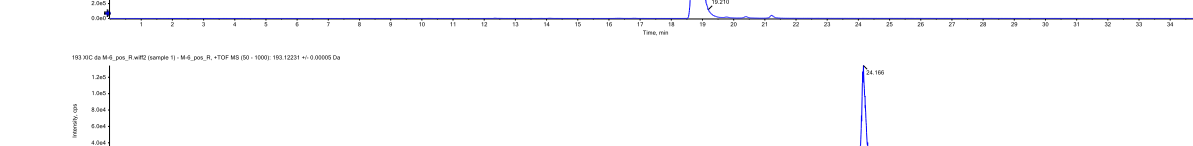
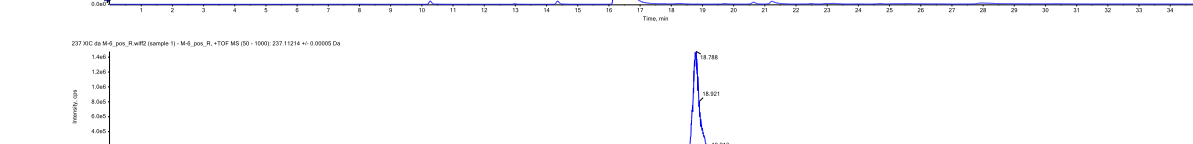
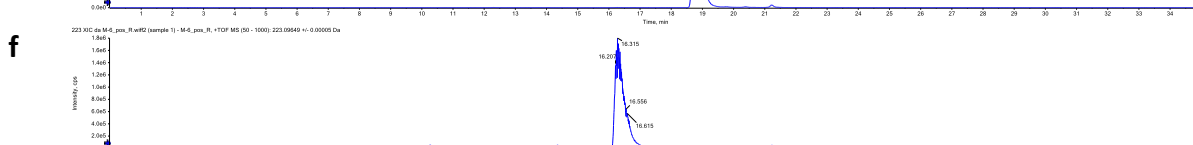
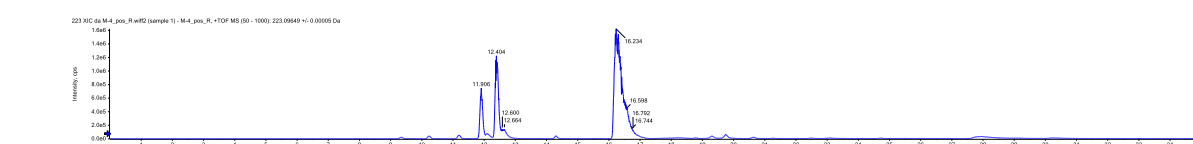
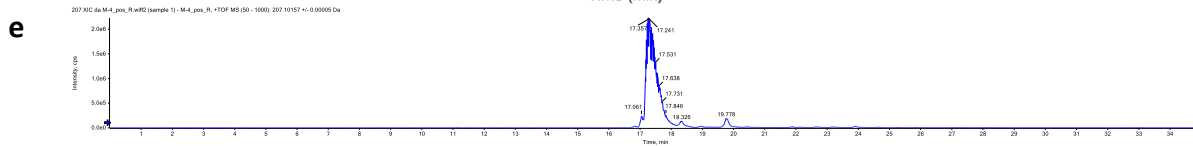
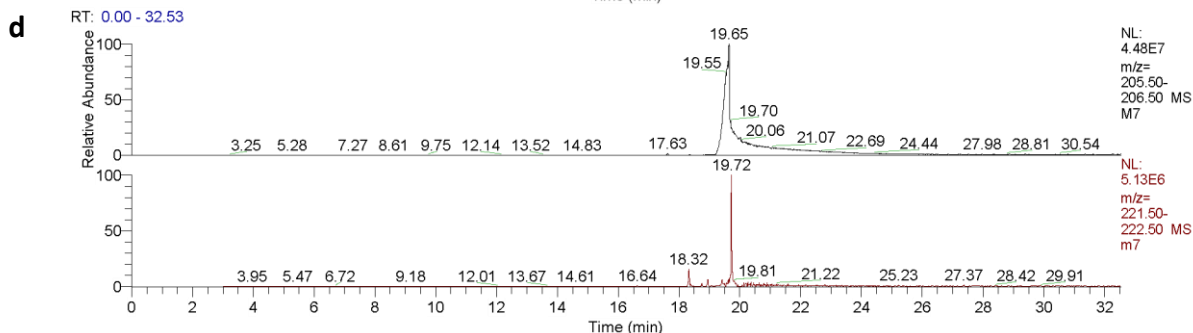
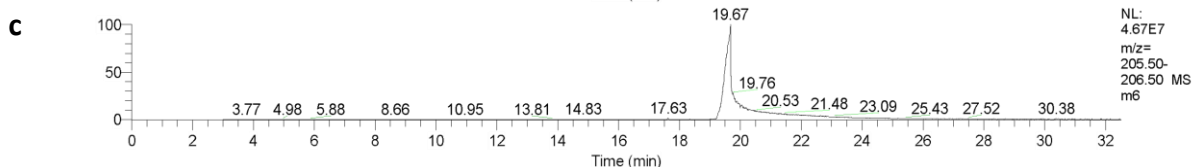
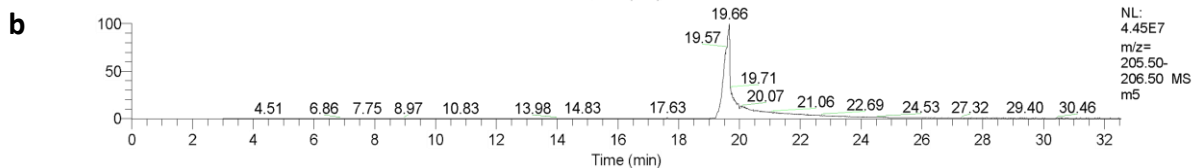
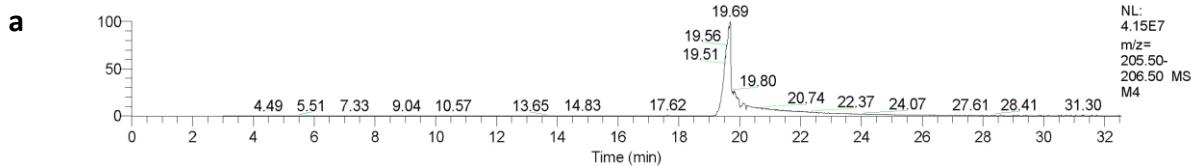
**Supplementary Figure 6. COQ4 and COQ6 proposed mechanisms of action for C<sub>1</sub> modifications. a.** Proposed mechanism for the Zn-dependent decarboxylase activity of COQ4 based on the inspection of the predicted AlphaFold model and mutagenesis experiments. **b.** Proposed mechanism for C<sub>1</sub>-hydroxylase activity of COQ6, based on previous literature on flavin dependent monooxygenases [25,27].



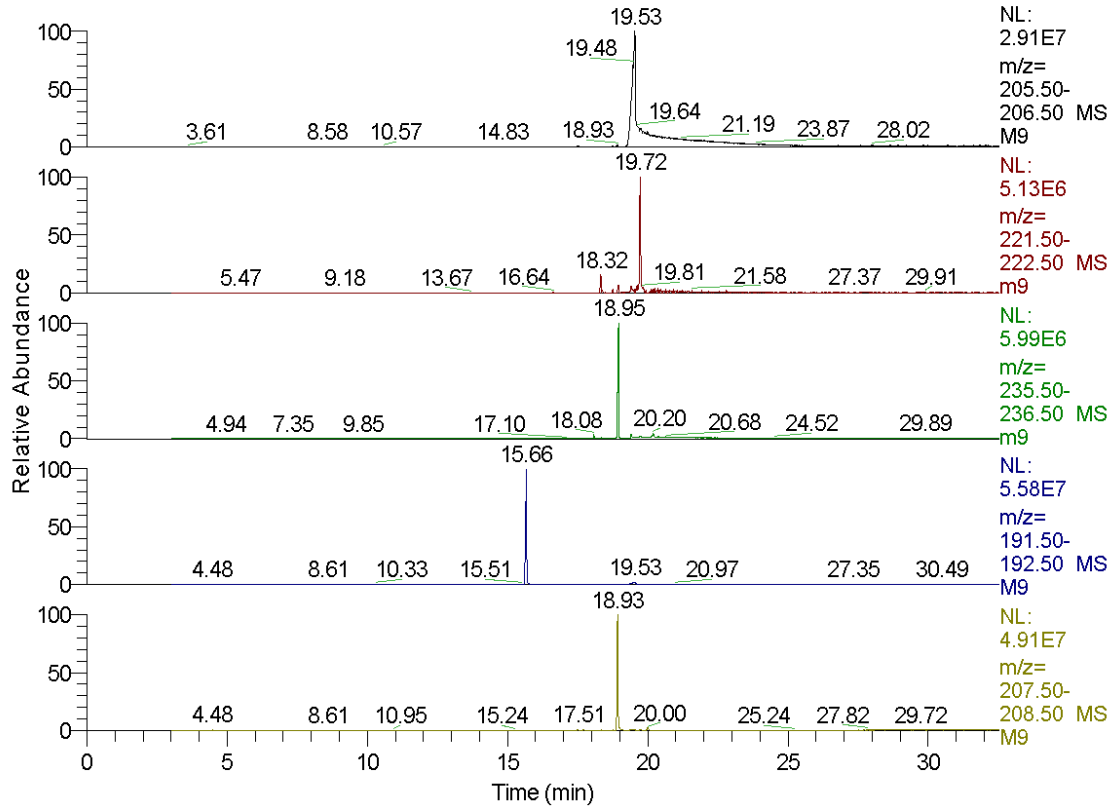
**Supplementary Figure 7. COQ6 is responsible for C<sub>1</sub>-hydroxylation.** **a.** Comparison of the GC/MS analysis of the overnight reaction of **4a** to **4b** with the blank injection. The extracted-ion chromatograms of **4b** recorded after the injection of 500 ppm analytical standard (red), blank injection (violet), reactions performed by truncated (green) and full-length (black) COQ6 are shown above. The full-scan mass spectra of corresponding to the peak of **4b** in the standard injection (red) and in the reaction by full-length COQ6 (black) are shown below. **b.** Visible spectra of 4-aminoantipyrine adducts with the analytical standards of **4a** (black) and **4b** (red).



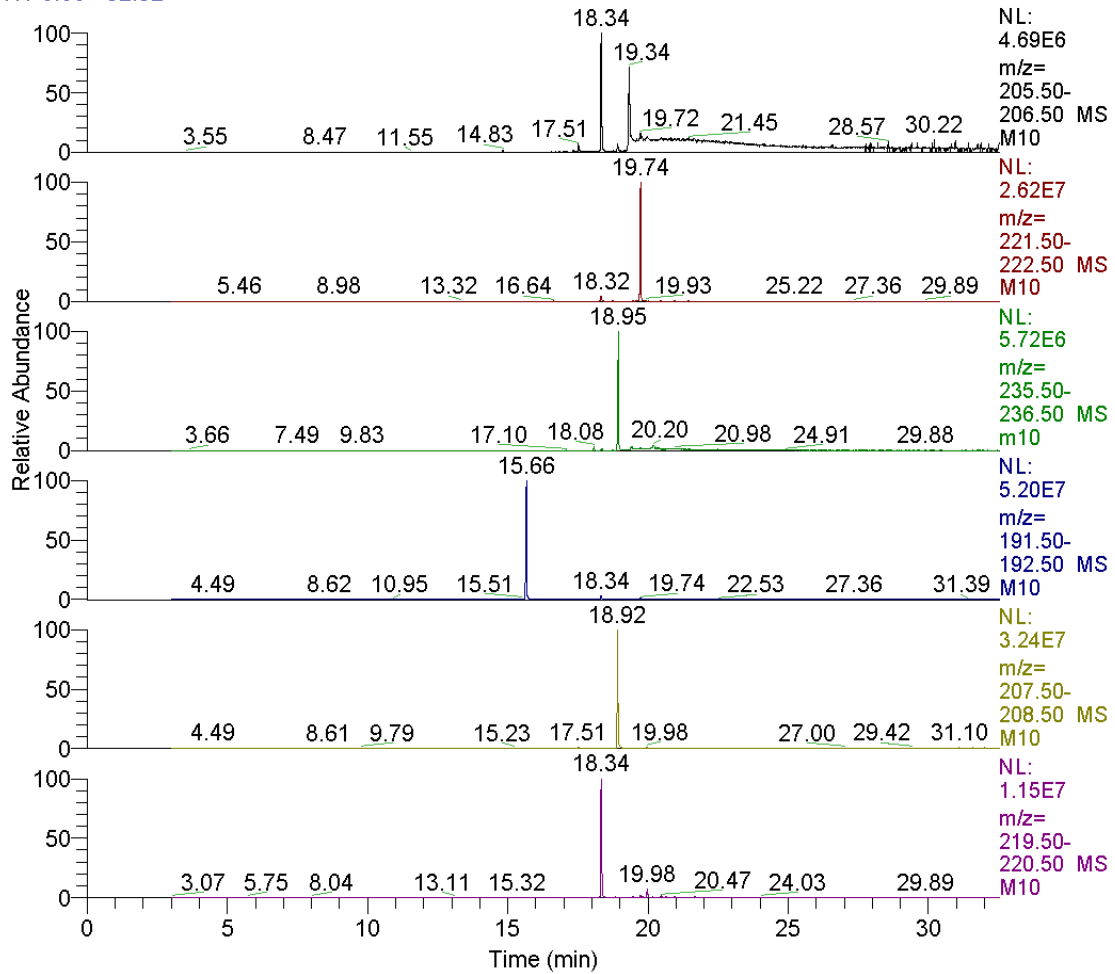
**Supplementary Figure 8. Native PAGE analysis of the purified COQ metabolon.** The gel filtered metabolon (see [Extended Data Fig. 7](#)) was submitted to Native PAGE analysis (14% polyacrylamide) at increasing concentrations (from left to right, 10, 25 and 50  $\mu$ M, respectively, see [Supplementary Fig. 16](#)). The two top bands visible in the gel were found to contain all the COQ proteins as shown by peptide mapping (see [Supplementary Table 2](#)). In-house MW marker was prepared by vortexing Bovine Serum Albumin and generating dimeric and trimeric oligomeric states. Dashed lines indicate where lanes have been moved and rearranged in the gel to ease visual comparison; original gels can be found in [Supplementary Figure 16](#). Native-PAGE analyses were repeated in  $n = 2$  independent experiments.

