Genòmica funcional en *Sparus aurata*: aplicacions biotecnològiques

Seminaris de Recerca 2014. Facultat de Farmàcia.

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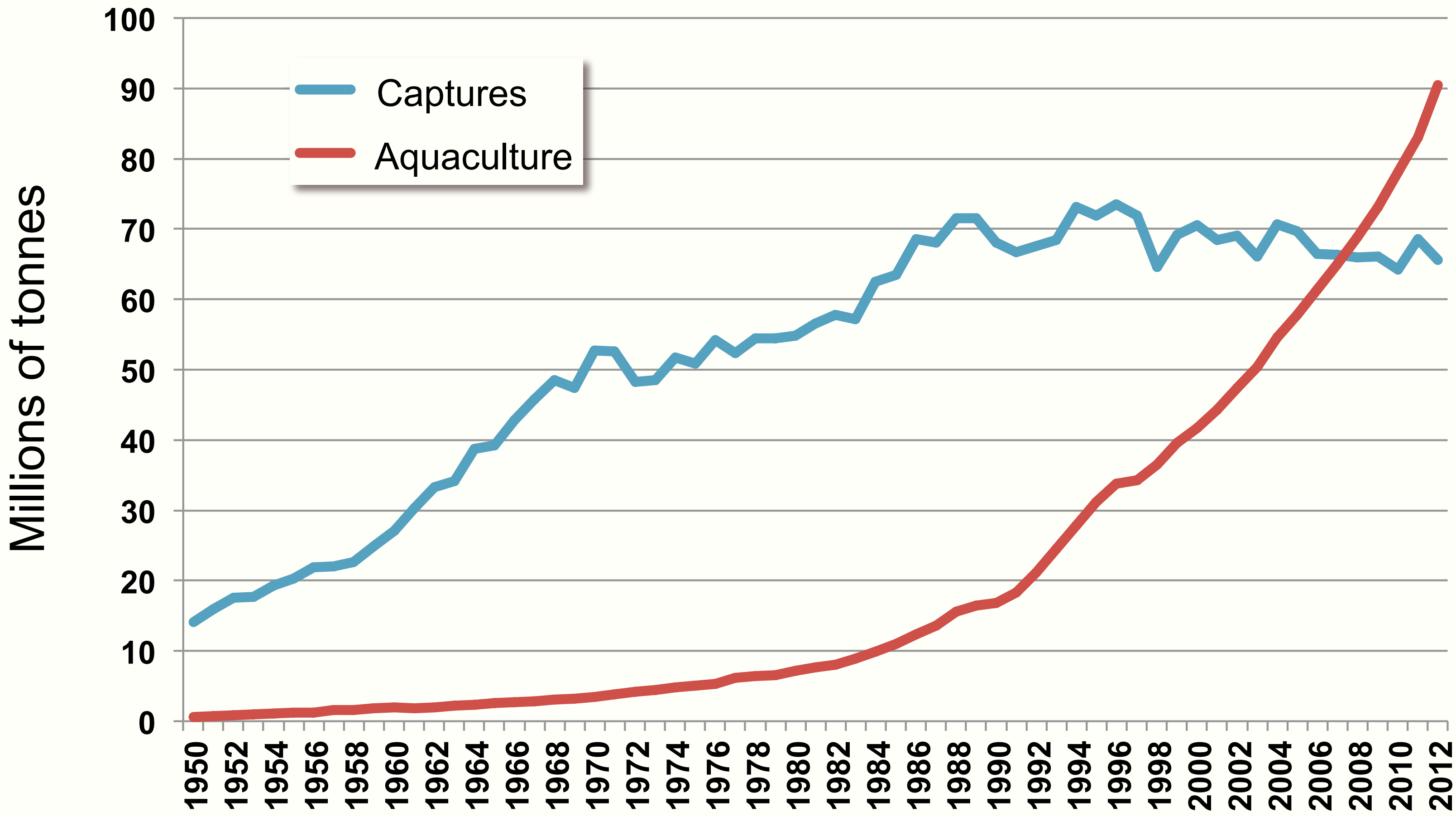
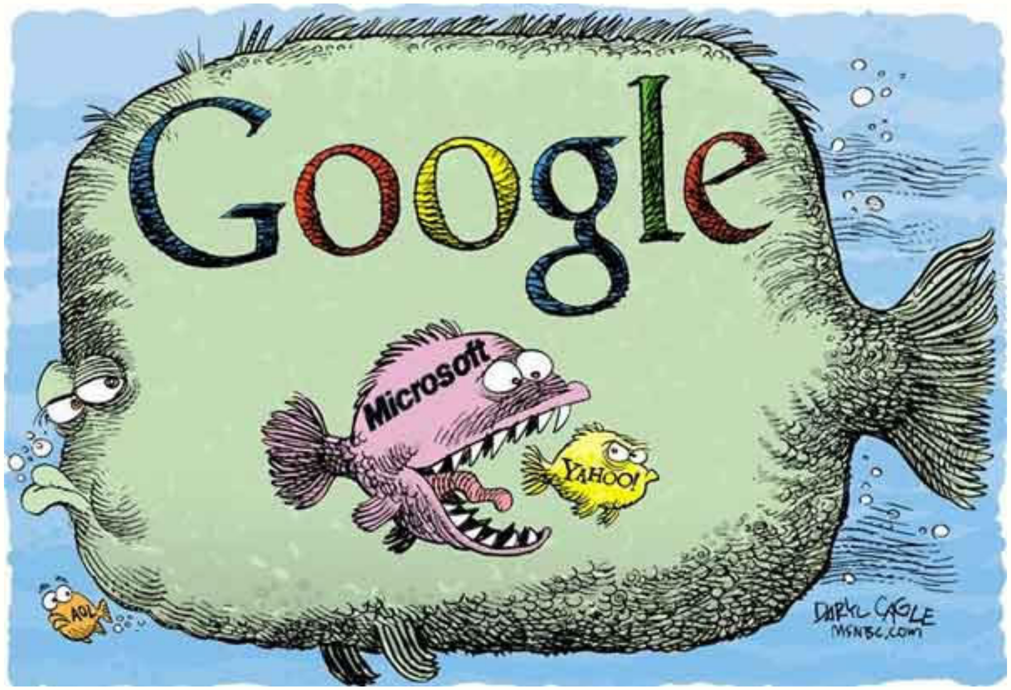
Fish captures and aquaculture in a global world



Fisheries and Aquaculture Department. Food and Agricultural Organization of the United Nations, 2012

Main concerns of aquaculture

- ✓ **High production cost of fishmeals for carnivorous fish (essentially fish flour from low quality fish).**



Main concerns of aquaculture

- ✓ High production cost of fishmeals for carnivorous fish (essentially fish flour from low quality fish).

- ✓ Susteinability of aquaculture:
  - ➢ How to reduce dependence on marine fisheries (often overexploited)?
  - ➢ Environmental impact of extensive aquaculture: Nitrogenous waste causes local water eutrophication.

Main concerns of aquaculture

- ✓ **Partial replacement of dietary protein by other nutrients…**

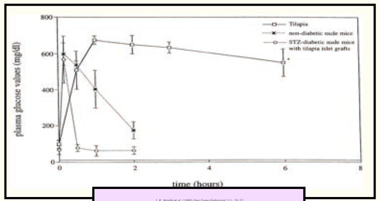
Main concerns of aquaculture

✓ **Partial replacement of dietary protein by other nutrients…**

…carnivorous fish are glucose intolerant!!

