

Genetic bypass of Aspergillus nidulans crzA function in calcium homeostasis

Ricardo S. Almeida[†]*, Omar Loss^{**†††}, Ana Cristina Colabardini[†], Neil Andrew Brown[†], Elaine Bignell^{†††}, Marcela Savoldi[†], Sergio Pantano^{††}, Maria Helena S. Goldman[‡], Herbert N. Arst Jr^{†††}, and Gustavo H. Goldman[†]***

⁺Faculdade de Ciências Farmacêuticas de Ribeirão Preto, [‡]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil; ⁺⁺Biomolecular Simulations Group, Institut Pasteur de Montevideo, Uruguay; ⁺⁺⁺Section of Microbiology, Imperial College London, London SW7 2AZ, United Kingdom; ***Laboratório Nacional de Ciência e Tecnologia do Bioetanol – CTBE, Caixa Postal 6170, 13083-970 Campinas, São Paulo, Brazil

 * Departamento de Microbiologia, Universidade Estadual de Londrina, Londrina, Brazil;
 ** Current address: Medical Research Council (MRC), Cell Biology Unit and Laboratory for Molecular and Cell Biology, Department of Cell and Developmental Biology, University College London, London WC1E 6BT, UK

Corresponding author: Dr. Gustavo H. Goldman Departamento de Ciências Farmacêuticas Faculdade de Ciências Farmacêuticas de Ribeirão Preto Universidade de São Paulo Av. do Café S/N CEP 14040-903, Ribeirão Preto, São Paulo, Brazil Phone: 0055-016-36024280 Fax: 0055-016-36024280 e-mail address: ggoldman@usp.br

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Figure S1 Structural superposition of Human Calcineurin with the *A. nidulans* model. The comparative model of the regulatory subunit of *A. nidulans* calcineurin is presented with the same colouring scheme as Figure 5 (main text). Residues Asp81 and Gly120 are shown in balls and sticks. The structure of the regulatory subunit of the human homologue is superimposed and shown as grey, semi-transparent cartoon. The catalytic subunit is shown in blue. The helix attached to the regulatory subunit corresponds to the autoinhibitory segment. The acidic residue (Asp15 in the human protein, corresponding to Glu35 in A. *nidulans*), which is in the close neighborhood of Gly12 is shown in sticks. The inset shows a closer view of this interaction in a slightly different orientation.