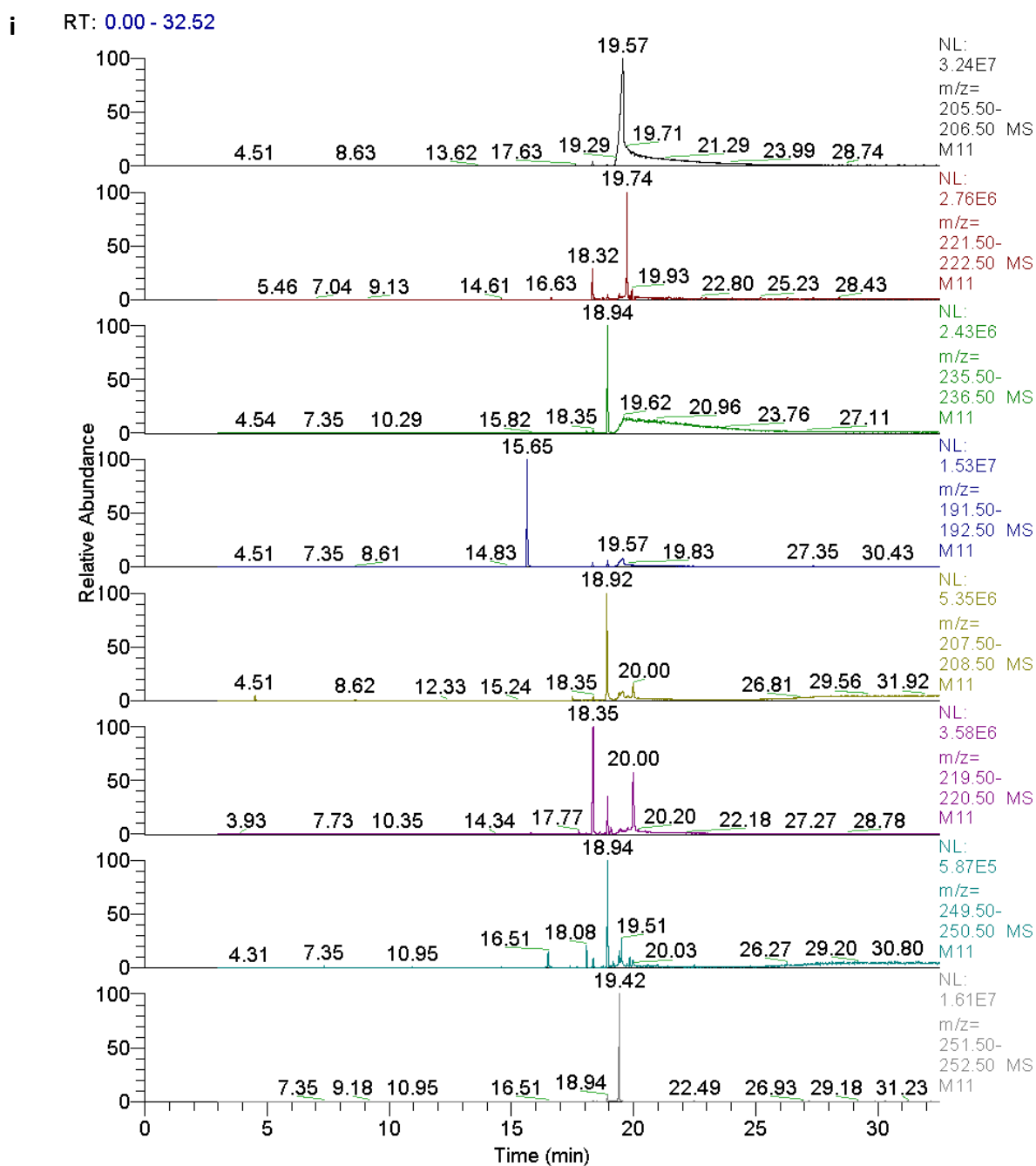


**g** RT: 0.00 - 32.53

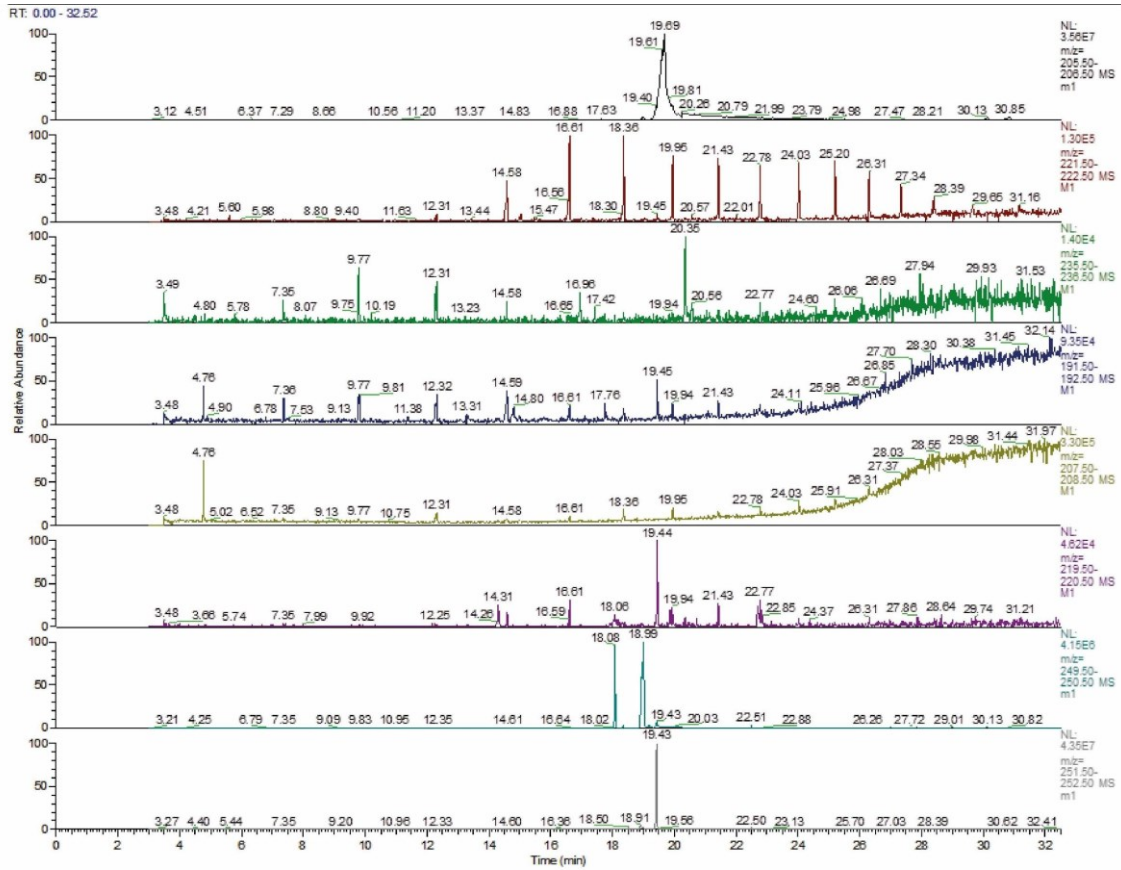
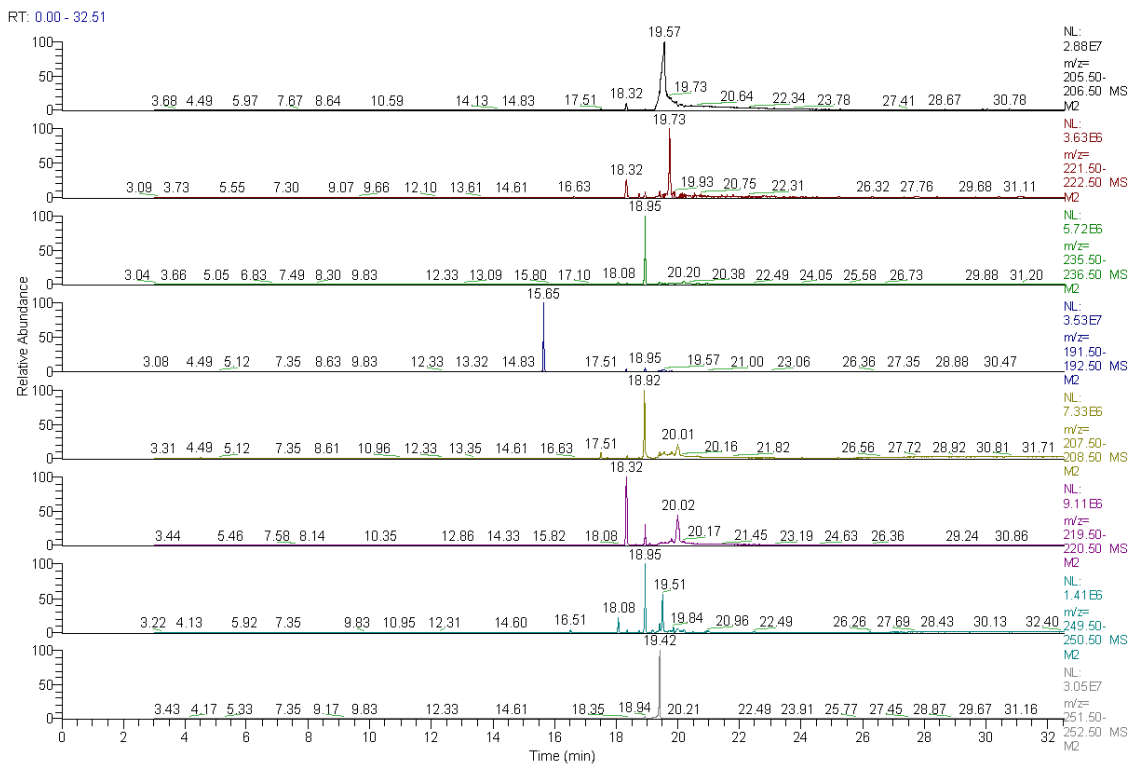


**h** RT: 0.00 - 32.52

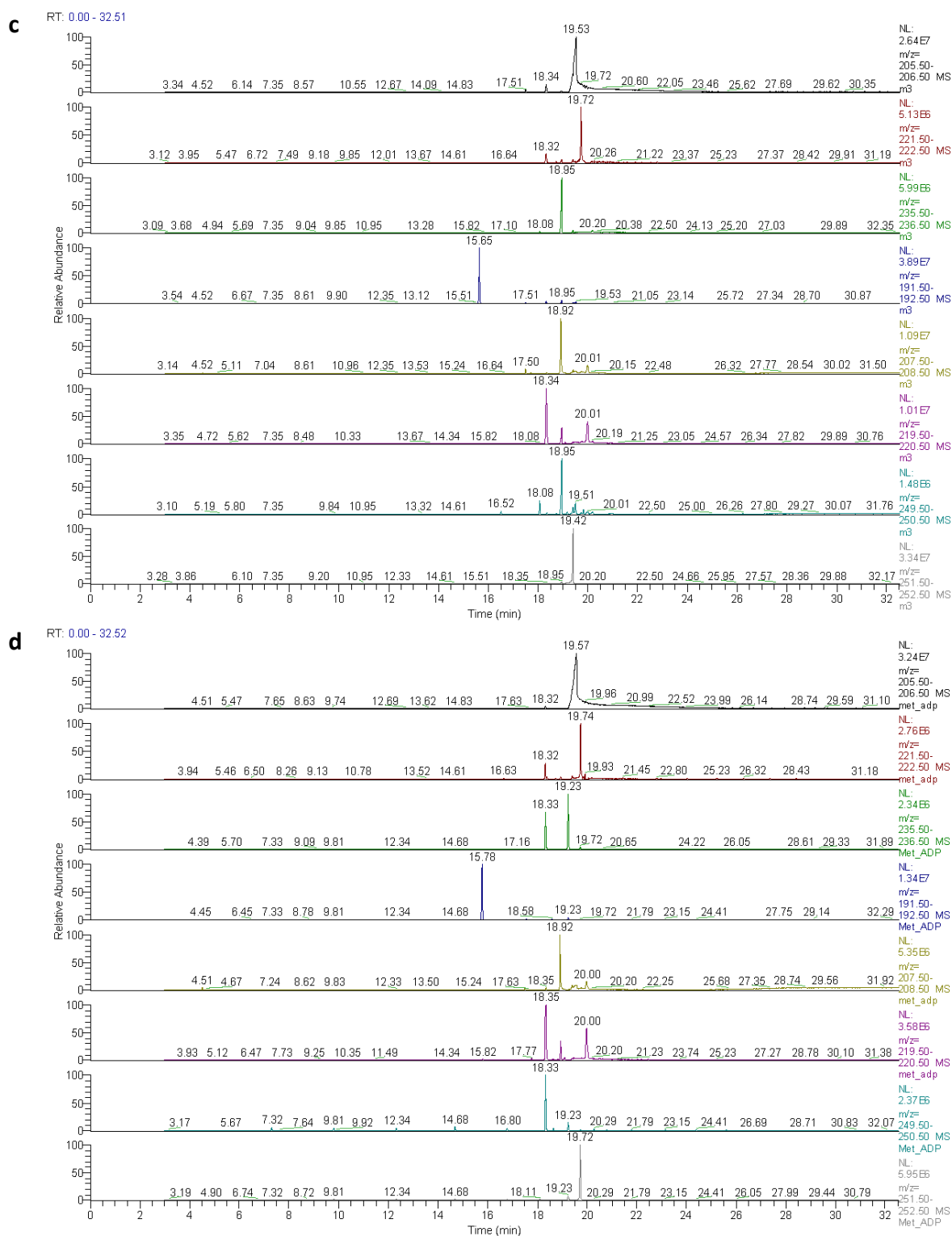




**Supplementary Figure 9. Chromatograms of experiments reported in Figure 6b.** **a.** Metabolon without FDXR: from top to bottom, GC XIC of **1** ( $m/z=206$ ). **b.** Metabolon without FDX2: GC XIC of **1** ( $m/z=206$ ). **c.** Metabolon without COQ6: GC XIC of **1** ( $m/z=206$ ). **d.** Metabolon without COQ3: GC XIC of **1** ( $m/z=206$ ) and **2** ( $m/z=222$ ). **e.** Metabolon without COQ4: UHPLC/ESI+ XIC of **1** ( $m/z=207$ ), **2** ( $m/z=223$ ) and **3** ( $m/z=237$ ). **f.** Metabolon without COQ6 starting from **2**: UHPLC/ESI+ XIC of **2** ( $m/z=223$ ), **3** ( $m/z=237$ ) and **4a** ( $m/z=193$ ). **g.** Metabolon without COQ5: GC XIC of **1** ( $m/z=206$ ), **2** ( $m/z=222$ ), **3** ( $m/z=236$ ), **4a** ( $m/z=192$ ) and **4b** ( $m/z=208$ ). **h.** Metabolon without COQ7: GC XIC of **1** ( $m/z=206$ ), **2** ( $m/z=222$ ), **3** ( $m/z=236$ ), **4a** ( $m/z=192$ ), **4b** ( $m/z=208$ ) and **5** ( $m/z=220$ ). **i.** Metabolon without COQ9: GC XIC of **1** ( $m/z=206$ ), **2** ( $m/z=222$ ), **3** ( $m/z=236$ ), **4a** ( $m/z=192$ ), **4b** ( $m/z=208$ ), **5** ( $m/z=220$ ), CoQ<sub>1</sub> ( $m/z=250$ ) and CoQ<sub>1</sub>H<sub>2</sub> ( $m/z=252$ ).