Carnivororus fish as a glucose intolerant model

- ✓ **Natural diet: high protein / low carbohydrates.**
- ✓ **Low ability to metabolize dietary carbohydrates.**
- ✓ **Low ability to control blood glucose levels.**

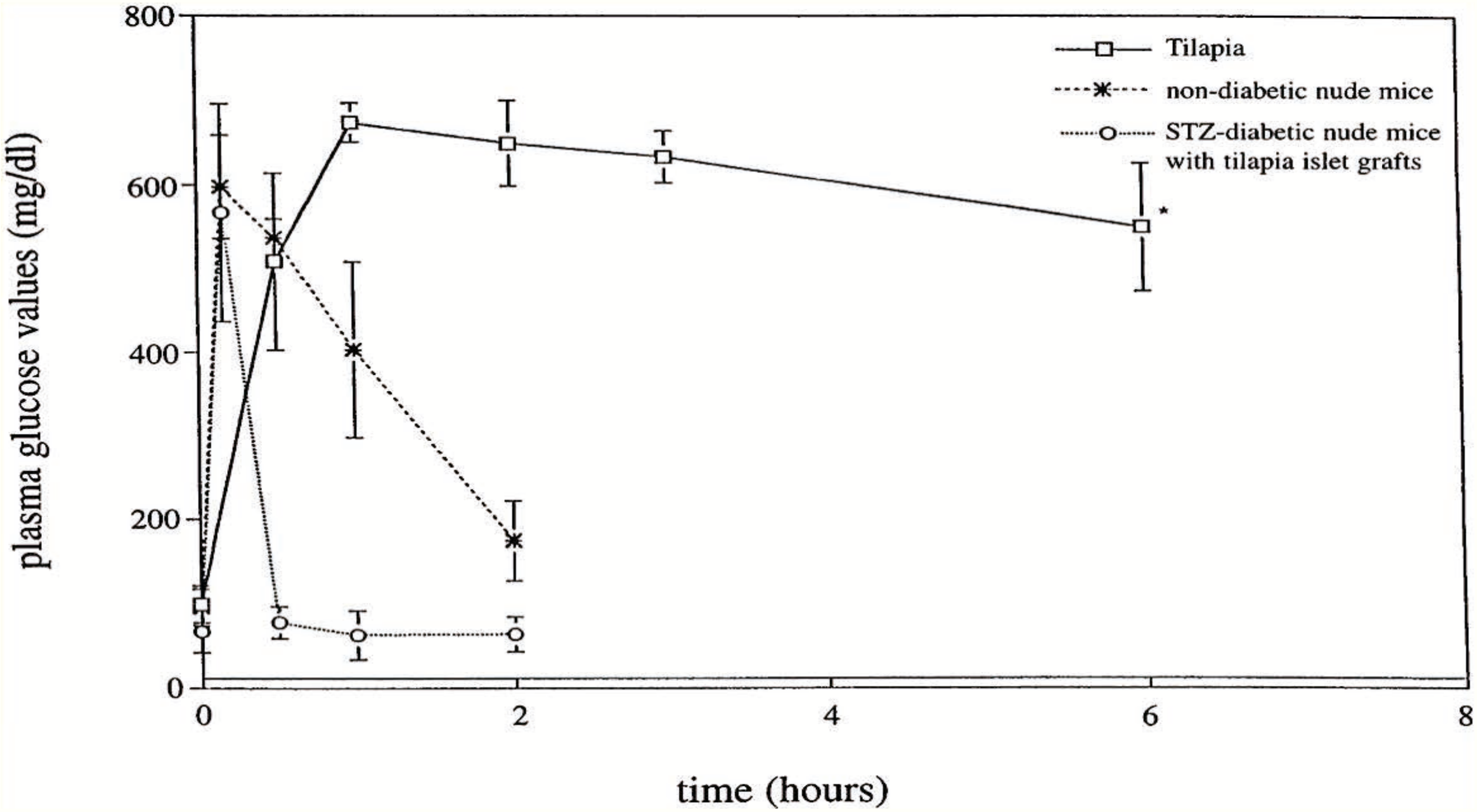


J. R. Wright et al. (1998) *Gen Comp Endocrinol* 111: 20-27

- ✓ **Alternative model to study glucose intolerance and type 2 diabetes.**

Carnivororus fish as a glucose intolerant model

- ✓ Natural di
- ✓ Low ability
- ✓ Low ability
- ✓ Alternativ



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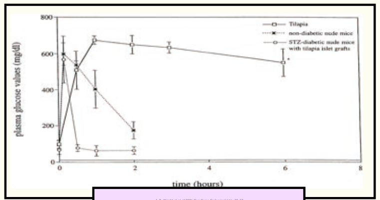

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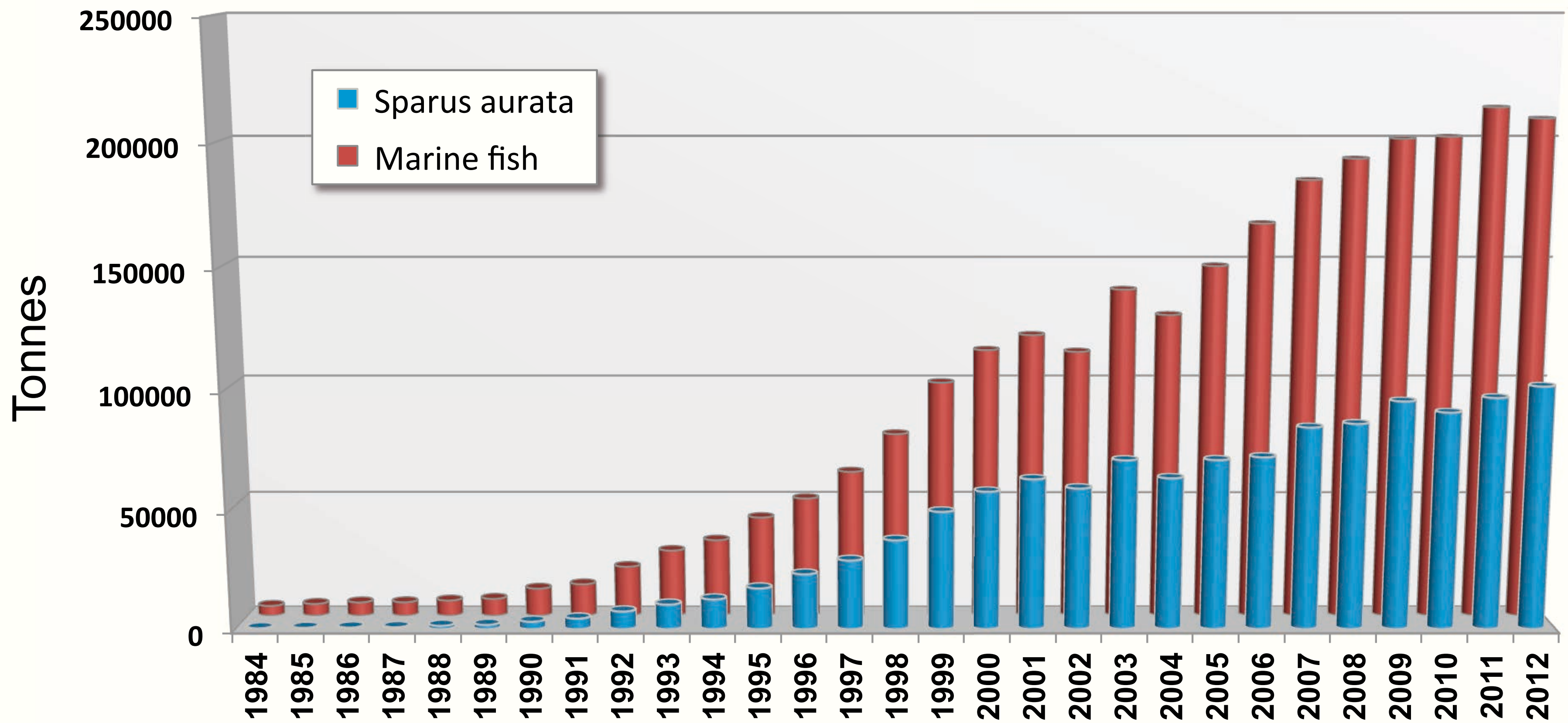
- ✓ **Alternative model to study glucose intolerance and type 2 diabetes.**