		10 20 30 40 50 60 70 80 90	
Q5B3A1 fol	.A) 1 :	: MAEORVNVHIPSRPAVLDSVRIANN OF PIEA-APDPWHRNGKPOPCTASIKTSYSSAV-AAANADDVSLSLDYCKLYRRISEDIRNMTAGSK :	90
04wxn0	1	: MAEORYNYN IP SHPAYL <mark>D</mark> SWOLENTIOL PLEV - AP DPWHEDGKPOPCTASLKU SYSSAU - AAAAADD YSLSLDYCKU YRRUPEDU RWMGKHSL ;	90
02111.55	1	MARORYNYN TR SHRAWL DSWRIARD TRI DT DA ARRAWHRLCK SOPCTASIACI SYSSAW A SANADDYSLST DYCH ARRABEDT RTMGOHER	90
7007177	1	MADADUMUUT CUDDUT CCUDUT DE DATE DE DATE AND DE DUDE CUCODE TASE VE SUCCEDUT ANTADO VETSE DA ATADO VETSE DA AT	
AZK/K/	1		
QUCP50	1		90
AICKBI	:	MA-EHLSIQIPSPTTVDDSWORKDIQIPIPIGPDPWHRAGKPQPCTASIKDSYSSAIAAAAADDVSLSIDWEKDWRRDBDDRHMGTHGP	89
B8M/H5	1 :	:NLRPATRPSIV <mark>O</mark> NVSIGNINICHOPAADDP/HRSGKSQC/TAAVKISYSSAVAAANADD/SLSIDYCKIYRRUSAAVRDSVKPTS :	84
B6Q3T7	1 :	:NLRPATRPSIV <mark>D</mark> YUSIRNINI.HFPAAPDPMHRPGKSQPCTAAVKLSYSSAVAAANADDVSLSLDY-KLYRRISTAAVRDSVKPQD :	84
C5P9W2	1 :	:VDYINLENTEFPFELIVDPDAWGEPNEPOPAILSLEVAPPRSEINEAAENDHVETTLYSALYKTEPONIEGAIAEAV :	78
C6HRU7	1 :	:PSGF <mark>D</mark> TLLERNINFEFPISFGPDAWDRFGKPQPATLSIRLSYPRPIIAQCGGSDDVSFTLSYEELYRKLOQVIKDATIGED :	81
D4AMK2	1 :	:DYTOLRDVOLPVEVYFQTDAWERTNKPOPAJFSVRISYPAALISLAAENDTVGHILNYGTLYRSIESAAISSNKDTI :	77
F2SEW1	1 :	:smc <mark>d</mark> ytolrdvolpvevylordawrrtnkpopalfsvrlsypaalislaaendtvghfldyctlyrstesatissnkdat :	80
F2PW87	1 :	:DYIOLRNVOLPVPVYLOTDAWRRTNKPOPAUFSVRISYPAALISLAAENDIVGHPLNYGTLYRSUSSAWISSNKDAV :	77
C4JYU6	1 :	:POLDYFVIDYTNIGRNIGFFUPLAFT DAWNRPKKPOPAVISURVAVPRSUIKVASEVDDTPSOLNYSAUYRRUSSAUTRVASAT ;	84
	a) or		
02B3A1 (tol	AJ 91 :	: PSGTTPGQHMISVDG <u>SRREEMMRAELGODVRLTPGIVANCGLGUIDETAAGVRRMAHVHQSRRRS</u> SA <u>SASE</u> ARAANFNSSTSSSST :	176
Q4WXN0	91 :	:SPSQRMVSADGSRRDEMMRTEIGQDVRLTAGIVANCGLGDDETAAGIRRMSHVHCHPGSRRGSASAEIQA-PLAGLSLASP :	171
Q2UL55	91 :	:HPGKRMISLEGSRRNSMMKNDVGQDVRLTAATVANCSLGDDETTAGVRRMSHLHNAASQAPASTS :	156
A2R7K7	91 :	:NPGHRMISVDGSRRDEMLASDVGQDVRLTAGIVAHCGLGLIDETAAGVRRMSHVHNTQRRSSASAGSQSQALAAIFNSASTS :	172
Q0CP50	91 :	:HPGHQLISVEGGRRNSMMETEVGQDVRLTAALVANCALGLIDETAAGVRRMSHVHQGPRHSNLLGTFDFSNSSSTS :	166
A1CKB1	90 :	:AAGQHMVSADGARREEMQRAEPGQDVRLTAGIYANCGLGLLDETAAGIRRMSHVHQSGRGSASASASASAAAAAAAAAAAAAAAAAAAAAA	174
в8м7н5	85	GGHLSOAAETTLSNDVRVIAGLIANCGLGULDETIAGVRRMDHVOAHPESPSRRRASOASRRLS-GVSSSVPKELOTATS	163
B603T7	85		163
C5D9W2	79		118
C6UPII7	82		113
D4MW2	70		126
DARIKZ	10 .	PDENAGDE STR.SS I SKUTUDV ABI I SDAAMKV I WSE I GALEKADODDATT ODKWE I EKKE	130
FZSEWI	81 3	PDENAGDPSTRPSHSKUTVD/ALTISDAAHKVTWSEIGATEHAKDDAVILDHWTTEKKE	139
F2PW87	18	Second State Sta	136
C4JYU6	85 :	:ERAEPADAGEDGNLYLGGLIDLCEEAARAEQDELLSRYGMERAEERAEERAE	124