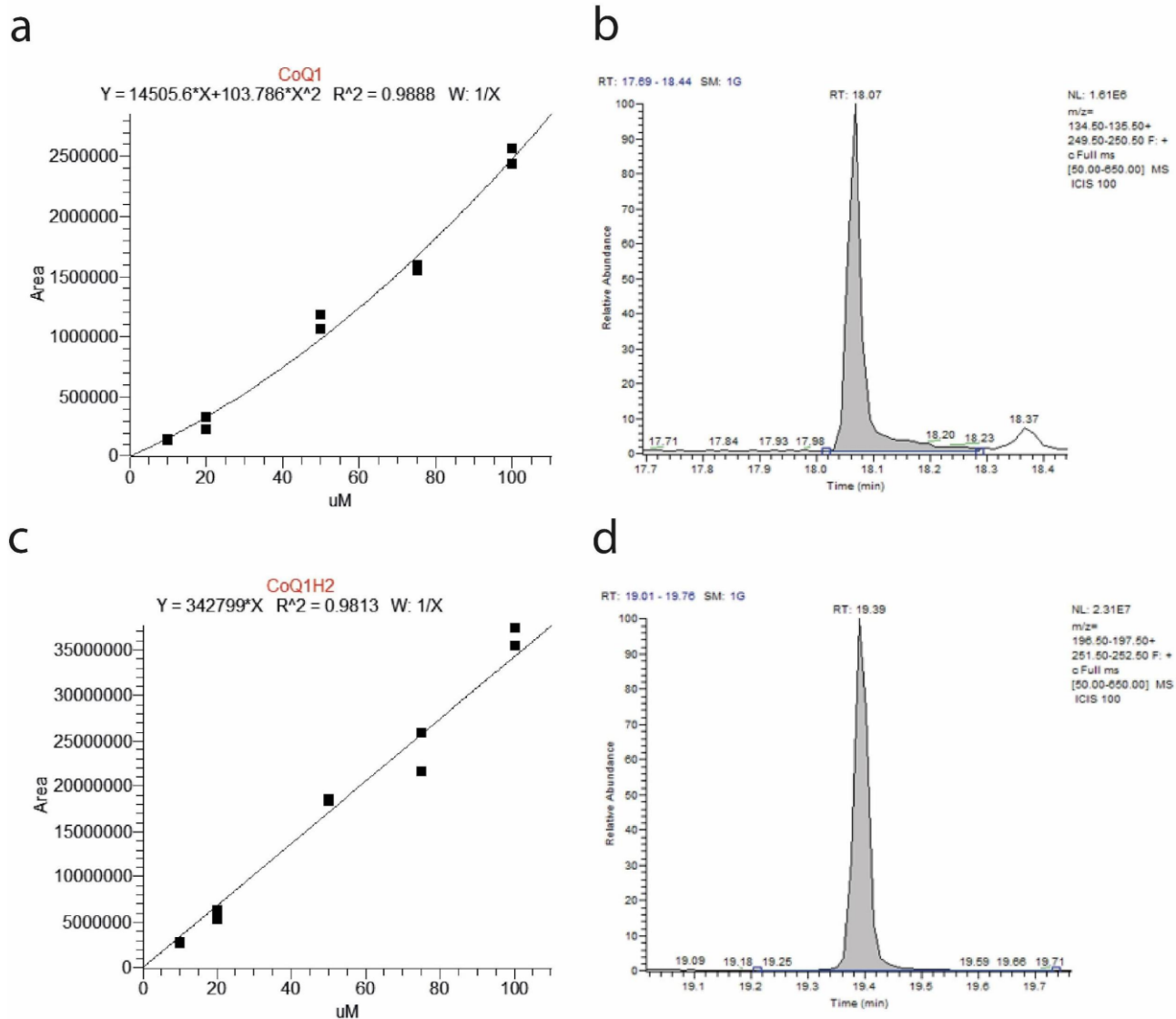
**a****b**



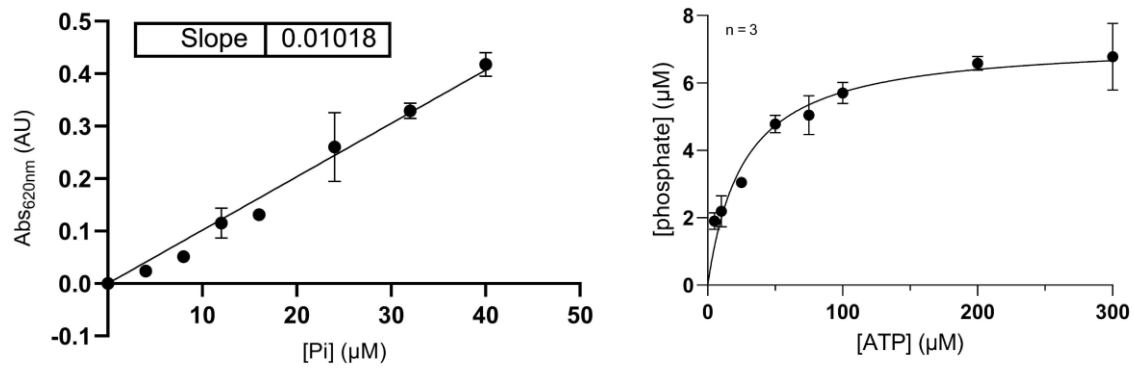


**Supplementary Figure 10. Chromatograms of experiments reported in Figure 6c. a.** Metabolon with COQ8B and ATP: from top to bottom, GC XIC of **1** ( $m/z=206$ ), **2** ( $m/z=222$ ), **3** ( $m/z=236$ ), **4a** ( $m/z=192$ ), **4b** ( $m/z=208$ ), **5** ( $m/z=220$ ), CoQ<sub>1</sub> ( $m/z=250$ ) and CoQ<sub>1</sub>H<sub>2</sub> ( $m/z=252$ ). **b.** Metabolon with COQ8B without ATP: GC XIC of **1** ( $m/z=206$ ), **2** ( $m/z=222$ ), **3** ( $m/z=236$ ), **4a** ( $m/z=192$ ), **4b** ( $m/z=208$ ), **5** ( $m/z=220$ ), CoQ<sub>1</sub> ( $m/z=250$ ) and CoQ<sub>1</sub>H<sub>2</sub> ( $m/z=252$ ). **c.** Metabolon without COQ8B and ATP: GC XIC of **1** ( $m/z=206$ ), **2** ( $m/z=222$ ), **3** ( $m/z=236$ ), **4a** ( $m/z=192$ ), **4b** ( $m/z=208$ ), **5** ( $m/z=220$ ), CoQ<sub>1</sub> ( $m/z=250$ ) and CoQ<sub>1</sub>H<sub>2</sub> ( $m/z=252$ ). **d.** Metabolon with COQ8B and ADP: GC XIC of **1** ( $m/z=206$ ), **2**

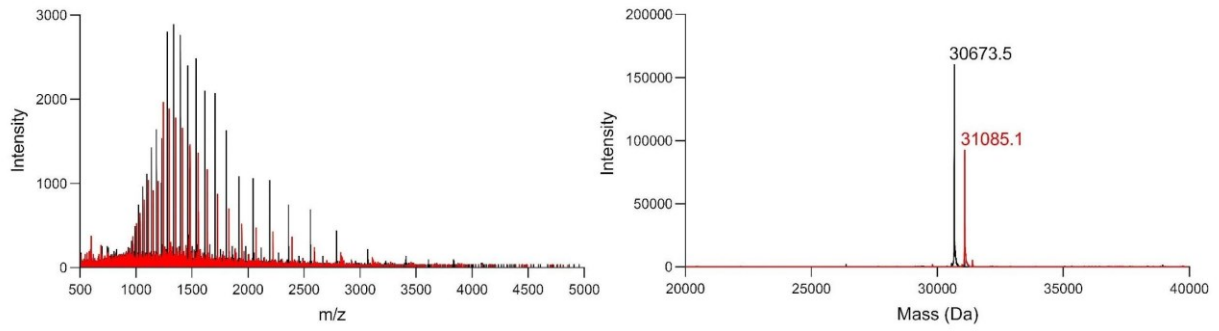
( $m/z=222$ ), **3** ( $m/z=236$ ), **4a** ( $m/z=192$ ), **4b** ( $m/z=208$ ), **5** ( $m/z=220$ ), CoQ<sub>1</sub> ( $m/z=250$ ) and CoQ<sub>1</sub>H<sub>2</sub> ( $m/z=252$ ).



**Supplementary Figure 11. Calibration curves and automated peak integration of CoQ<sub>1</sub>(H<sub>2</sub>).** **a.** Quadratic calibration curve employed for CoQ<sub>1</sub> quantitation. **b.** CoQ<sub>1</sub> peak integration showed for a 100  $\mu$ M analytical standard. **c.** Linear calibration curve employed for CoQ<sub>1</sub>H<sub>2</sub> quantitation. **d.** CoQ<sub>1</sub>H<sub>2</sub> peak integration showed for a 100  $\mu$ M analytical standard.



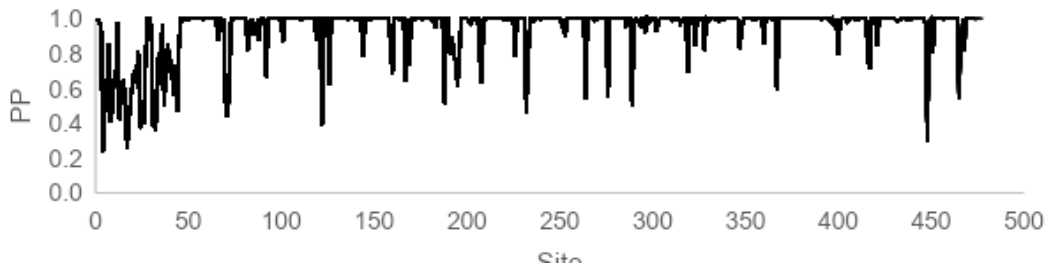
**Supplementary Figure 12. Monitoring inorganic phosphate production using Malachite green assay.** Calibration of the inorganic phosphate adduct with Malachite Green dye and Michaelis-Menten curve describing the  $K_M$  of ATP for COQ8B. All data are presented as mean values  $\pm$  s.d. The error bars correspond to the standard deviations in  $n = 3$  independent measurements for each datum.



**Supplementary Figure 13. COQ3 is phosphorylated by COQ8B.** Intact protein mass-spectrometry analysis shows that COQ3 is phosphorylated by COQ8B in five positions. Raw and deconvoluted mass spectra collected after the injection of 50  $\mu\text{g}$  of the reaction (red) and of the negative control (black) are shown.

## COQ6

---



$\underline{PP} = 0.94$

Length= 478 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 41 (8.6%)

Sequence identity to human COQ6 = 75.3%

Deposited sequence – tAncCOQ6\_tr – with the N-terminal cleavage site shown in bold and red.

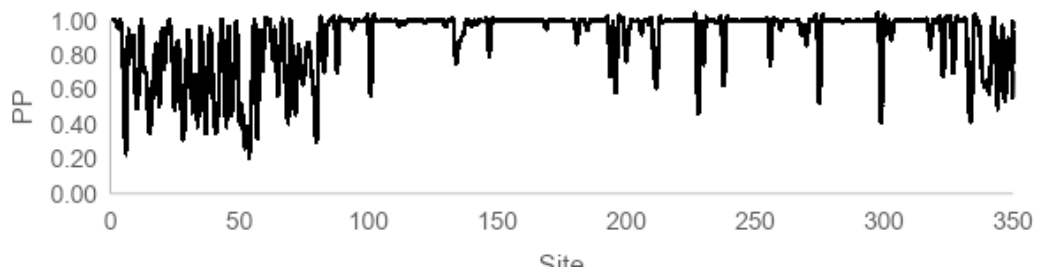
Accession code: OQ859713

>Tetrapod ancestor COQ6

```
MAARLGLSGWGRRRLRLRCGARLSLARRGLARCCRRRSSSGGPAVYDVVISGGGMVGTAMACALGYDPHFQDKKILLEA
GHKKVFDQLPESYSNRVSSITPGSATLLSSFGAWDHICNMRFKPFRMQVWDACSDAMITFDKENLEDMGYIVENDVIM
AALTKQLEAVSDRVEVLYRSRAVGYTWPLPYHTAEANPWVQIELADGRRLQTKLLIGADGQNSMVRKAAGIQNVQWNY
DQSAVVATLHLSEATDNNVAWQRFLPTGP IALLPLSDTVSSLVWSTSHEHAAELLSMDEESFVDAINS AFWSENENHSEFID
TAGSMFRSALSLLMPSGTSARQLPPSVARVDPKSRAMFPLGLGHATEYVRHRVALIGDAAHRVHPLAGQGVMNGFGDV
ACLTHHLSQAAFNGKDLGSTRHLLLEYETERQRHNLPLMAAIDLLKRLYSTKVAPFVLLRTLGLQATNALSPVKEQIMAFASK
```

## COQ3

---



$\underline{PP} = 0.89$

Length= 362 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 44 (12.15%)

Sequence identity to human COQ3 = 64.7%

Deposited sequence – tAncCOQ3\_tr – N-terminal cleavage site shown in bold and red.

Accession code: OQ859710

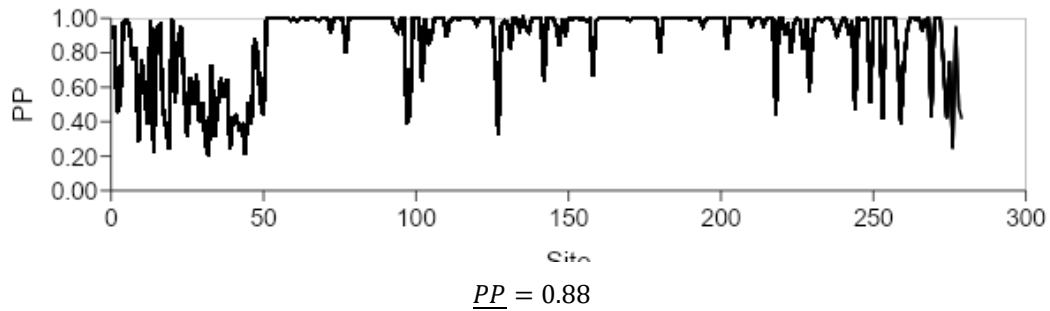
>Tetrapod ancestor COQ3

```
MWGGGRGSRAGRLLVALRGRSRGRGAGCRRLS LAAAAGNHYGWTLQMAPRFKSSNRTMWLKS NSTTFA
SLTKMKSSRS AVKRMYSTSQTTPDKEMKKFQALAHKWWDEQGEYAALHSMNDLRV PFIRD TLLNMSGDH
QLGSPLSGMKILDVGC GGGLLSEPLGRLGASVTGIDPLEDNIRTAELHKS FDPVL DKRIQYKACSL EEEV EAT
```

ETFDAVVASEVVEHVADVETFIKCCYQVLKPGGSLFITTINKTQLSYALGIVVAERIMGIVPAGTHDWEKFISPE  
ELERLLESNGFSVETVNGMLYNPLSGSWWIENTSINYALHAVKSKVQEQSDSTEPPSEQEQEQHQEAETST  
STTV

## COQ4

---



Length= 279 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 21 (7.5%)

Sequence identity to human COQ4 = 77%

Deposited sequence – tAncCOQ4\_tr – N-terminal cleavage site shown in bold and red.