Sparus aurata (gilthead seabream, orada)

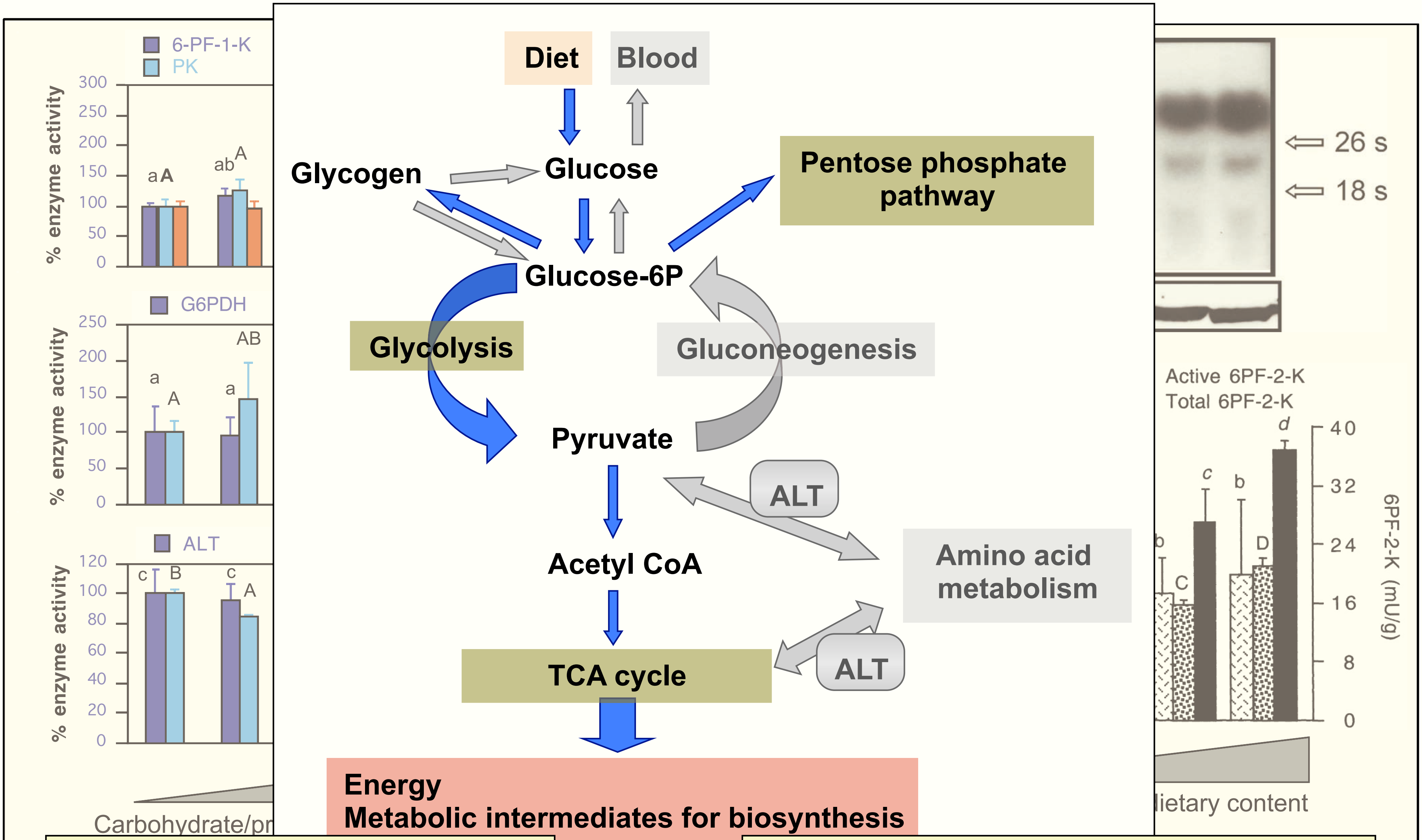
- ✓ **Carnivorous fish**
- ✓ **The most cultured marine fish in Europe**

Marine fish and *Sparus aurata* production in Europe



Fisheries and Aquaculture Department. Food and Agricultural Organization of the United Nations, 2012

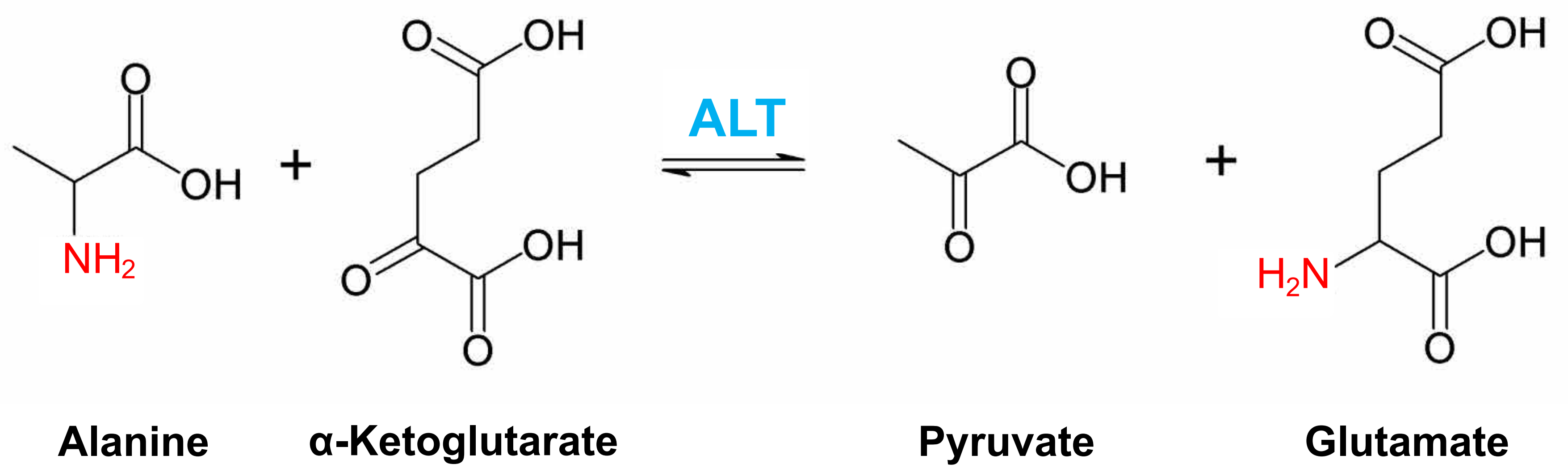
Sparus aurata tolerates partial substitution of dietary protein by carbohydrates