		•	
,	->	90 1 200 1 210 1 220 1 230 1 240 1 250 1 260 1 270 1 280	
Q5B3A1[fol.	AJ177 :	: GGPIDDVYGQCEVW <mark>LHUDVAUDVAENGLHYR</mark> SSIVWGYRQGSTA-GSDV <mark>SSSER</mark> CPVVLEEGFRIECHVCY <mark>CILGVNSHER</mark> VEKQVVIISIEFK :	269
Q4WXN0	172	:PIDSVYGQCEVWLQLPKALLRAEE <mark>GU</mark> KYRSVTVWGYRQGDESTALDGERCPVVLEEEFRIDGIRC <mark>HCILGVN</mark> SHERVEKQAVIISLEFK :	260
Q2UL55	157 :	:PIDGIFGRCEVWLHUPKALLRAEEGLKYRSVUVWGYKQENEA-AGNLQDSERCPVVLEEEFRIEGIKGH <mark>CILGVNSHERVEKO</mark> AVIVSLEFK :	247
A2R7K7	173 :	:PIDGVFGQCEVSDHIPKALLRAEE <mark>GL</mark> KYRSVTVWGYRQANEAITDDVGESERCPVVLEEEFRIDGIRCY <mark>CILGVN</mark> SHERVEKQAVIVSDEFK :	264
Q0CP50	167 :	:PIDGVFGQCEVWLHLPKALLRADG <mark>CL</mark> HYRSVTRWGYRQPNGE-TPSGETSERCPVVLEEEFRIEGIRCHCILGVNSHERVEKQAVIVSLTFQ :	257
A1CKB1	175 :	:PIDAAFGRCEVWLTUPKALL:AEEGLKYRSVVWGYRQGESGQALDSDRCAVVLEEEFKIDCIRCHCILGVNSHERVEKQAVIVSLEFK :	263
в8м7н5	164	PEVINGMEGECEVILHIPNAHLINGGELSERTVOTWVYADDSAS-LEIAVESSROVAIVEOEFRVEGINGHCHGUNSHERTEKOAVIITIDDER	256
B603T7	164		256
C5D9W2	119	ENT BENTING DVANLE BACHIECT DE CARENAL DU SCESSON DE EREPTEDIR CYCLIAUNEUER RODVOVAL TEX	186
C6HBII7	114		204
D47MW2	127		210
D9 ABIK2	140		210
FZSEWI	127		213
FZPW87	137 :	ETT OF THE AT INVERSE OF CTLUT	210
C4JYU6	125	:KRECTURYPTANICAAG OR CVGSSTGRGREKRECTURNIKGY <mark>CIILCIN</mark> EHBREKOTVDITUVEN :	192
,	->	-290 - 300 - 310 - 320 - 330 - 340 - 350 - 360 - 360	
Q5B3A1[fol	AJ270 :	: G-PCQLAWGSTVVDTYQAWTRAVAERVEETSFQTVEALATFVARIVTVDYANERVTVRVEKPSALAFVEASGVEVTRSQAFFERTA : 354	
Q4WXN0	261 :	: G-PCQLAWGSTVVDTYQETTREVAEQVEETAFQTVEALATFVARIVTVQFANERVTVRVEKPSALSFVERSGIEITRSQAFFEKS- : 344	
Q2UL55	248	: G-PGQLAWGSTVVDTYQAMTRAVAERVEETSFQTVEALATFVARIVTVEFANERVTVRVEKPSALAFVGRSGIEITRSQSFFERS- : 331	
A2R7K7	265	: G-PGQLPWGSTVVNSYQAMTRAVAERVETISFQTVEALATFVARIVTVEFGNERVTVRVEKPSALAFVEGSGVEITRSQAFFE : 346	
Q0CP50	258	: G-PCQLAWGSTVMDTYQA <mark>M</mark> TRAVAEKVETITIFQTVEALATFVARIVTVDEKNERVTVKVEKPSALAFVERSGVEITRSQAFFE : 339	
A1CKB1	264	: G-P-COLAWGSRVVDTYQQMTRTVAERVDEISFOTVEALATFVARIVTVEFGNERVTVRVEKPSALAFVDRSGVEITRSOAFFE : 345	
в8м7н5	257	G-SGEASWASTFLNTYCOMVRVIAERVENTSYOTVEALATFTARTATYDEGNDSVTVLVEKPSAMAFVERAEVOITRSKAFF : 337	
в603т7	257	G-SCEVAWASTFLNTYOEWVRTLAE	
C5P9W2	187		
C6HBII7	205		
D/AME?	200 0	$\frac{1}{2} = \frac{1}{2} = \frac{1}$	
DAREKZ	211		
FZSEW1	Z14 3	: QSR GK THRUTPKET QHOW SKV AESV DKU'S YET VEALATI HAAK I VLUEE PLDEVT VKVE KPSAMAT VAFSG LETTRAASET : 292	
F2PW87	211 :	: QSRCKERMIPKEYQTWSKVAESVDKUSYETVEALAVHAAKIVLVEPLDEVTVRVEKPSAMAFVAFSGLEITRAASFF: 289	
CATVII6	193		