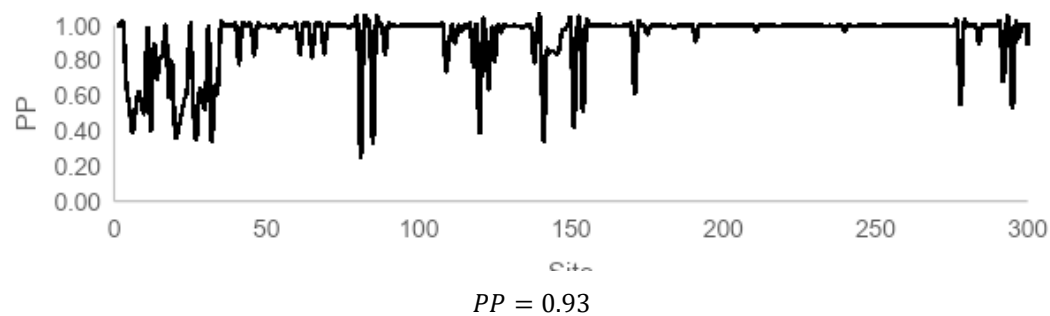
Accession code: OQ859711

>Tetrapod ancestor COQ4

MATLLRRARGLLPLLRPVLGSPGLTGRALCMRQA**PA**ADSPLRAAEEGYGPLYPGHIPTSPLQKALLAAGSAC  
MALYNPYRHDMVAVLGETTGHLALQNLDRMRNDPEGYQLQERPRIRLSTLDLAKLRSLPDGSFGREYVR  
FLDDNRVSPDTRAPVKFVDDEELAYVIQRYREVHLLHTLLGMPTNMLGEVVVKWFEAVQTGLPMCILGAFF  
GPIRLSARKLQVLVTELPWAVQNGRNARCVLNIYYERRWEQSLESREELGITPPPIRVTGLA

## COQ5

---



Length= 312 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 23 (7.4%)

Sequence identity to human COQ5 = 75%

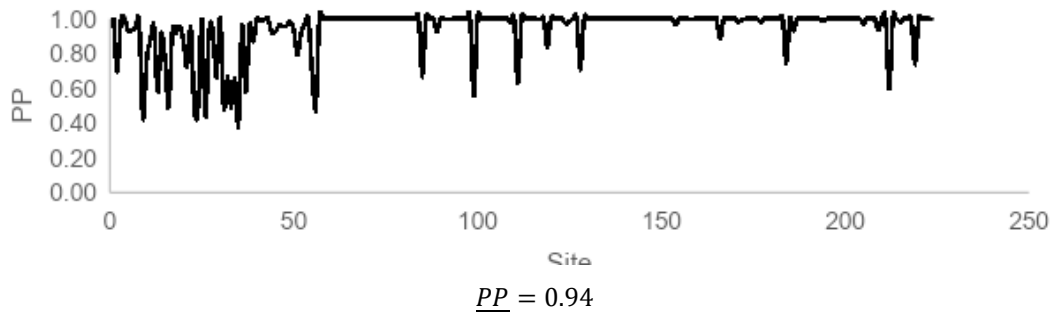
Deposited sequence – tAncCOQ5\_tr – N-terminal cleavage site shown in bold and red.

Accession code: OQ859712

>Tetrapod ancestor COQ5

MAASMRCACSRALCGCRSGARVCCRAHSTE**A**AEKETHFGFQTVSEEEKGEKVYQVFENVAKKYDIMNDAM  
 SLGIHRLWKDALLHQMNYPYPTQLLDVAGGTGDIAFRFINYVRSQRERQVRQELKSHQNLQSWQEISKSYQE  
 EEQDSLGGSSQAVICDINKEMLKVGKQKAQQLGYSEGLSWVVGNAEELPFDDDKFDVYTIAFGIRNVTHIDQA  
 LQEAYRVLKPGGRFLCLEFSQVNNPLISRLYDLYSFQVIPVLGEVIAGDWKSYQYLVESIRRFPSQEEFKAMIE  
 DAGFFKVEYHNLTSGIVAIHSGFKL

### COQ7



Length= 224 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 20 (8.9%)

Sequence identity to human COQ7 = 77.7%

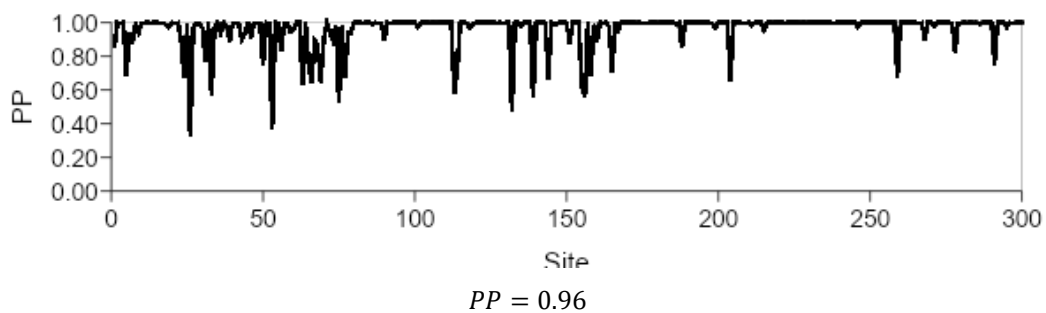
Deposited sequence – tAncCOQ7\_tr – N-terminal cleavage site shown in bold and red.

Accession code: OQ859714

>Tetrapod ancestor COQ7

MERAAAAVRRGWRAHCRRRLRLGAGPRRPCCAQARRTSVR**F**CSTGMTLDNVDKAVIDRII  
 RVDHAGEYGANRIYAGQMAVLGRTSVGPVIQMQMWDQEKEHLKKNELMVAHRVRPTILMP  
 FWNVAGFVLGAGTALLGKEGAMACTVAVEESISEHYNNQIRTLMEEDPEKYKELLQIIKK  
 FRDEELEHHDGTGLEHDAELAPAYSLLKNVIQIGCKAAIYLSERI

### COQ9



Length= 318 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 18 (5.7%)

Sequence identity to human COQ9 = 84.3%

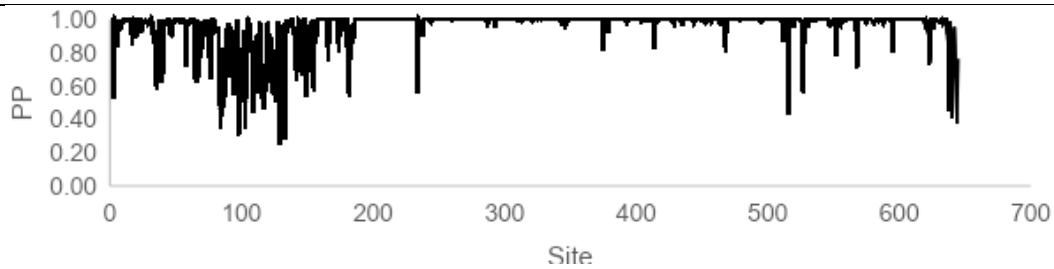
Deposited sequence – tAncCOQ9\_tr\_N79 – N-terminal cleavage site shown in bold and red.

Accession code: OQ859717

>Tetrapod ancestor COQ9

MMAAAVAGLLRRAGWRLQLRCRVVVRQQLSPVQRAFHASAVLRRVSDEQKQQPPSSSSQQHSESQPT  
EPDPESQRS**H**PSYTDQGGEESEDYEESEQLQHRILTAALFVPEHGWTAEAIAEGAKSLGLSAAAAGMFGN  
DGSDLILHFVSQCNSKLSLELEEHLVQLGQAEEKKTDQFLRDAVEARLRMLIPYIEKWPQALSILLPHNIP  
ASLNLLTSMVDDMWHYAGDQSTDINWYTRRAVLAGIYNTTELVMQDSSPDFEDTWRFLFNINDAMNMG  
HTAKQVKSTGEALVQGLMGAAVTLKNLTGLNQRR

### COQ8A



$$\underline{PP} = 0.95$$

Length= 645 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 47 (7.3%)

Sequence identity to human COQ8A = 73.9%

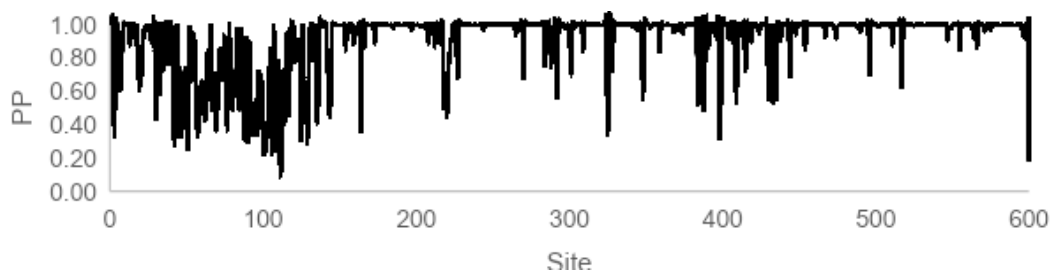
Deposited sequence – tAncCOQ8A\_tr – N-terminal cleavage site shown in bold and red.

Accession code: OQ859715

>Tetrapod ancestor COQ8A

MAGDAIMLMRGLAKLSKAVLETQAGQLRLGGEAVAIARTWQATAEEGFSAAMGKMQLGKQENLSDIGE  
DFGSEYDFSGPESSANKDFSSPSGQPHEHSGAEGPAYSYATNGPFRNTGDSSRADSPVSAKNGKLF  
GFRDPGNPFAAA**F**GQTRAFHQDHSSVGGTLAEDIEKAREAKANPENKPHKQMLSERARERKVPVTRIGRLA  
NFGGLAVGLGIGALAEVAKKSLRPEERNKKAVALDSSPFLSEANAERIVRTLCKVIRGAALKLQMLSIQDDAF  
INPQLQKIFERVRQSADFMPKQMMKTLNNDLGNWRDKLEFFEERPFAAASIGQVHLARLKDGREVAMKIQ  
YPGVAQSINSDVNNLMTVLSMSNALPEGLFPEHLIEVLSRELALECDYKREAAKAKFKELLKDHPFFYVPAV  
VDELCSQHVLTTTELVSFGFPLDQAEGLSQEIRNEICHNILVCLRELFEFRFMQTDPNWSNFFYDPQLHKVALL  
DFGATRGFDEDFTDIYIEVIKAAADQDRERVLKKSIEMKFLTGYESKAMENAHLDVILGEAFASEEPDFGS  
QSTTERIHGLIPVMLKHRLVPPPEETYSLHRKMGGSLFLICKLAKIPCKNMFQEAYSKYWSRRAKKQEQ

### COQ8B





$PP = 0.89$

Length= 629 amino acids

Ambiguously reconstructed sites (alternative state  $PP > 0.2$ ) = 77 (12.2%)

Sequence identity to human COQ8B = 63.8%

Deposited sequence – tAncCOQ8B\_tr – N-terminal cleavage site shown in bold and red.

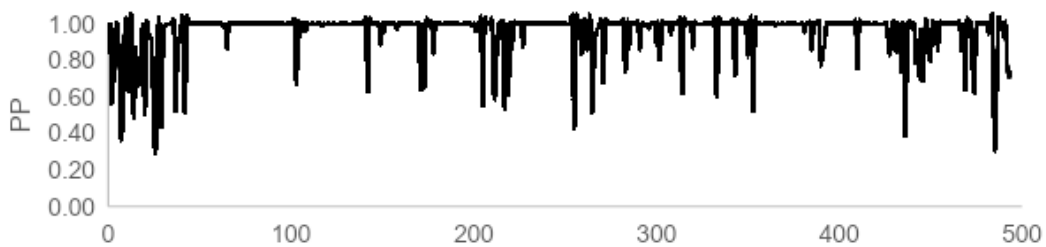
Accession code: OQ859716

>Tetrapod ancestor COQ8B

```
MWSEVGSVLRGAGRVGQAFETQGEQLRLMARSSALGAGLKRAQESVEQCLSSLLASRQRGARDEFSEA  
SEEDASRWGVASEMPPDISLPEAAAGAGSAQSPGGRPHPPAHGARGPGWPSGSPSFSGRGPGMGQTR  
SFHQDAAVRGLTAEDIKKAREAKQKQSKPPRQKLSEARERKVPASRISRLANFGGLAVSLGLGALAEVAKK  
SLNGEQPKDTRSLLDSSPFLSEANAERIVDTLCKVRGAALKIGQMLSIQDNSFISPQLQKIFERVRQSADFM  
PAWQMMKVLAEELGPDWREKLASFEERPFAAASIGQVHLGVLRDGREVAMKIQYPGIAQSIRSDVDNLLSVL  
KMSVWLPEGLFADNSIQVLQRELEWECYKREAAACARRFRQLLKDDPFFYVPEVIDELTTKRVLTMELVSGV  
PLDQCVGLDQDIRNEICFNILRLCLRELFEFRFMQTDPNWSNFFYDAEKHKVTLDFGASREFGKEFTDHYIE  
VVKAAADGDRAKVLQKSKDLKFLTGFETKVFEEAHVDAVMILGEAFASPEPFDGFTQNTTRRIQNLIPLVMLKH  
RLTPPEESYSLHRKMAGSFLICAKLGAVIPCREMFQEIYGRYWARERAAPLEAATA
```

## FDXR

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$PP = 0.94$

Length= 494 amino acids

Ambiguously reconstructed sites (alternative state  $PP > 0.2$ ) = 47 (9.5%)

Sequence identity to human FDXR = 73%

Deposited sequence – tAncFDXR\_tr – N-terminal cleavage site shown in bold and red.