I. Metón et al. (1999) *Br J Nutr* 82: 223-232

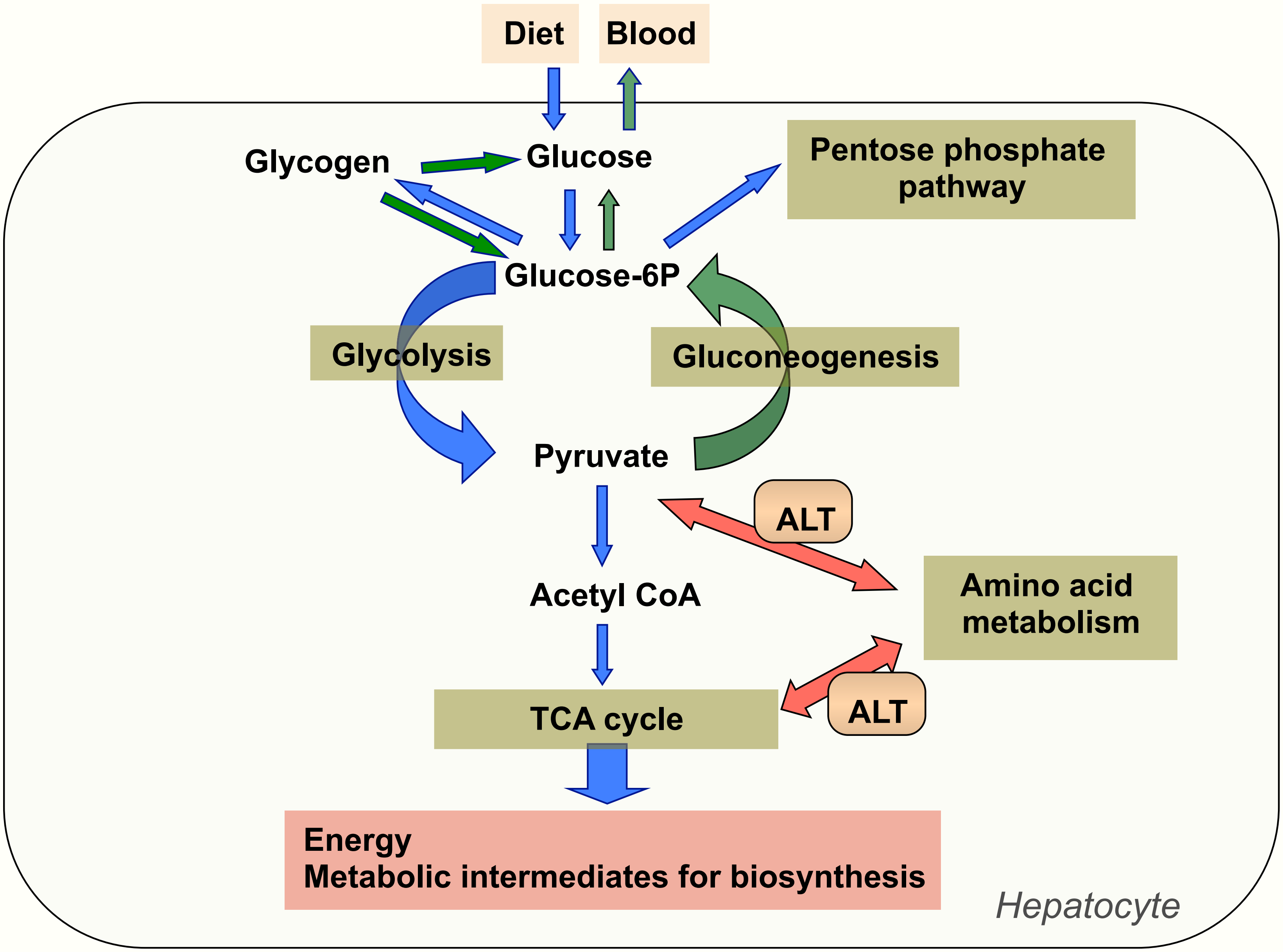
F. Fernández et al. (2007) *J Exp Mar Biol Ecol* 343: 1-10

Alanine aminotransferase (ALT, GPT)



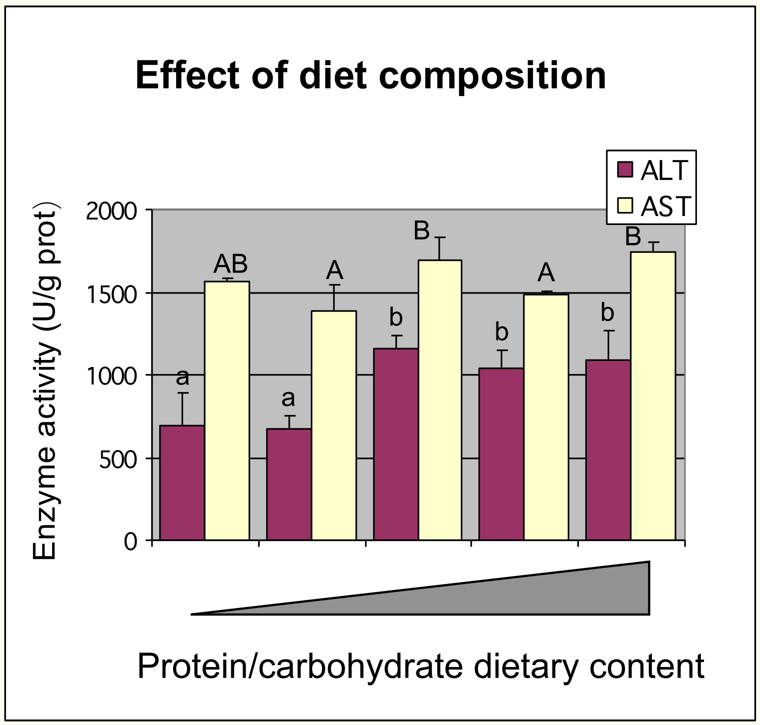
- ✓ Biochemical evidence supports existence of cytosolic and mitochondrial isoforms; their functional role remain unclear.
- ✓ Widely used as a marker for liver injury (hepatitis, cirrhosis, hepatotoxicity). Increased serum activity is also associated to other clinical conditions (fatty liver disease, diabetes, obesity, muscle diseases).
- ✓ ALT is a marker of protein utilization in fish.

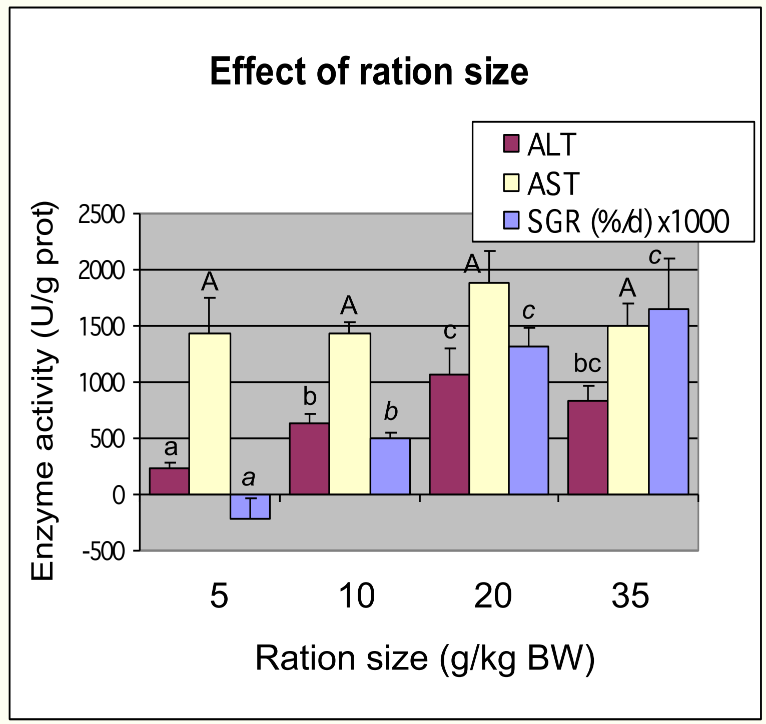
ALT is a molecular link between carbohydrate, amino acid and energy metabolism



Hepatic ALT activity as a biomarker of nutritional status in *Sparus aurata*

Sparus aurata





I. Metón et al. (1999) *Br J Nutr* 82: 223-232

Hypothesis

- ✓ Control of ALT expression may improve the use of dietary carbohydrates for energy purposes while preserving proteins for growth in *Sparus aurata* (protein-sparing effect).