Figure S2A Sequence conservation analysis of FolA.

A PSI-BLAST search was made using the primary sequence of FolA on the UniProt database (<u>www.uniprot.org</u>). Sequences were selected up to E-value = 0.0001. The list of aligned sequences, coloured by conservation, are shown below. Black, dark grey, light grey and white background shading indicate decreasing degrees of conservation. The conserved Asp18, which is mutationally substituted by Asn is indicated by a red background. Underlined residues are sequence motifs which are potentially phosphorylatable by PKA (pink), PKC (green) and casein kinase II (brown).



Figure S2B The low level of identity between FolA and experimentally characterised protein homologues makes the construction of theoretical models difficult. However, comparison of the position occupied by Asp18 with analogous residues in different homologues reveals that this conserved aspartic acid establishes an intermolecular salt bridge with a conserved arginine (Arg207 in FolA) located in a neighbouring subunit (see below). This arginine is indicated by a blue arrow in the multiple sequence alignment. Although it is unlikely that the rather conservative D18N mutation alters the protein structure significantly, it might play a role in the formation of its functional oligomeric form. The figure shows the crystal structure of 7,8-dihydroneopterin aldolase from *Staphylococcus aureus* (PDB id:1DHN, **Crystal structure and reaction mechanism of 7,8-dihydroneopterin aldolase from** *Staphylococcus aureus*. Hennig, M., D'Arcy, A., Hampele, I.C., Page, M.G., Oefner, C., Dale, G.E. Nat. Struct. Biol. (1998) 5: 357-362). Each of the eight protein monomers is represented by a different colour. Asp3 and Arg118 (corresponding to Asp18 and Arg207 in FolA, respectively) are shown using balls and sticks.



Figure S3 Phylogenetic tree based on the amino acid alignment of AN8823 homologues. Phylogenetic tree calculated using the Bootseq, Protdist and Neighbour programs in the Phylip package. The bootstrap values calculated are shown beside their respective branches.

	`
2	1
a	
_	

Consensus	PxIxIT
VIVIT	PVIVIT
NFAT1	PRIEIT
TRESK	PQIIIS
Crz1	PIISIQ
Slm1	PNIYIQ
Slm2	PEFYIE
Hph1	PVIAVN
RCAN1	PSVVVH
Rcn1	GAITID
Rcn2	PSITVN
Rcn1 (Dm)	PAIIVH
folA (mut)	PAVL <mark>N</mark> S

b)

NFAT1	ESILLVPPTWPKPLVP
NFAT2	DOYLAVPOHPYOWAKPKPLSP
NFAT3	MDYLAVPSPLAWSKARIGGH
NFAT4	DQFLSVPSPFTWSKPKPGHT
DSCR1	KQFLISPPASPPVGWKQVEDATP
folA	LRNIQLPLPAAPDPWHRNGKPQP

Figure S4 a) Calcineurin docking sequences in various interacting proteins (adapted from Interaction of calcineurin with substrates and targeting proteins. Huiming Li, Anjana Rao and Patrick G. Hogan, Trends Cell Biol., 2011, 21: 91-103). The mutated residue encoded by *folA1* (D18N) is highlighted in red. b) Sequence alignment of the calcineurin-binding region B in human NFAT1 to -4 and the calcineurin inhibitor DSCR1 (taken from Transcriptional regulation by calcium, calcineurin, and NFAT. Patrick G. Hogan, Lin Chen, Julie Nardone, et al. *Genes Dev.* 2003, 17: 2205-2232). These sequences were realigned including the sequence of FolA using T-coffee with standard options at (<u>http://www.tcoffee.org/</u>XXREF T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. Paolo Di Tommaso, Sebastien Moretti, Ioannis Xenarios, Miquel Orobitg, Alberto Montanyola, Jia-Ming Chang, Jean-François Taly and Cedric Notredame, Nucl. Acids Res. 2011, 39 (suppl 2): W13-W17).

File S1

Mapping of crzA suppressor mutations using classical genetic techniques

The standard parasexual and sexual cycle mapping techniques (Clutterbuck, 1974) were used in conjunction with Clutterbuck's genetic map (www.fgsc.net/Aspergillus/gene_list/index.html) and the (partially) annotated genome sequence (www.fgsc.net/Aspergillus/gene_list/index.html) and the (partially) annotated genome sequence (www.fgsc.net/Aspergillus/gene_list/index.html) and the (partially) annotated genome sequence (www.aspgd.org/) to determine map positions relative to those of identified genes.

Genetic mapping of *cnaB***:** The thermosensitive mutation provisionally designated *scr*A1 and now designated *cna*B1 and the mutation provisionally designated rev2 and now designated *cna*B2 were both located to linkage group I using the parasexual cycle. As both of these mutations showed close linkage to *paba*A1 in preliminary crosses and might therefore be allelic, further localization was done only with *cna*B1. Using selected progeny a cross of genotype *bi*A1 *lys*F51 x yA2 *cna*B2 *paba*A1 was analysed. Of 51 *paba*A⁺ *lysF*⁺ progeny, 3 carried *cna*B2, 9 carried yA2 and 40 carried *bi*A1; of 51 *cna*B⁺ *lys*F⁺ progeny, 24 carried *paba*A1, 15 carried yA2 and 36 carried *bi*A1; of 12 *cna*B+ *paba*A⁺ progeny, 11 carried *lys*F51, 11 carried yA2 and 3 carried *bi*A1. These data clearly indicate the order *bi*A— *y*A—*cna*B—*paba*A—*lys*F.