Accession code: OQ859718

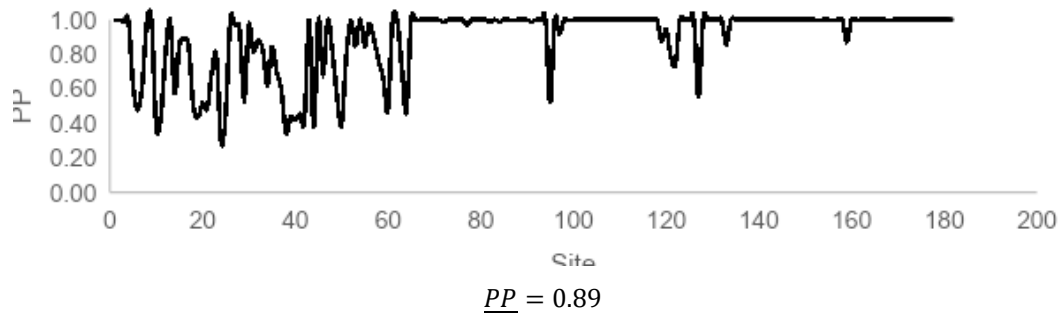
>Tetrapod ancestor FDXR

```
MGAPRGAVCWLWGVRSLSLARSLSRPRAGSPGVRRLLSTASPTPQICIVGSGPAGFYTAQHLLKHHKQAQVDIYE  
KLPVPFGLVRFVAPDHPEVKNVINTFTQTAHSDRCSFYGNVTVGKDVTVVEELQQAYHAVVLSYGAEDNRT  
LGIPGENLPGVYSARAFVWYNGLPENRDLNPDLSSETAVILGQGNVALDVARILLSPLDLLKKTIDITQHSLEA  
LAQSKVKRVWLVGRRGPLQVAFTIKELREMINLPGTRPLLDPSDFEGLGDAIKDLPRPRKRLTELMIKTALEK  
PGEKEAARWASATREWGLRFLRSPVEVLPSADGKRAAGIRLAVTRLEGSGESARAVPTGEVEDIECGLILSSI
```

GYKSLPIDPSVPFDPKQGIIPNSMGRVQGAPGLYCSGWVKRGPTGVIITTMNDSFDTAQSVLEDLQSGVLDV  
SAPKPGFQAIRALLQQRGVHPVVSFSDWEKIDAAETARGKAVGKPREKILDVEEMLQLASQ

### FDX2 - tAncFDX2

---



Length= 182 amino acids

Ambiguously reconstructed sites (alternative state PP > 0.2) = 22 (12.1%)

Sequence identity to human FDX2 = 70.5%

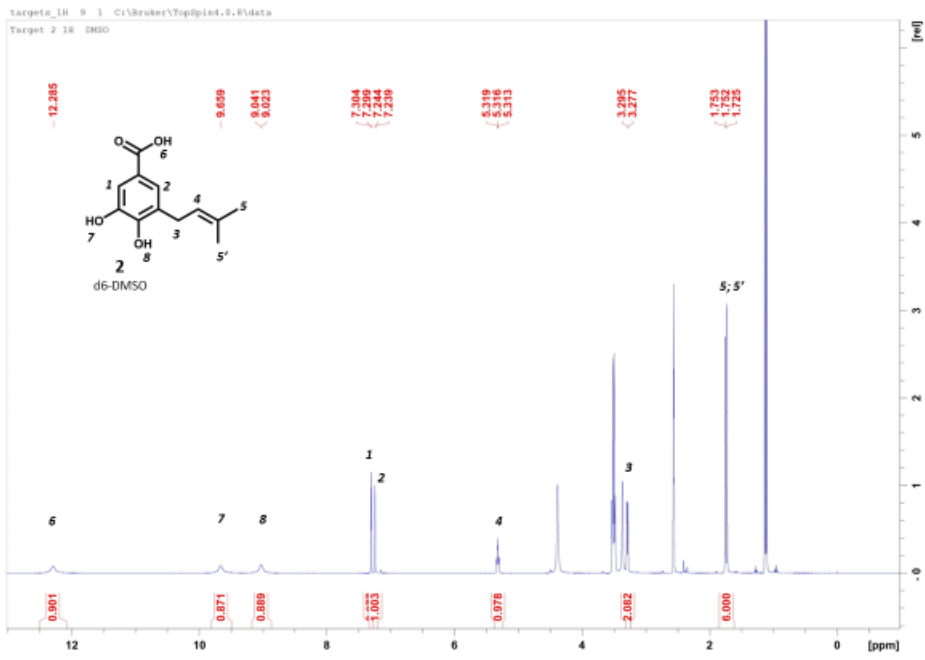
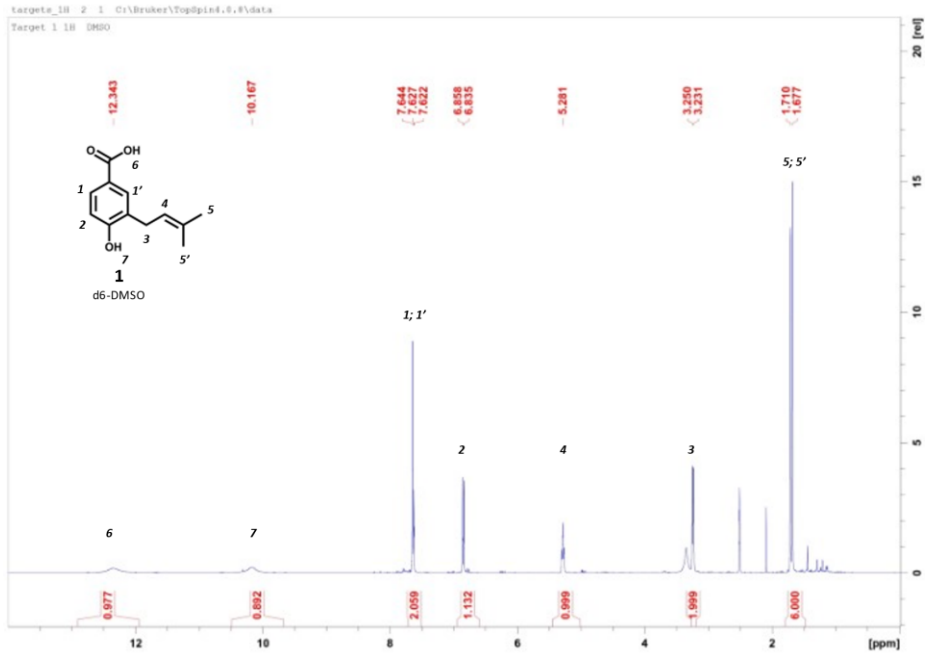
Deposited sequence – tAncFDX2\_tr – N-terminal cleavage site shown in bold and red.

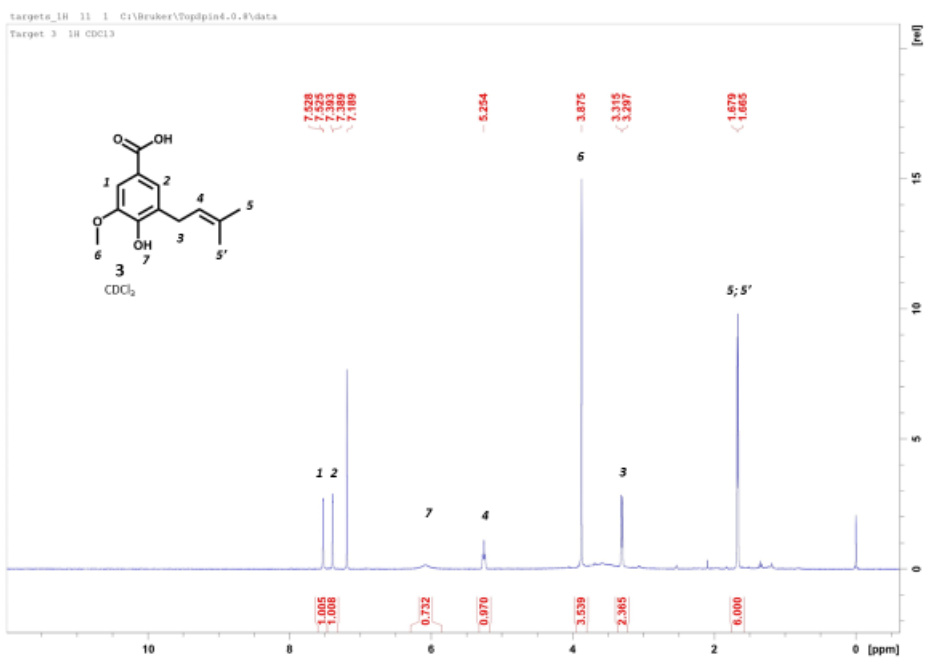
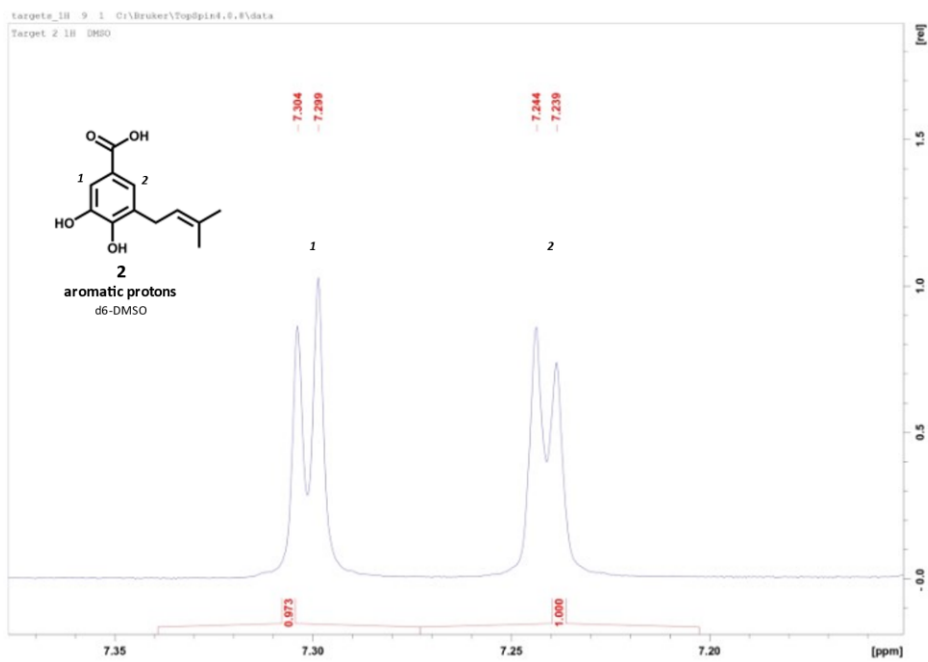
Accession code: OQ859719

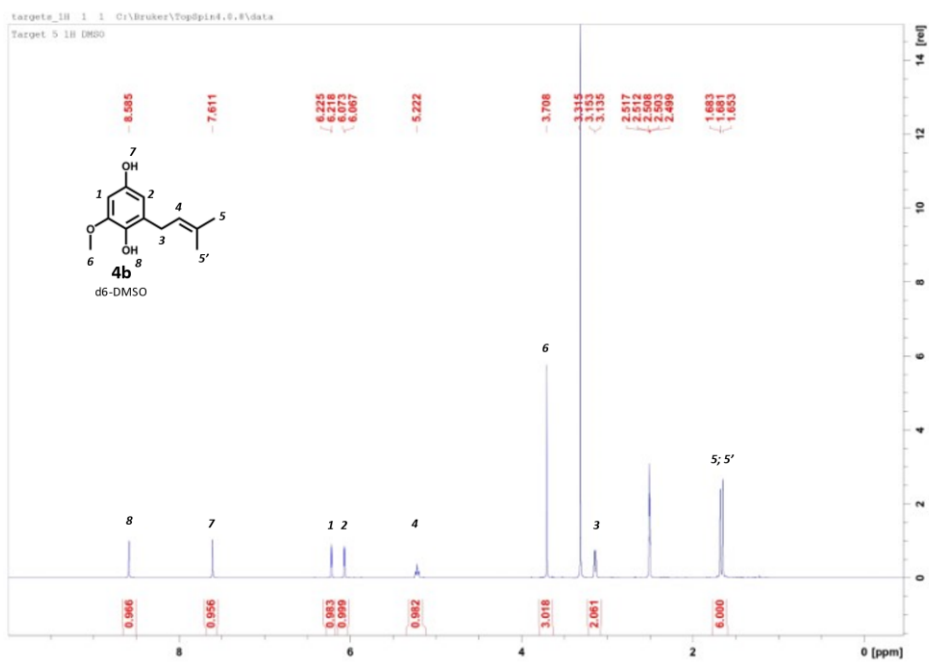
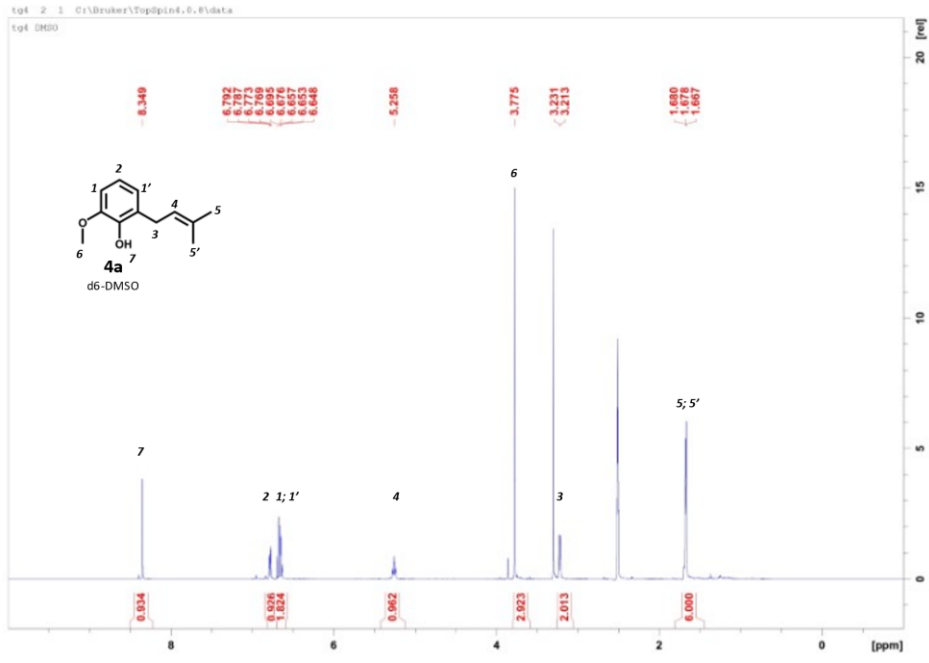
>Tetrapod ancestor FDX2