- ✓ This would allow a significant replacement of dietary protein by carbohydrates in aquafeeds for fish farming, and thus reduce the production cost and the environmental and biodiversity impacts of extensive aquaculture.

Objectives

- ➢ **Global objective:**
  - ✓ **To increase the current knowledge on the regulation of the expression of ALT isozymes in *Sparus aurata* to enable a biotechnological action to spare protein and improve the use of dietary carbohydrates.**
- ➢ **Specific objectives:**
  - ① **Cloning and molecular and kinetic characterization of cytosolic and mitochondrial isoforms of ALT from *Sparus aurata*.**
  - ② **Characterization of ALT gene promoters.**
  - ③ **Identification of proteins that may interact with ALT isoforms and eventually regulate the enzyme activity.**
  - ④ **Effect of ALT inhibition on the intermediary metabolism of *Sparus aurata*.**

Specific objective 1

- **Global objective:**
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  ③ Identification of proteins that may interact with ALT isoforms and eventually regulate the enzyme activity.

  ④ Effect of ALT inhibition on the intermediary metabolism of *Sparus aurata*.

Molecular cloning of cALT1 and mALT from *Sparus aurata*

```
Spa cALT  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~MSHQAAN GVPCRGKVLT   17
Spa mALT  MSATRMQLLS PRNVRLLSRG RSELFAGGSG GGPRVRSLIS PPLSSSSPGR ALSSVSATRR GLPKEKMTEN GVSSRAKVLT   77