Genetic mapping of *folA*: The thermosensitive mutation provisionally designated *scr*B2 and now designated *fol*A1 was located to linkage group III using the parasexual cycle. As *scr*C is also in linkage group III (see below), a *fol*A1 x *scr*C3 cross was analysed and indicated free recombination as did a *fol*A1 cross to a strain carrying the *scr*C-linked *hal*A24 and *cbx*A17 mutations. Following crosses indicating free recombination between *fol*A1 and a number of other linkage group III markers, random progeny from a cross involving *mea*B6, *cnx*H3 and *s*C12 suggested linkage and the order *fol*A—*mea*B—*cnx*H—*s*C. A cross involving *fol*A1 and *gal*E9, *mea*B6, *cnx*H3 and *s*C12 gave inconclusive results with regard to gene order with a slight and misleading (owing to insufficient numbers of progeny) indication that *fol*A might lie between *gal*E and *mea*B. Analysis of progeny able to grow at 42° C from a cross of partial genotype *gal*E9 *fol*A1 *mea*B6 *s*C12 x *nud*R825 established that *fol*A is centromere-distal to *nud*R. Finally, analysis of 56 *gap*A⁺ *fol*A⁺ progeny from a cross of relevant partial genotype *gap*AΔ x *fol*A1 *gal*E9 *mea*B6 *cnx*H3 *s*C12 showed that the *gap*A to *gal*E map distance is greater than either the *gap*A to *fol*A to *gal*E map distance. Taken together these mapping crosses plus the genome sequence indicate the gene order as *gap*A—*fol*A—*gal*E—*nud*R—*mea*B—*cnx*H—*s*C.

Genetic mapping of scrC: Mutations provisionally designated rev1 and rev3 and now designated scrC4 and scrC3, respectively, were located to linkage group III using the parasexual cycle. As these suppressor mutations have similar phenotypes, meiotic analysis was done only with scrC3. The first cross to be analysed established close linkage between scrC3 and cbxA17. A subsequent cross involving scrC3, cbxA17 and halAΔ established that scrC lies between cbxA and halA in close proximity to halA.

Table S1 Primers and Lux probes used in this work

An8823 pRS426 5F	5' GTAACGCCAGGGTTTTCCCAGTCACGACGGTTCACAGGTGGATGGA
An8823 pyro 5R	5' GACCCAACAACCATGATACCACTCGGCCACTTATCACTCAAC 3'
An8823 pyro 3F	5' CTGTCGATCATGTGGATGCTGTTGGCTATCAGAATTCTGGGTTTAG 3'
An8823 pRS426 3R	5' GCGGATAACAATTTCACACAGGAAACAGCCTATCCTTATTCGCAACTCCCTGC 3'
An8823_RL	5′ GAGATACGAGACGCAGGTCCG[FAM]G 3′
An8823_FL/RL	5' CCCGTGGCTTCCAACAACAT 3'
An8823 pRS 5F	5' GTAACGCCAGGGTTTTCCCAGTCACGACGGTTCACAGGTGGATGGA
An8823 pyro 5R	5' GACCCAACAACCATGATACCACTCGGCCACTTATCACTCAAC 3'
An8823 pyro 3F	5' CTGTCGATCATGTGGATGCTGTTGGCTATCAGAATTCTGGGTTTAG 3'
An8823 pRS 3R	5' GCGGATAACAATTTCACACAGGAAACAGCCTATCCTTATTCGCAACTCCCTGC 3'
pmcA probe Lux	5'-CGGACCTTCAATGCCTGGTTGTC[FAM]G 3'
pmcA primer Lux	5'-GGGAGGCGTTCAAGTTCGAT-3'
pmcB probe Lux	5'-CGGATTCTTCCAACCCAGACATC[FAM]G 3'
pmcB primer Lux	5'-CCAGACAAAGGTGTTGAAGACGA-3'

Table S2 Analyses of the sexual crossings between the suppressors with wild-type strains

		Phenotypes				
I	Parentals	Ca ^R ts⁺	Ca ^R ts⁻	Ca ^s ts⁺	Ca ^s ts⁻	Total
folA1 crzA∆ cnaB1 crzA∆	GR5 R21	108 255	131 22	53 19	0 0	292 296

Ca^R ts⁺, calcium-resistant and thermoresistant to 44°C. Ca^R ts⁻, calcium-resistant and thermosensitive to 44°C. Ca^S ts⁺, calcium-sensitive and thermosensitive to 44°C. Ca^S ts⁻, calcium-sensitive and thermosensitive to 44°C.