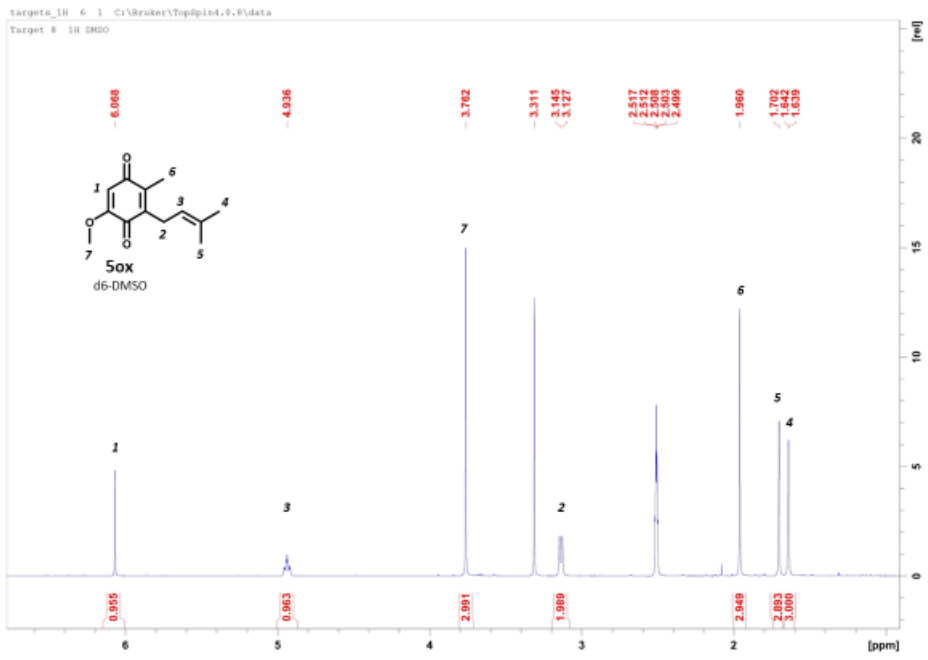
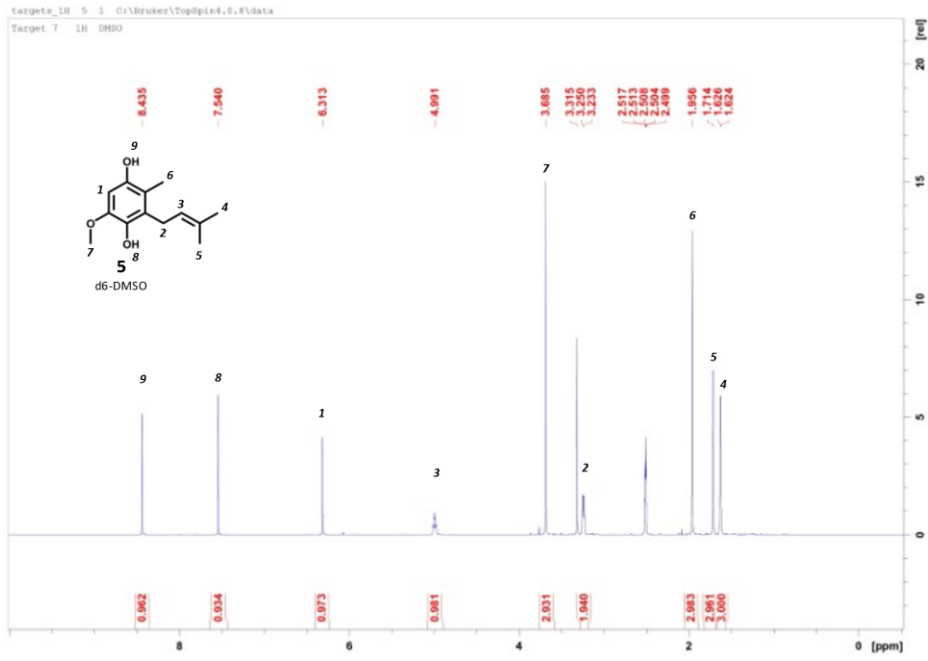
MAASMVARGVAAGCLLRACGGCLFSRPGGCRRRSRAASRAPARGFQTA**G**AHQAEESQAPEPSDDVVNV  
VFIDRSGQRIPVKGVGDNVLYLAHKHGIDLEGACEASLACSTCHVYVSEEFLDKLEPDEREDDMLDMAPL  
LQENSRLGCQIILTKELEGAFTLPKITRNFYVDGHVPKPH

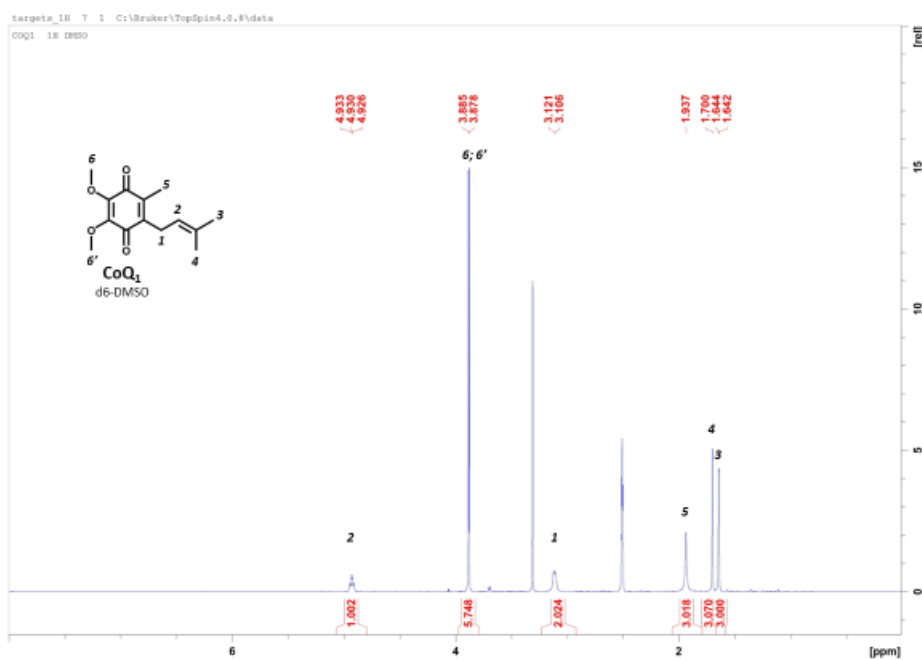
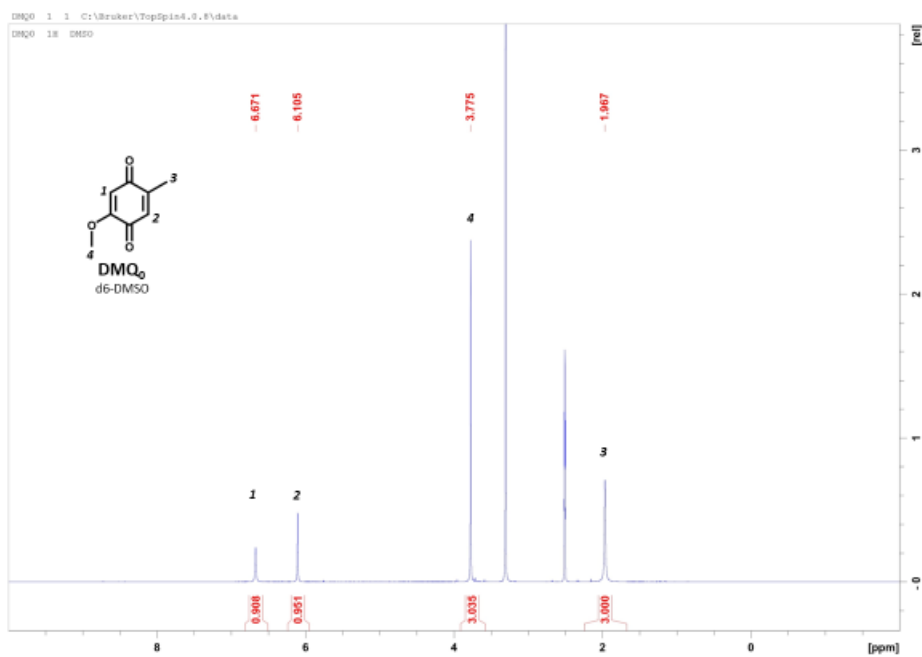
**Supplementary Figure 14. List of reconstructed sequences.** Each ancestral sequence used in this study is shown separately including: the posterior probability for reconstruction, final amino acid length, the number of ambiguously reconstructed sites, sequence identity compared to the human orthologue, accession code and sequence description, and final sequence. The amino acid labelled in red corresponds to the final residue removed in the truncated construct. The graphs shown illustrate the posterior probability for the reconstructed amino acid at each site across the polypeptide. The final posterior probability for the entire reconstruction is shown as PP.



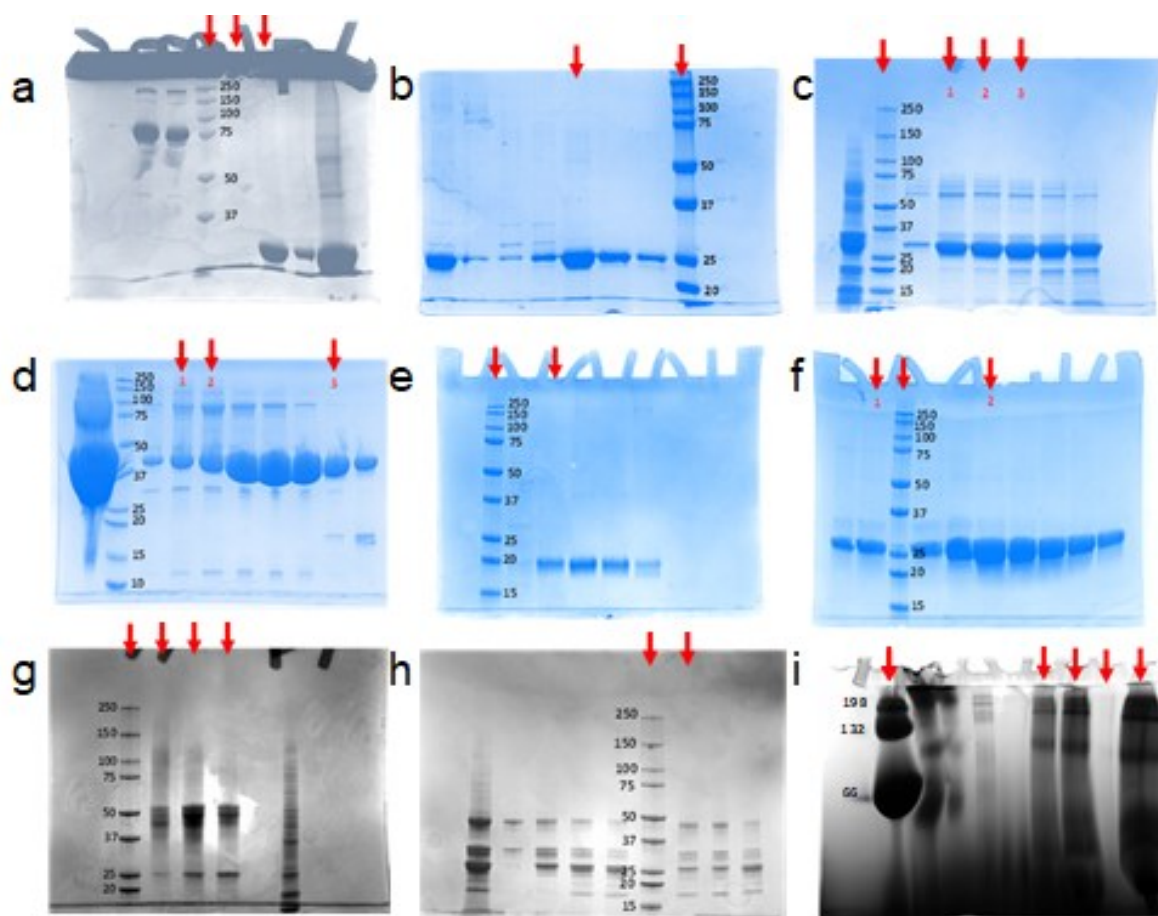








**Supplementary Figure 15. NMR spectra of the CoQ compounds.** NMR spectra of all compounds employed in this study: **1**; **2**; close-up spectrum for the aromatic H atoms illustrating their  $J$  coupling ( $J = 2$  Hz); **3**; **4a**; **4b**; **5**; **5<sub>ox</sub>**; **5<sub>ox</sub>** non-prenylated (denoted as **5<sub>ox0</sub>**); CoQ<sub>1</sub>.



**Supplementary Figure 16. SDS- and Blue Native-PAGE gels.** Each lane used in a separate figure is indicated with a red arrow. The molecular weight ladder has its bands labelled with their individual respective molecular weights (kDa) shown. Where necessary, gels that have individual lanes cut out and reassembled for a separate figure are labelled and numbered in red in accordance with their respective figure panel. Expected molecular weights for each COQ protein are shown in [Supplementary Table 2](#), except for COQ8B which has an expected molecular weight of 55.553 kDa. Panels a-g can be found in [Extended Data Fig. 1](#); Panel h is displayed in [Extended Data Fig. 7](#); Panel i is present in [Supplementary Fig. 8](#). **a.** SDS-PAGE analysis of COQ3 after size exclusion chromatography. **b.** SDS-PAGE analysis of COQ4 after size exclusion chromatography. **c.** SDS-PAGE analysis of COQ5 after size exclusion chromatography. **d.** SDS-PAGE analysis of COQ6 truncated after size exclusion chromatography. Due to the presence of a large band skewing the molecular weight ladder, in [Extended Data Fig. 1d](#) an identical ladder is used to depict the molecular weights **e.** SDS-PAGE analysis of COQ7 after size exclusion chromatography. **f.** SDS-PAGE analysis of COQ9 after size exclusion chromatography. **g.** SDS-PAGE analysis of COQ8B after size exclusion chromatography. **h.** SDS-PAGE analysis of the COQ metabolon after size exclusion chromatography. **i.** Blue Native-PAGE analysis of the COQ metabolon after size exclusion chromatography.



## Supplementary Tables

Target mutation	Mutagenesis cycle number	Target strand	Sequence
H142A	1	F	AGAGAAGTAGCGGACTTATTGCATACGTTACTTGG
H142A	1	R	CAATAAGTCCGCTACTTCTCTATAACGCTGGATCAC
H146A	2	F	GACTTATTGGCGACGTTACTTGGCATGCCAACTAA
H146A	2	R	AAGTAACGTCGCCAATAAGTCGTGTACTTCTCTAT
D143A	1	F	GAAGTACACGCGTTATTGCATACGTTACTTGGCATG
D143A	1	R	ATGCAATAACGCGTGTACTTCTCTATAACGCTGG
E158A	2	F	ATGTTAGGAGCGGTCGTCGTAAAGTGGTTTGAAGC
E158A	2	R	TACGACGACCGCTCCTAACATATTAGTTGGCATG
Sequencing primer		F	AACAGAGTCTCGCCAGAC