Hum ALT1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MASS TGDRSQAVRH GL..RAKVLT   22
Rat ALT1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MASR VNDQSQASRN GL..KGKVLT   22
Hum ALT2  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~MQRAAALVR RGCGPRTPSS WGRSQSSAAA EASAVLKVRP ERSRRERILT   46

Spa cALT  VDNMNPRVKK VEYAVRGPVV QRAVQIEKEL REGVKKPFTE VIKANIGDAH AMGQKPITFF RQVLAMCSYP ELLKDNMFPE   97
Spa mALT  IDTMNPTVKK VEYAVRGPIV QRAMELEKEL SEGMKKPFAE VIKANIGDAH AMGQQPITFF RQVLALCSYP ELLNDSTFPE  157
Hum ALT1  LDGMNPRVRR VEYAVRGPIV QRALELEQEL RQGVKKPFTE VIRANIGDAQ AMGQRPITFL RQVLALCVNP DLLSSPNFPD  102
Rat ALT1  LDTMNPCVRR VEYAVRGPIV QRALELEQEL RQGVKKPFTE VIRANIGDAQ AMGQRPITFF RQVLALCVYP NLLSSPDFPE  102
Hum ALT2  LESMNPQVKA VEYAVRGPIV LKAGEIELEL QRGIKKPFTE VIRANIGDAQ AMGQQPITFL RQVMALCTYP NLLDSPSFPE  126

Spa cALT  DAKQRARRIL EACGGHSIGA YSASQGIECI RQDVARYIEK RDGGIASNPD NIYLSTGASD AIVTILKLLV RGEGCDRTGV  177
Spa mALT  DAKSRARRIL QSCGGNSMGS YSASQGIDSV RHDVARYTER RDGGVPCDPD NIYLTTGASD GIVTMLKLLV CGEGATRTGI  237
Hum ALT1  DAKKRAERIL QACGGHSLGA YSVSSGIQLI REDVARYIER RDGGIPADPN NVFLSTGASD AIVTVLKLLV AGEGHTRTGV  182
Rat ALT1  DAKRRAERIL QACGGHSLGA YSISSGIQPI REDVAQYIER RDGGIPADPN NIFLSTGASD AIVTMLKLLV SGEGRARTGV  182
Hum ALT2  DAKKRARRIL QACGGNSLGS YSASQGVNCI REDVAAYITR RDGGVPADPD NIYLTTGASD GISTILKILV SGGGKSRTGV  206