**Supplementary Table 1.** List of primers employed for site-directed mutagenesis of COQ4 (GST construct).

Protein	m/z	z	Peptide	Mass (Da)	Mr (Calc)	Delta	Start	End
<b>COQ6</b> Score 99% Coverage 32.3% 48.295 kDa	384.2254	2	RLYSTK	766.4362	766.4337	0.002502441	404	409
	425.743	2	FKPFR	849.4715	849.49725	-0.025756836	84	89
	457.7953	2	VAPFVLLR	913.5761	913.5749	0.001159668	410	417
	511.7891	2	VALIGDAAHR	1021.564	1021.56683	-0.003234863	333	342
	528.2665	3	KVFDQLPESYSNR	1581.778	1581.7786	-0.000976563	46	58
	535.6498	3	HNLPLMAAIDLLKR	1603.928	1603.9233	0.004394531	391	404
	554.6213	3	AMFPLGLGHATEYVR	1660.842	1660.8396	0.002441406	316	330
	559.6355	3	QLEAVSDRVEVLYR	1675.885	1675.8892	-0.004394531	127	140
	561.3557	2	ILLLEAGHKK	1120.697	1120.6968	0	37	46
	595.2949	2	HLLEYETER	1188.575	1188.5776	-0.002319336	380	388
	687.3651	2	LLIGADGQNSMVR	1372.716	1372.7131	0.002441406	175	187
	695.8727	2	SALSLLMPSGTSAR	1389.731	1389.7285	0.002319336	288	301
	706.9097	2	TLGLQATNALSPVK	1411.805	1411.8032	0.001708984	418	431
	724.92	2	HNLPLMAAIDLLK	1447.826	1447.8223	0.003295898	391	403
	727.8485	2	VFDQLPESYSNR	1453.682	1453.6836	-0.001220703	47	58
806.7721	3	TLGLQATNALSPVKEQIMAFASK	2417.294	2417.2986	-0.004150391	418	440	
838.95	2	QLEAVSDRVEVLYR	1675.885	1675.8892	-0.00378418	127	140	
<b>COQ3</b> Score 99% Coverage 32.2% 31.086 kDa	316.2001	2	VPFIR	630.3857	630.3853	0.000366211	45	49
	349.6957	2	TAELHK	697.377	697.37585	0.001098633	103	108
	359.5301	3	SFDPVLDKR	1075.569	1075.5662	0.002441406	109	117
	407.7319	2	FQALAHK	813.4492	813.4497	-0.000488281	20	26
	518.5967	3	IMGIVPAGTHDWEK	1552.768	1552.7708	-0.002319336	190	203
	557.2978	3	LGASVTGIDPLEDNIR	1668.872	1668.8682	0.003417969	87	102
	560.2889	2	FISPEELER	1118.563	1118.5608	0.002563477	204	212
	760.4287	2	TQLSYALGIVVAER	1518.843	1518.8403	0.002441406	176	189
	835.4404	2	LGASVTGIDPLEDNIR	1668.866	1668.8682	-0.001953125	87	102
	1051.008	2	DTLLNMSGDHLQGSPLSGMK	2100.001	2099.998	0.002441406	50	69
<b>COQ4</b> Score 99% Coverage 23.7% 27.871 kDa	430.7578	2	LSTLDLAK	859.5011	859.5014	-0.000305176	91	98
	468.2332	2	SLPDGDFGR	934.4518	934.45087	0.000976563	101	109
	557.2978	3	NARCVLNIIYERR	1668.872	1668.8518	0.01977539	212	224
	664.8837	2	CVLNIYERR	1327.753	1327.6707	0.08215332	215	224
	742.3789	3	AAEEGYGPLYPGHIPTSPQLK	2224.115	2224.1165	-0.001708984	13	33
	798.8998	2	FVDDEELAYVIQR	1595.785	1595.783	0.002075195	130	142
	835.4404	2	NARCVLNIIYERR	1668.866	1668.8518	0.014404297	212	224
<b>COQ5</b> Score 99% Coverage 20.3% 32.653 kDa	363.7337	2	VLKPGGR	725.4529	725.4548	-0.00189209	194	200
	553.2802	3	NVTHIDQALQEAYR	1656.819	1656.822	-0.003417969	180	193
	598.8177	2	VYQVFENNAK	1195.621	1195.6238	-0.002929688	26	35
	629.3242	2	SYQLVESIR	1256.634	1256.6401	-0.006347656	239	248
	842.4482	3	LYDLYSFQVIPVLGEVIAGDWK	2524.323	2524.3252	-0.002441406	217	238
<b>COQ7</b> Score 91% Coverage 20.3% 20.581 kDa	589.8202	2	IYAGQMAVLGR	1177.626	1177.6277	-0.001831055	32	42
	790.0207	2	YKELLQIHKFR	1578.027	1577.9656	0.061279297	130	141
<b>COQ9</b> Score 15% Coverage 22.6% 26.686 kDa	553.2802	3	INDAMNMGHTAKQVK	1656.819	1656.8076	0.010986328	198	212
	559.6355	3	KKTDQFLRDAVEAR	1675.885	1675.9004	-0.015625	97	110
	621.3946	2	LVQLGQAEKKK	1240.775	1240.7502	0.024414062	88	98
	838.95	2	KKTDQFLRDAVEAR	1675.885	1675.9004	-0.015014648	97	110
	1051.008	2	QVKSTGEALVQGLMGAAVTLK	2100.001	2100.1614	-0.16088867	210	230

**Supplementary Table 2. Peptide mapping analysis of the purified COQ metabolon.** The table lists the peptides obtained from the labelled band excised from the Blue Native Gel shown in **Supplementary Figure 8**. COQ9 shows a weaker signal suggesting that it could be less well retained by the metabolon during migration.

## Supplementary Methods

*Construct design, cloning, mutagenesis and expression of the COQs, FDXR and FDX2* - The ancestral sequences were submitted to the Mitofates server for inspection [1]. Ancestral COQ systems - COQ6, COQ3, COQ4 and COQ7 - were all truncated at the end of their mitochondrial targeting sequences. Differently, COQ9 was aligned against the human construct, COQ9\_N79 [2], as a reference to determine the cleavage site in the highly disordered N-terminal region. FDXR, FDX2, COQ5, COQ8A and COQ8B were all designed after the release of AlphaFold2 [3]. They were constructed based on a combination of both AlphaFold2 and Mitofates to predict the mitochondrial targeting sequences and additional disordered regions at N-terminus or C-terminus. All sequences have been deposited in the NCBI database (see Data Availability and [Supplementary figure 14](#)).

COQ6 was constructed as two forms: COQ6, accounting for the full-length sequence and truncated COQ6 bearing a 35 residue C-terminal truncation. The determination of this truncation was based on limited proteolysis by trypsin digestion experiments that suggested this region was highly flexible. Additionally, the truncation was very similar to that introduced for the successfully crystallized bacterial homologue Ubil [4].

All genes were synthesised, codon optimized for expression in *E. coli* and cloned directly into several different destination vectors (see below) by Genscript. COQ4, truncated COQ6, COQ6 and COQ7 were synthesised with an N-terminal His<sub>6</sub>-SUMO tag and cloned into pET-11d pre-destination vectors bearing ampicillin resistance. COQ3, COQ4 and COQ9 were synthesised with N-terminal His<sub>6</sub>-SUMO tag and cloned into pET-24a(+) pre-destination vectors possessing Kanamycin resistance. COQ5 was synthesised with an N-terminal Twinned-Strep tag bearing a PreScission protease cleavage site and cloned into a kanamycin-resistant pET-24(a)+ pre-destination vector. COQ3, COQ4, COQ5, COQ7, COQ8A, COQ8B, FDXR and FDX2 were all synthesised as tag-less proteins and inserted into a pGEX-6P-1 destination vector possessing an N-terminal GST-tag and PreScission protease cleavage site together with ampicillin resistance.

COQ4 double mutants were obtained by two subsequent cycles of site-directed mutagenesis PCR using the pGEX-6P-1 construct as template. Primers were purchased from Metabion ([Supplementary Table 1](#)). Mutagenesis was performed using the QuickChange methodology, as reported [5]. Briefly a PCR mix (25 µl) was prepared as follows: 12.5 µl PFuUltra II Hotstart PCR Master Mix (Agilent), 1 µl primer forward 10 µM, 1 µl primer reverse 10 µM, 1 µl of template plasmid 100 ng µl<sup>-1</sup>, 0.4 µl DMSO and 9.1 µl MQ water. Mutations were checked at each cycle by Sanger sequencing (Mycrosynth).

Plasmids containing COQ6 (both full-length and truncated), COQ3, COQ4 (wild-type and double point mutants), COQ7 and COQ9, were transformed by heat shock into *E. coli* BL21 cells (25 s, 42 °C). COQ5, FDXR, FDX2, COQ8A and COQ8B were transformed by heat shock into *E. coli* BL21-CodonPlus-RP cells (25 s, 42 °C). Cells from the resulting colonies were pre-inoculated into 100 ml of LB broth containing 100 µg ml<sup>-1</sup> of ampicillin and grown overnight at 37 °C. The following morning, 10 mL of the precultures were then transferred to 1 L Terrific Broth and grown at 37 °C, 200 r.p.m. for *ca.* 3 h until the optical density (OD<sub>600</sub>) reached 0.5-0.7. The protein expression was then induced with isopropyl β-D-1-thiogalactopyranoside, (0.1 mM final), and incubated for 16 h at various temperatures at 200 r.p.m. More specifically, COQ6 full-length, truncated COQ6, COQ3, COQ4 (wild type and double point mutants), COQ7 and COQ9 were expressed at 30 °C; COQ8B, FDXR and FDX2 were expressed at 24 °C; COQ5 and COQ8A were expressed at 16 °C. Cells were harvested by centrifugation (5,000 *g*, 15 min, 10 °C), flash frozen in liquid nitrogen, and stored at -80 °C.

*Purification of GST-tagged FDXR, FDX2, COQ3, COQ4, COQ7, COQ8A and COQ8B* - Cells (*ca.* 25 g) were resuspended 1:5 in Buffer A (50 mM HEPES pH 7.2, 250 mM NaCl, 10% (v/v) glycerol) supplemented with protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 10 µM leupeptin, 10 µM pepstatin) and 5 µg/g of cells of DNase I. The cell resuspension was incubated at 4 °C under stirring for 30 min prior to cell lysis performed with a high-pressure homogeniser (Emulsiflex c-3, ATA Scientific) in three cycles.

For soluble proteins (FDXR, FDX2, COQ3) the cell lysate was centrifuged (4 °C, 56,000 *g*, 1 h) using an Avanti J-26 XP centrifuge equipped with a JA-25.15 rotor (Beckman Coulter). The resulting supernatant was filtered through a 0.45 µm filter and loaded onto a gravity column containing Glutathione Sepharose 4B resin (Cytiva) pre-equilibrated with Buffer A. The crude extract was incubated with the resin for 1 h at 4 °C on a rotating plate. Subsequently, the resin was washed with at least five column volumes of Buffer A. The protein was then eluted out with at least ten column volumes of Buffer A supplemented with 50 mM of freshly prepared reduced glutathione. The purified protein sample was concentrated using an Amicon Ultra (Merck) with an appropriate cut-off to 2.5 ml. The reduced glutathione was then removed with a PD-10 desalting column (Cytiva) pre-equilibrated with Buffer A. To cleave the GST-tag the desalted sample was concentrated down to 1 ml and 1 U of GST-tagged PreScission Protease (Cytiva) was added and the sample was incubated overnight at 4 °C on a rotating wheel. The sample was then loaded onto an Äkta Pure system (Cytiva) equipped with a GSTrap-HP 5 ml column (Cytiva). The elution of the tag-less protein

was monitored by following the absorbance at 280 nm for all the proteins. To monitor the flavin cofactor incorporation, the absorbance at 370 nm and 450 nm for FDXR was monitored. The 2Fe-2S cluster absorption at 340 nm and 414 nm for FDX2 was measured.

For membrane proteins (COQ4, COQ7, COQ8A and COQ8B), the cell lysate was exposed to a low-speed centrifugation step (4 °C, 1,200 g, 10 min) to remove cell debris using a C0650 rotor and an Allegra X-30R centrifuge (Beckman Coulter). The supernatant was centrifuged (4 °C, 56,000 g, 45 min) to collect membrane pellet. Pellets were homogenised in Buffer A with a Potter-Elvehjem homogeniser. The total protein concentration was assayed with Biuret reagent based on a calibration line with BSA and adjusted to 10 mg/ml. *n*-Dodecyl  $\beta$ -D-maltoside (DDM, Anatrace) was added to the membrane suspension at 1% (w/v) final concentration and incubated overnight at 4 °C. The detergent solubilised extract was obtained by centrifugation (4 °C, 56,000 g, 45 min) collecting the supernatant. The recombinant GST-tagged membrane COQ proteins were then purified as described above for soluble systems, supplementing 0.05% (w/v, final) DDM to all buffers.

The purified proteins were then concentrated down to about 150  $\mu$ M, assessing the concentration using a NanoDrop ND-100 UV/Vis spectrophotometer (Thermo Scientific) based on the absorption of the prosthetic groups ( $\epsilon_{450 \text{ nm}} = 10.9 \text{ mM}^{-1}\text{cm}^{-1}$  for FDXR,  $\epsilon_{414 \text{ nm}} = 11 \text{ mM}^{-1}\text{cm}^{-1}$  for FDX2) or on the absorption at 280 nm for the other proteins ( $\epsilon_{280 \text{ nm}}$  predicted by ProtParam tool - ExPASy). Purity of the samples were evaluated by SDS-PAGE. The purified proteins were then aliquoted, flash frozen in liquid nitrogen and stored at -80 °C.

*Purification of HisSUMO tagged COQ6, COQ3, COQ4, COQ7 and COQ9* - Cells (ca. 25 g) were resuspended 1:5 in Buffer A (50 mM HEPES pH 7.2, 250 mM NaCl, 10% (v/v) glycerol) supplemented with protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 10  $\mu$ M leupeptin, 10  $\mu$ M pepstatin) and 5  $\mu$ g/g of cells of DNase I. The cell resuspension was incubated at 4 °C under stirring for 30 min prior to cell lysis performed with a high-pressure homogeniser (Emulsiflex c-3, ATA Scientific) in three cycles.

For soluble proteins (truncated COQ6, COQ3, and COQ9) the cell lysate was centrifuged (4 °C, 56,000 g, 1 h) using an Avanti J-26 XP centrifuge equipped with a JA-25.15 rotor (Beckman Coulter). The resulting supernatant was filtered through a 0.45  $\mu$ m filter and loaded onto a gravity column containing Nickel Sepharose High Performance IMAC resin (Cytiva) pre-equilibrated with Buffer A. The crude extract was incubated with the resin for 1 h at 4 °C on a rotating plate. Subsequently, the

resin was washed with at least five column volumes of Buffer A. The protein was then washed with 5 column volumes of Buffer A supplemented with increasing concentrations of imidazole including 5- and 30-mM wash steps. The protein was then eluted out with at least ten column volumes of Buffer A supplemented with 300 mM imidazole. The purified protein sample was concentrated using an Amicon Ultra (Merck) with an appropriate cut-off to 2.5 ml. The imidazole was then removed with a PD-10 batch desalting column (Cytiva) pre-equilibrated with Buffer A. To cleave the His-SUMO tag the desalted sample was concentrated down to 1 ml and the sample was then mixed with a 6×His-tagged SUMO protease (1.2 mg ml<sup>-1</sup>) to a volume ratio of 100:1 and incubated overnight at 4 °C on a rotating well. The sample was then loaded onto an Äkta Pure system (Cytiva) equipped with a Ni HiTrap-HP 5 ml column (Cytiva) pre-equilibrated in Buffer A. Proteins were eluted out using an imidazole concentration gradient of 2% buffer B (Buffer A supplemented with 300 mM imidazole) corresponding to 6 mM imidazole. The elution of the tag-less protein was monitored with a multiwavelength detector following the absorbance at 280 nm for all the proteins. For COQ6, the absorption at 370 nm and 450 nm for COQ6 were also measured to account for FAD retention.

For membrane proteins (COQ3, COQ4, COQ6, COQ7) the cell lysate was exposed to a low-speed centrifugation step (4°C, 1,200 *g*, 10 min) to remove cell debris using a C0650 rotor and an Allegra X-30R centrifuge (Beckman Coulter). The supernatant was centrifuged (4 °C, 56,000 *g*, 45 min) to collect membrane pellet. Pellets were homogenised in Buffer A with a Potter-Elvehjem homogeniser. The total protein concentration was assayed with Biuret reagent based on a calibration line with BSA and adjusted to 10 mg/ml. *n*-Dodecyl β-D-maltoside (DDM, Anantrace) was added to the membrane suspension at 1% (w/v) final concentration and incubated overnight at 4 °C. The detergent solubilised extract was obtained by centrifugation (4 °C, 56,000 *g*, 45 min) collecting the supernatant. The recombinant His-SUMO-tagged membrane COQ proteins were then purified as described above for soluble systems, supplementing 0.05% (w/v, final) DDM to all buffers.

The purified proteins were then concentrated down to about 150 μM, assessing the concentration using a NanoDrop ND-100 UV/Vis spectrophotometer (Thermo Scientific) based on the on the absorption at 280 nm ( $\epsilon_{280}$  nm predicted by ProtParam tool - Expasy). Purity of the samples was evaluated by SDS-PAGE. The purified proteins were then aliquoted, flash frozen in liquid nitrogen and stored at -80 °C.

*Purification of Strep tagged COQ5* - Cells (ca. 15 g) were resuspended 1:5 in Buffer A (50 mM HEPES pH 7.2, 250 mM NaCl, 10% (v/v) glycerol) supplemented with protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 10  $\mu$ M leupeptin, 10  $\mu$ M pepstatin) and 5  $\mu$ g/g of cells of DNase I. The cell resuspension was incubated at 4 °C under stirring for 30 min prior to cell lysis performed with a high-pressure homogeniser (Emulsiflex c-3, ATA Scientific) in three cycles.

The cell lysate was exposed to a low-speed centrifugation step (4°C, 1,200 g, 10 min) to remove cell debris using a C0650 rotor and an Allegra X-30R centrifuge (Beckman Coulter). The supernatant was centrifuged (4 °C, 56,000 g, 45 min) using an Avanti J-26 XP centrifuge equipped with a JA-25.15 rotor (Beckman Coulter) to collect membrane pellet. Pellets were homogenised in Buffer A with a Potter-Elvehjem homogeniser. The total protein concentration was assayed with Biuret reagent based on a calibration line with BSA and adjusted to 10 mg/ml. FOS-choline 12 (Anatrace) was added to the membrane suspension at 1% (w/v) final concentration and incubated overnight at 4 °C. The detergent solubilised extract was then obtained the following day by centrifugation (4 °C, 56,000 g, 45 min) using an Avanti J-26 XP centrifuge equipped with a JA-25.15 rotor (Beckman Coulter) and collecting the supernatant. The crude extract was loaded on 1 ml of Strep-Tactin XT Sepharose (Cytiva) after an incubation of approximately 1 h. Subsequently, the resin was washed with at least five column volumes of Buffer A + 0.05% (w/v, final) DDM. The protein was then eluted with 5 column volumes of Buffer A supplemented with 0.05% (w/v) DDM and 5 mM Biotin. The purified protein sample was concentrated using an Amicon Ultra (Merck) with an appropriate cut-off to 2.5 ml. The Biotin was then removed with a PD-10 batch desalting column (Cytiva) pre-equilibrated with Buffer A + 0.05% (w/v, final) DDM.

To cleave the Twinned-Strep-Prescission tag the desalted sample was concentrated down to 1 ml and the sample was then mixed with 1 U of GST-tagged Prescission Protease (Cytiva) and incubated overnight at 4 °C on a rotating well. The sample was then loaded onto an Äkta Pure system (Cytiva) equipped with a GSTrap-HP 5 ml column (Cytiva) attached *in-tandem* to a Strep-XT-Trap 5 mL column (Cytiva). The tag-less protein eluted in the flow-through using Buffer A + 0.05% (w/v) DDM and monitored with a multi-wavelength detector following the absorbance at 280 nm. The purified protein was then concentrated down to about 150  $\mu$ M, assessing the concentration using a NanoDrop ND-100 UV/Vis spectrophotometer (Thermo Scientific) based on the on the absorption at 280 nm ( $\epsilon_{280}$  nm predicted by ProtParam tool - ExPASy). Purity of the sample was evaluated by SDS-PAGE. The purified protein was then aliquoted, flash frozen in liquid nitrogen and stored at -80 °C.

*UV/Visible Spectrometry* - UV/Vis spectra of FDXR and FDX2 were recorded using a Diode Array 8453 UV/Vis Spectrophotometer (Agilent) equipped with 10.00 mm quartz cuvettes (Hellma). About 30  $\mu\text{M}$  FDXR was reduced with a five-fold excess of sodium dithionite. Oxidation was attempted by adding oxygenated buffer. After 30 minutes an equimolar concentration of FDX2 was added to attempt FDXR oxidation. Separately, about 30  $\mu\text{M}$  FDX2 was reduced with a five-fold excess of sodium dithionite. Oxidation was elicited by adding oxygenated buffer. UV/Vis spectra were recorded at each step.

*4-aminoantipyrine spectrophotometric method of phenols/quinones analysis (HRP assay)* - HRP endpoint assay was performed after 10 min reaction of 5  $\mu\text{M}$  truncated COQ6 final volume of 150  $\mu\text{l}$  in Buffer A with 5  $\mu\text{M}$  FDXR, 5  $\mu\text{M}$  FDX2, 1 mM **4a**, 50  $\mu\text{M}$  FAD and 1 mM NADPH at 30 °C 200 rpm in a bench-top incubator. The reaction mixture was supplemented with 2 mM hydrogen peroxide, 0.01 mg ml<sup>-1</sup> HRP, 0.1 M 4-aminoantipyrine. The quenching reaction was carried out at 30 °C 200 rpm for 30 min. The assay was exploited to catalyse the formation of a quinone-imide adduct between the amide moiety of 4- aminoantipyrine and the 4-hydroxy group of the CoQ<sub>1</sub> intermediates using HRP. The various adducts produced various chromophores: Adducts with 4-hydroxybenzoate (such as **3**) or phenols (such as **4a**) produce chromophores with maxima at  $\lambda = 490$  nm; Adducts formed with immature hydroquinones (such as **4b**) produce chromophores with  $\lambda = 450$  nm. Activity was assessed by comparing the shift of the adduct peaks after COQ6 reaction compared to the spectra of the adducts obtained with standard solutions of **4a** and **4b** ([Supplementary Fig. 7b](#)). Spectra were recorded using a Diode Array 8453 UV/Vis Spectrophotometer (Agilent) equipped with 10.00 mm quartz cuvettes (Hellma).

*Malachite Green ATPase assay* - Malachite Green assay was performed after 10 min of reaction of 1  $\mu\text{M}$  COQ8B with 0.1 mM ATP at 30 °C 200 rpm in a bench-top incubator. Activity was tested in the presence of 0.1 mM of each intermediate, or of 1  $\mu\text{M}$  of individual COQ proteins, or a combination of both. Moreover, it was assayed in the presence of the following COQ protein multimers: COQ6-COQ3, COQ7-COQ9 and the COQ metabolon. Basal ATPase activity of COQ8B was assayed by incubating with ATP only. The reactions were quenched by adding 2/3 final of the dye provided by the Malachite Green Phosphate Assay Kit (Sigma-Aldrich). After 10 min of incubation at RT in the dark the absorbance at 620 nm was measured using a ClarioStar plate reader (BMG Labtech) with Greiner 96 transparent flat wells plates. A dilution of the dye in the assay buffer was used as blank.



Absorbance values were converted into  $\mu\text{M}$  concentration of phosphate by building a calibration line using 4-40  $\mu\text{M}$  of phosphate analytical standard provided with the kit (Supplementary Fig. 12). Data were analysed by subtracting the basal activity of COQ8B in the absence of any ligand. Linear regression of the standards and data analysis was performed with GraphPad Prism 9. To determine the  $K_M$  of ATP for COQ8B, we incubated the enzyme (1  $\mu\text{M}$ ) with a range of ATP concentrations (5 – 300  $\mu\text{M}$ ) for 10 minutes at 30 °C at 200 rpm (Supplementary Fig. 12). The analysis was performed as described above. GraphPad Prism 9 was used to perform non-linear regression.

*Limited proteolysis and peptide mapping* - Protein bands were isolated, cut into small fragments and transferred into Eppendorf tubes. The sample was then incubated with (200  $\mu\text{l}$ ) of 100 mM ammonium bicarbonate buffer pH 7.8, 50% acetonitrile, to destain the gel fragments. Acetonitrile (100  $\mu\text{l}$ ) was then added to dehydrate the gel pieces. The organic solvent was removed by air-drying and 100  $\mu\text{l}$  of 10 mM dithiothreitol solution was added to reduce the sample (30 min at 37 °C). 100  $\mu\text{l}$  of 55 mM iodoacetamide was added in place of dithiothreitol and incubated for 45 min at 60 °C. The solution containing gel pieces was decanted and then immersed twice with 200  $\mu\text{l}$  of 100 mM ammonium bicarbonate with 10 minute incubation periods in between. This solution was decanted, and gel pieces re-dehydrated with acetonitrile (100  $\mu\text{l}$ ) until opaque-white in colour. The organic solvent was then removed and the fragments air-dried. Gels were immersed and rehydrated using 100 mM ammonium bicarbonate buffer pH 7.8 (100  $\mu\text{l}$ ), with the addition of sequencing grade trypsin (20 ng/ $\mu\text{l}$ ) (Promega, Madison, WI, USA) for digestion was left overnight at 37 °C. The resultant peptides were extracted using 50% acetonitrile, 5% formic acid solution in water (100  $\mu\text{l}$ ). This was performed twice with each step consisting of vortexing for 15 mins. The original supernatant and sequential extractions were collected, dried, and stored at -20 °C. MS analyses were conducted with peptides solubilized in water and 0.1% formic acid (50  $\mu\text{l}$ ).

A LC unit (ExionLC TM AD) was employed for all analyses. The unit possessed an accompanying column oven set at 40 °C, in addition to a temperature controlled autosampler (10 °C) and a binary gradient pump system. MS instrument comprises a high resolution QTOF mass spectrometer (AB Sciex X500B) with an accompanying Turbo V Ion source and a Twin Sprayer ESI (electrospray ionization) probe, controlled by SCIEX OS 2.1 software. Peptides were separated using Reverse phase (RP) HPLC using a Hypersil Gold (Thermo Fisher Scientific, USA) C18 column (150  $\times$  2.1 mm, 3  $\mu\text{m}$  particle size, 175 Å pore size) using a linear gradient (2-50% solvent B in 15 min), flow rate of 0.2 ml min<sup>-1</sup>, with solvents A and B being a 0.1% aqueous FA solution in water and a 0.1% FA solution

made up in acetonitrile, respectively. Positive polarity was employed for mass spectra generation using the following instrument parameters throughout: declustering potential 100 V, curtain gas 30 psi, temperature 350 °C, ion source gas 1 40 psi, ion source gas 2 45 psi, ion spray voltage 4500 V, collision energy 10 V. SCIEX OS 2.1 software was used to acquire spectra, and processed with Peaks studio 4.5 software for protein identification. Resulting masses were then initially searched and screened against the SwissProt and *ad hoc* databases, followed by specific peptide mapping against the ancestral sequences.

*Intact protein mass spectrometry* – 10 mg ml<sup>-1</sup> COQ3 was incubated O/N at 30 °C 200 rpm in a bench-top incubator with 1 µM COQ8B and 1 mM ATP in a final volume of 50 µl Buffer A with 0.05% DDM (w/v, final). A negative control reaction was performed by removing ATP from the mixture. Sample was diluted up to 2.5 ml Buffer A; buffer was exchanged to Buffer A with a PD10 batch desalting column (Cytiva) to remove any residual detergent. Sample was then concentrated up to 50 mg ml<sup>-1</sup> with Amikon Ultra 10K (Merck). Proteins were unfolded by diluting the sample up to 10 mg ml<sup>-1</sup> in water: acetonitrile 1:1 containing 0.1% FA. A LC unit (ExionLC TM AD) was employed for all analyses. The unit possessed an accompanying column oven set at 40 °C, in addition to an autosampler (10 °C) and a binary gradient pump system. MS instrument comprises a high resolution QTOF mass spectrometer (AB Sciex X500B) with an accompanying Turbo V Ion source and a Twin Sprayer ESI (electrospray ionization) probe, controlled by SCIEX OS 2.1 software. Injection volume was 5 µl (50 µg). Chromatographic separation was carried out with a bioZen™ WidePore C4 column (100 mm length X 2.1 mm diameter, 2.6 µm particle size; Phenomenex). The mobile phase consisted of water (A) and acetonitrile (B) both including 0.1% (v/v) formic acid. Flow rate was set at 0.35 ml min<sup>-1</sup>. Gradient elution was performed as follows: 15% B at 0.0-0.1 minutes, 15-98% B at 0.1-5.0 minutes, 98% B at 5.0-15.0 minutes, 98-15% B at 15.0-18.0 minutes, 15% B at 18.0-23.0 minutes. Positive polarity was employed for mass spectra generation using the following instrument parameters throughout: declustering potential 100 V, curtain gas 30 psi, temperature 350 °C, ion source gas 1 40 psi, ion source gas 2 45 psi, ion spray voltage 4500 V, collision energy 10 V. Mass deconvolution was performed using BiotoolKit extension of SciexOS 2.1 software: input m/z range 500-5000 Da, mass range 20-70 kDa, isotope resolution 30000.

*Micro-Scale Thermophoresis* - COQ3 was labelled with a 2<sup>nd</sup> generation RED-NHS dye (NanoTemper GmbH) following the kit instructions. Briefly, 10 µM protein was incubated at RT in the dark with

300  $\mu$ M dye. The excess of dye was removed with a B-column pre-equilibrated in buffer A with 0.05% DDM (w/v, final). The labelled protein concentration and the degree of labelling were evaluated based on the absorbance at 280 and 650 nm. Binding assays were carried out in Buffer A supplemented with 0.05% DDM (w/v, final), 0.05% (v/v, final) Tween-20 and 0.1% (w/v, final) Pluronic acid. Final protein concentration was 40 nm. **2** was titrated in serial dilution in the 5 mM - 150 nM range. Binding experiments were performed in the presence and absence of 150  $\mu$ M MgCl<sub>2</sub> and in the presence of 1 mM EDTA as a control.

*Nano Differential Scanning Fluorimetry* - Thermo-stability analyses were carried out using a TychoTMNT.6 system (NanoTemper GmbH). The proteins were diluted to 1 mg/ml in Buffer A. The protein was pre-incubated with ligands in ice for 15 minutes prior to experiments. Melting curves were obtained following the intrinsic fluorescence of Tryptophan and Tyrosine residues (emission 350 nm and 330 nm, respectively) applying a temperature gradient from 35 °C to 95 °C for a time range of three minutes. Data were analysed as F<sub>350</sub>/F<sub>330</sub> ratio and derived to determine the inflection temperature (T<sub>m</sub>). All measurements were performed in duplicate. The following conditions were tested: COQ3 in the presence of 150  $\mu$ M CuSO<sub>4</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub>, MnCl<sub>2</sub>, CaSO<sub>4</sub>, CaCl<sub>2</sub>, ZnSO<sub>4</sub>, ZnCl<sub>2</sub>, MgSO<sub>4</sub>, MgCl<sub>2</sub> and 1 mM EDTA; COQ4 in the presence of 25  $\mu$ M CuSO<sub>4</sub>, FeSO<sub>4</sub>, MnSO<sub>4</sub>, MnCl<sub>2</sub>, CaSO<sub>4</sub>, CaCl<sub>2</sub>, ZnSO<sub>4</sub>, ZnCl<sub>2</sub>, MgSO<sub>4</sub>, MgCl<sub>2</sub> and 1 mM EDTA; COQ4 in the presence of ZnCl<sub>2</sub> in the 0.095-24.4  $\mu$ M range. EC<sub>50</sub> values were determined by plotting the difference between melting temperature in presence and absence of ligand versus the logarithm of the molarity of the ligand and analysing the data by non-linear regression.

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