Spa cALT  MISIPQYPLY SAAITDLGAV QVHYYLDEAN CWSLDVAELR RALNAARQHC NPRVLCIINP GNPTGQVQSR QCIEDVIRFV  257
Spa mALT  MISIPQYPLY SAALAELGAV QINYYLNEQK CWSLDISELQ RSLDEARQHC NPRALCIINP GNPTGQVQSR QCIEDVIRFA  317
Hum ALT1  LIPIPQYPLY SATLAELGAV QVDYYLDEER AWALDVAELH RALGQARDHC RPRALCVINP GNPTGQVQTR ECIEAVIRFA  262
Rat ALT1  LIPIPQYPLY SAALAELDAV QVDYYLDEER AWALDIAELR RALCQARDRC CPRVLCVINP GNPTGQVQTR ECIEAVIRFA  262
Hum ALT2  MIPIPQYPLY SAVISELDAI QVNYYLDEEN CWALNVNELR RAVQEAKDHC DPKVLCIINP GNPTGQVQSR KCIEDVIHFA  286
                                                                 *
Spa cALT  KEEHLFLMAD EVYQDNVYAE GCKFQSFKKV LFEMGPEYSS TVEMASFHST SKCYMGECGF RGGYMEVINM DPEVKAQLTK  337
Spa mALT  AKERLFLMAD EVYQDNVYAE GCQFHSFKKV LFEMGPEYSN TVELVSFHST SKCYMGECGF RGGYMEIINL DSEVKAQLTK  397
Hum ALT1  FEERLFLLAD EVYQDNVYAA GSQFHSFKKV LMEMGPPYAG QQELASFHST SKGYMGECGF RGGYVEVVNM DAAVQQQMLK  342
Rat ALT1  FKEGLFLMAD EVYQDNVYAE GSQFHSFKKV LMEMGPPYST QQELASFHSV SKGYMGECGF RGGYVEVVNM DAEVQKQMGK  342
Hum ALT2  WEEKLFLLAD EVYQDNVYSP DCRFHSFKKV LYEMGPEYSS NVELASFHST SKGYMGECGY RGGYMEVINL HPEIKGQLVK  366

Spa cALT  LVSVRLCPPV SGQALMDLVV NPPQPDEPSY TTFMKERTAV LAELAEKARL TEQILNTVPG ITCNPVQGAM YSFPRITLPQ  417
Spa mALT  LVSVRLCPPV PGQALMDLVV NPPQPGEPSH EKFIKERTTT LCALAEKAKL TEQVLNTVQG ISCNPVQGAM YSFPCITIPE  477
Hum ALT1  LMSVRLCPPV PGQALLDLVV SPPAPTDPSF AQFQAEKQAV LAELAAKAKL TEQVFNEAPG ISCNPVQGAM YSFPRVQLPP  422
Rat ALT1  LMSVRLCPPV PGQALMDMVV SPPTPSEPSF KQFQAERQEV LAELAAKAKL TEQVFNEAPG IRCNPVQGAM YSFPQVQLPL  422
Hum ALT2  LLSVRLCPPV SGQAAMDIVV NPPVAGEESF EQFSREKESV LGNLAKKAKL TEDLFNQVPG IHCNPLQGAM YAFPRIFIPA  446

Spa cALT  KAIDKAKEAG HIPDMFYCMK LLEEEGICLV PGSGFGQREG TFHFRMTILP PTEKLKVLLQ RLRDFHQRFT QEFS  491
Spa mALT  KAIKEATDNG QKPDMFYCMK LLEETGICLV PGSGFGQRDG TYHFRMTILP PKDKLKILLT KVKEFHQKFT QQYS  554
Hum ALT1  RAVERAQELG LAPDMFFCLR LLEETGICVV PGSGFGQREG TYHFRMTILP PLEKLRLLLE KLSRFHAKFT LEYS  496
Rat ALT1  KAVQRAQELG LAPDMFFCLC LLEETGICVV PGSGFGQQEG TYHFRMTILP PMEKLRLLLE KLSHFHAKFT HEYS  496
Hum ALT2  KAVEAAQAHQ MAPDMFYCMK LLEETGICVV PGSGFGQREG TYHFRMTILP PVEKLKTVLQ KVKDFHINFL EKYA  523
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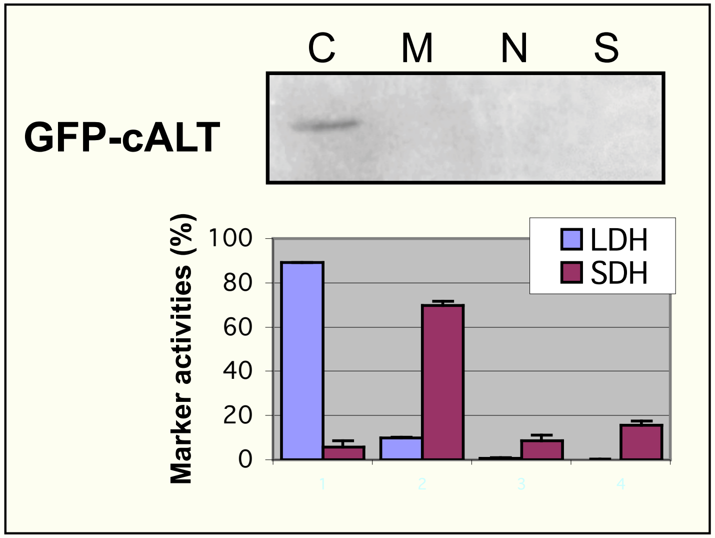
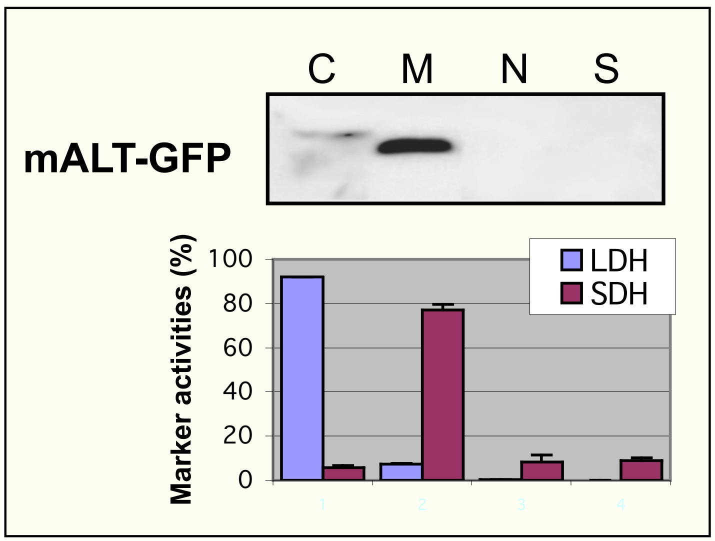
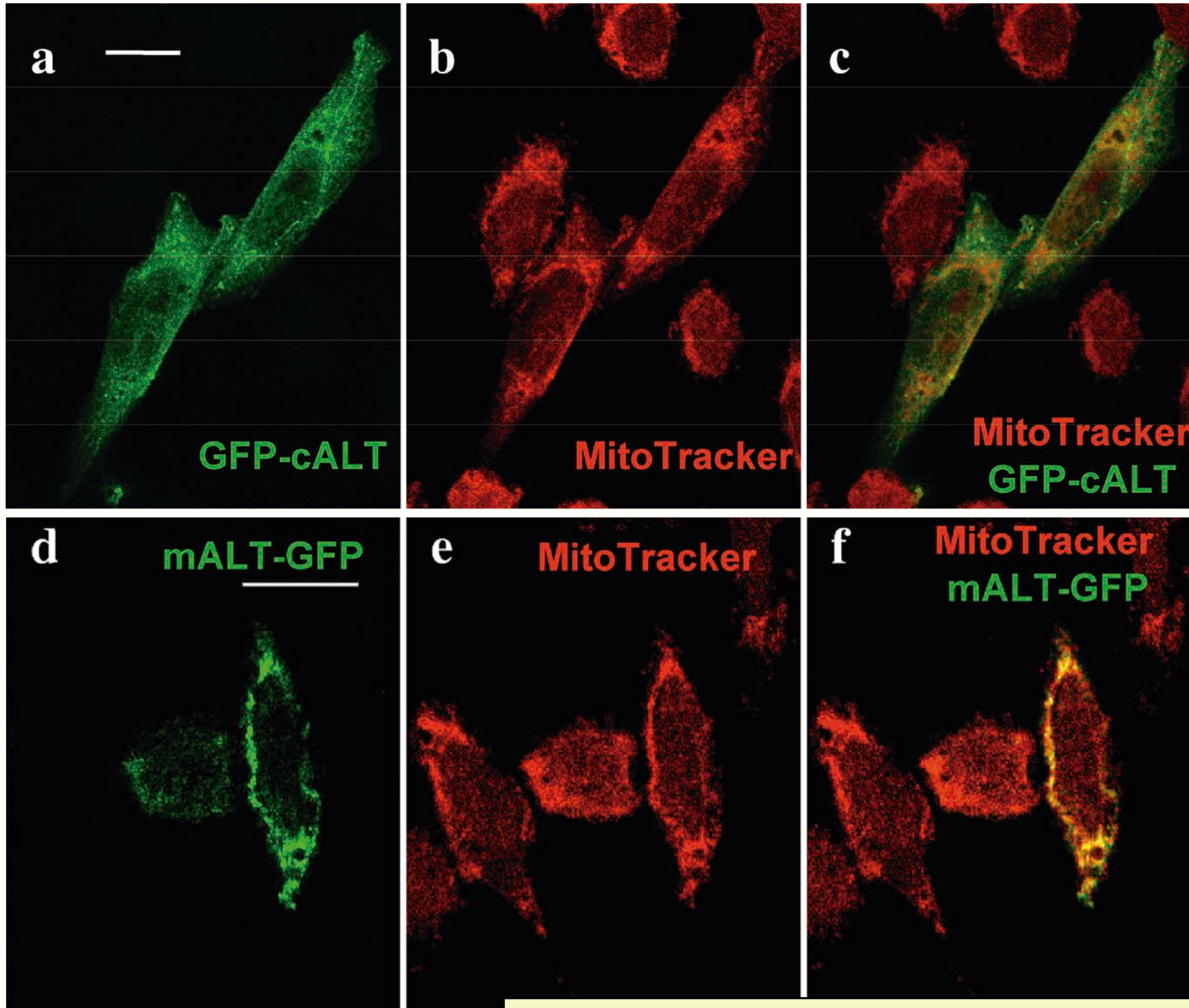
Subcellular localization of cALT1 and mALT

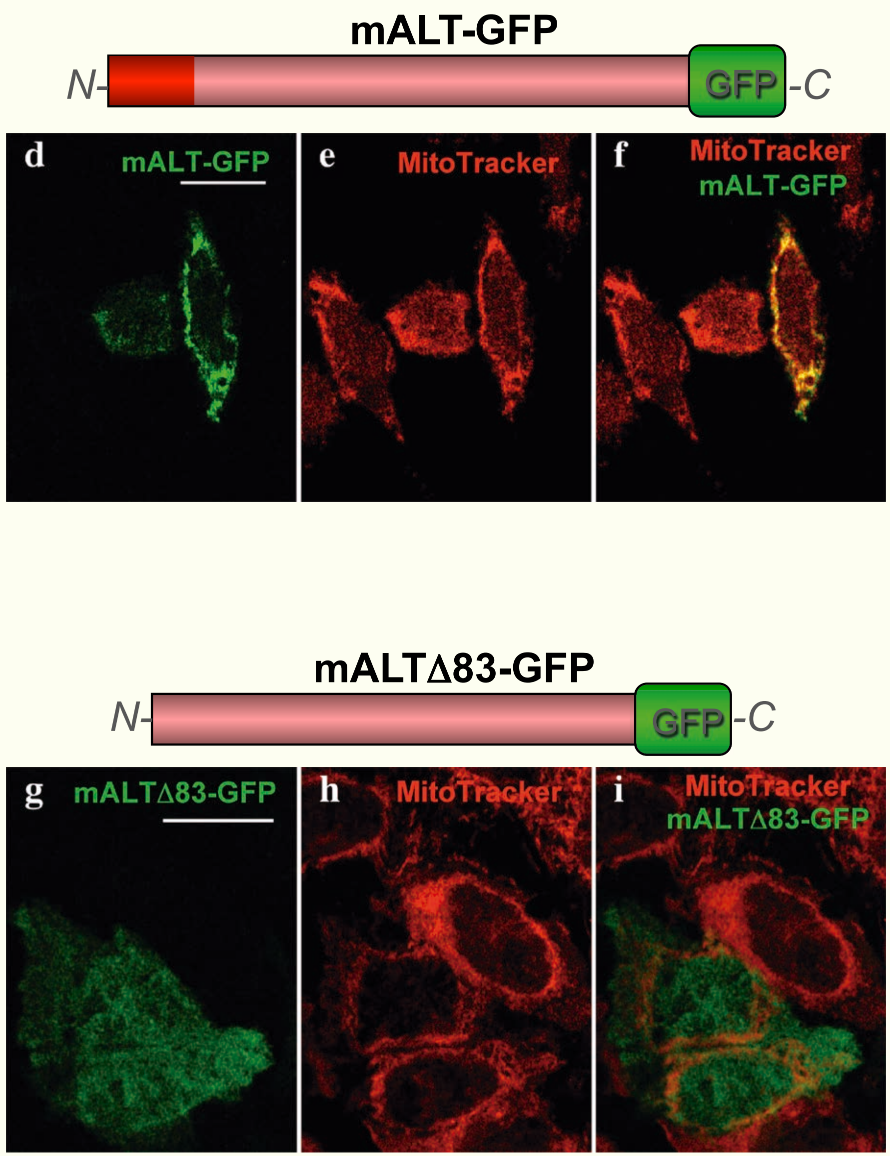




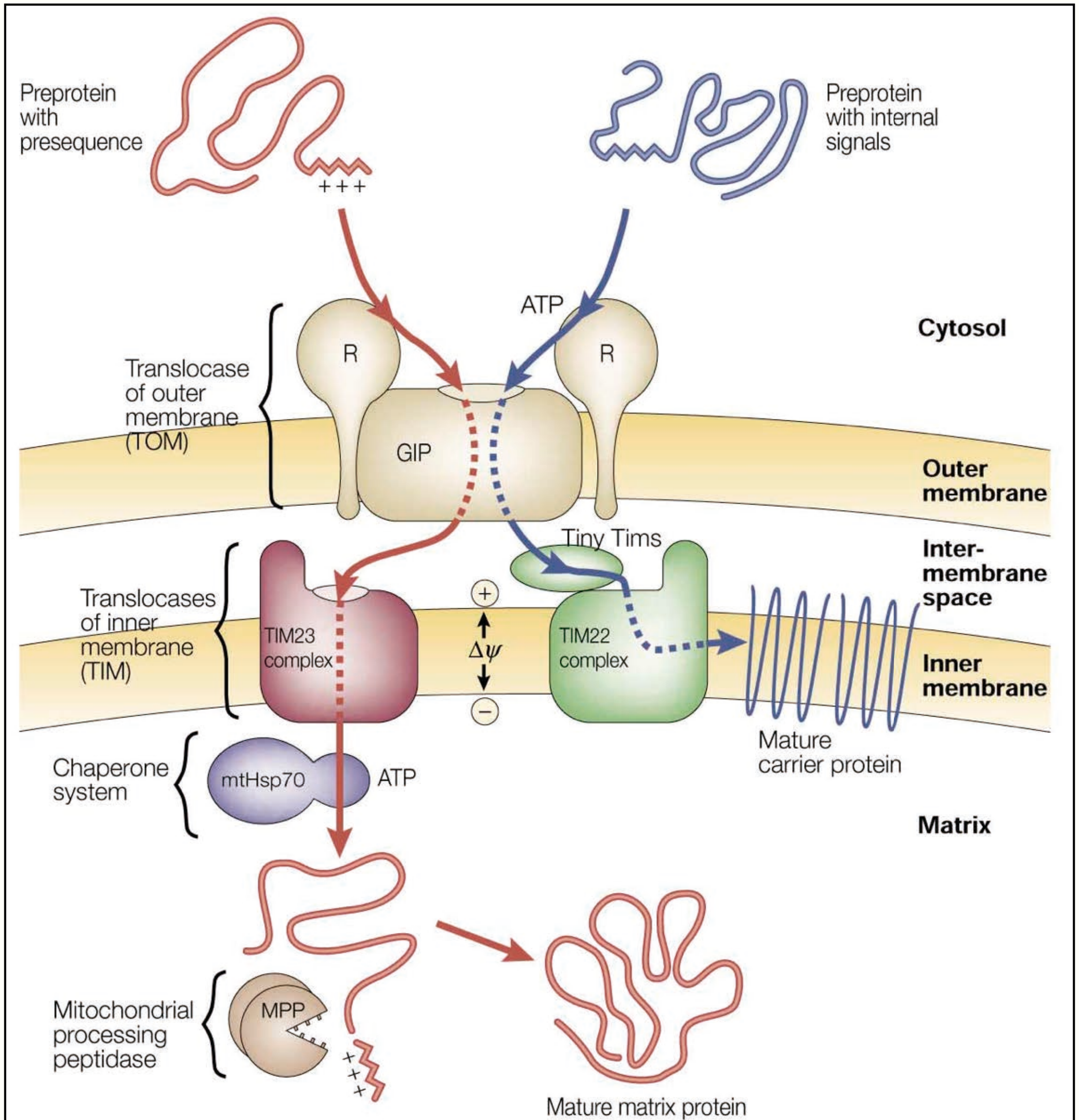


I. Metón et al. (2004) *FEBS Lett* 566: 251-254

Proposed mechanism of mitochondrial mALT import

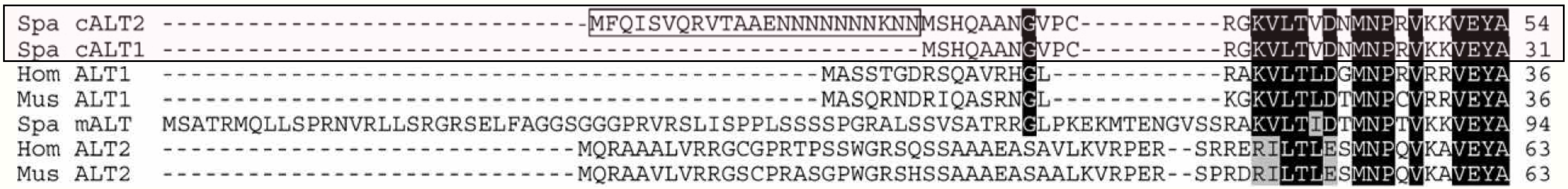


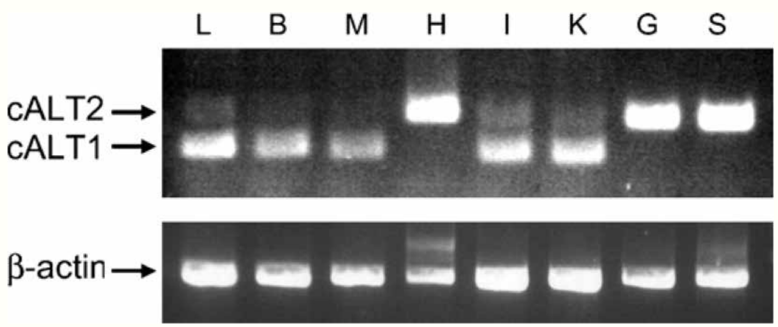
I. Metón et al. (2004) *FEBS Lett* 566: 251-254

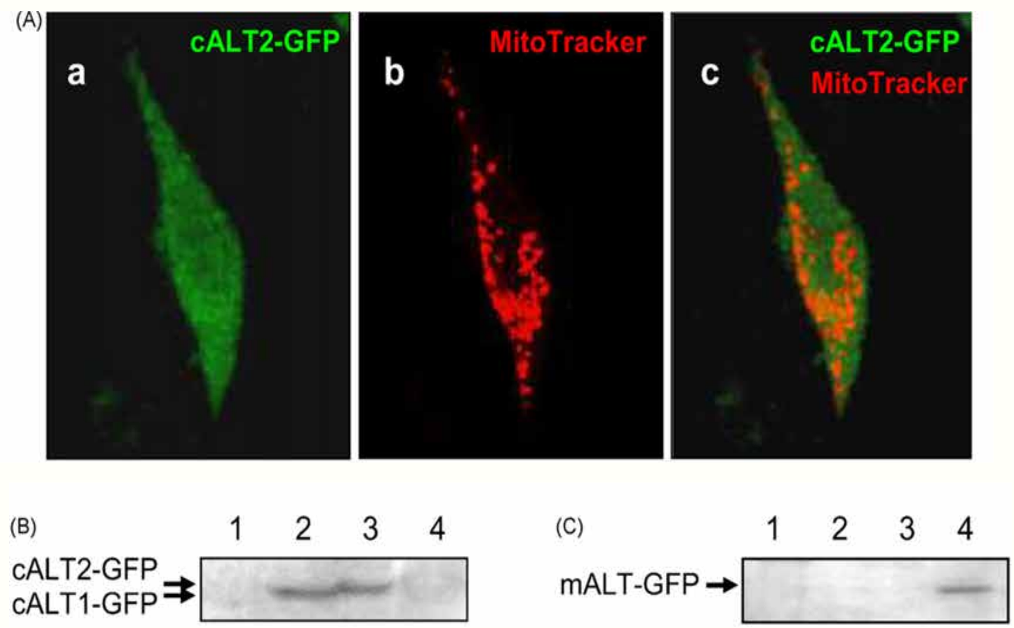


N. Pfanner & A. Geissler (2001) *Nature Rev* 2: 339-349

Alternative splicing generates two cytosolic isozymes: cALT1 and cALT2

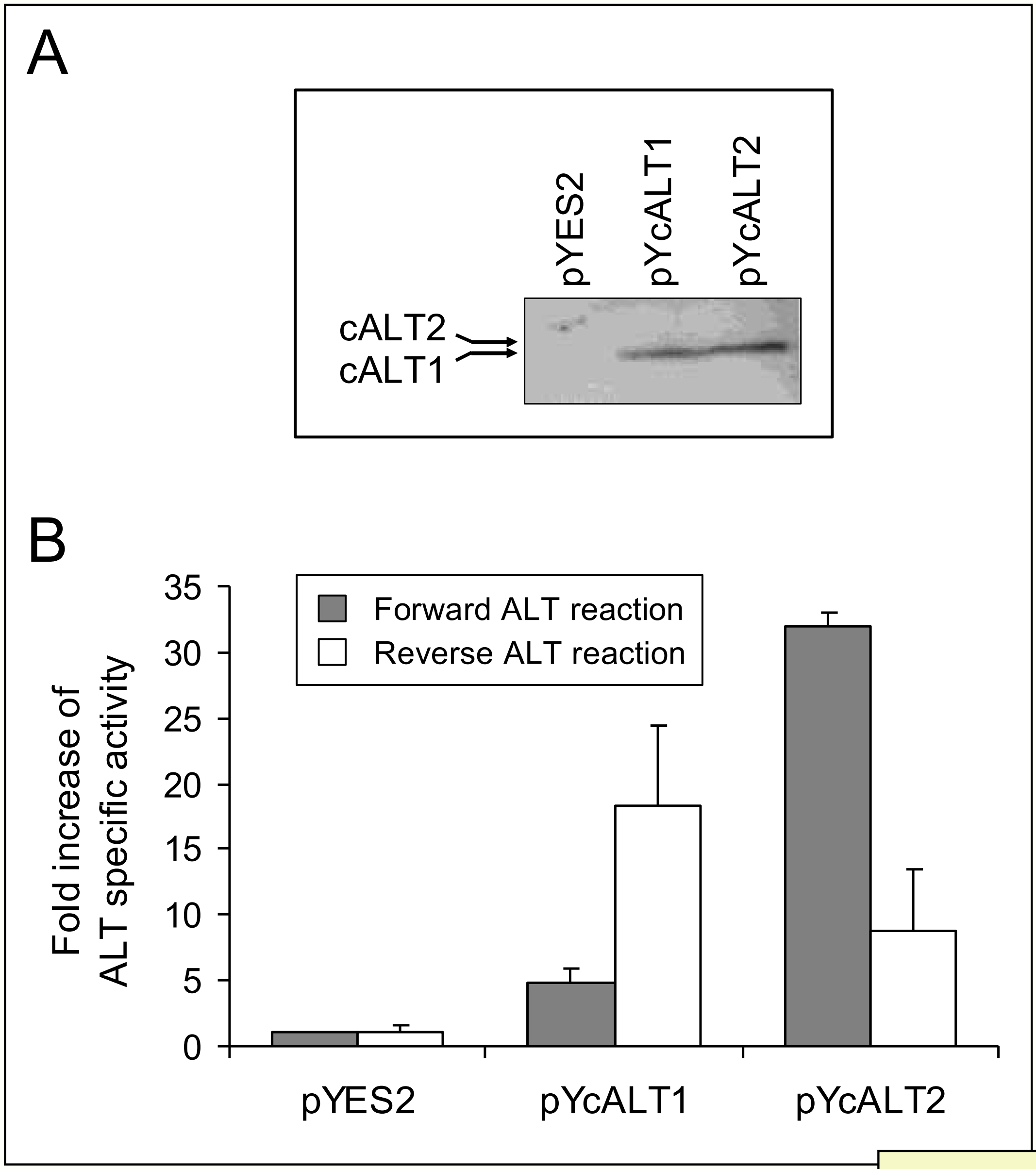




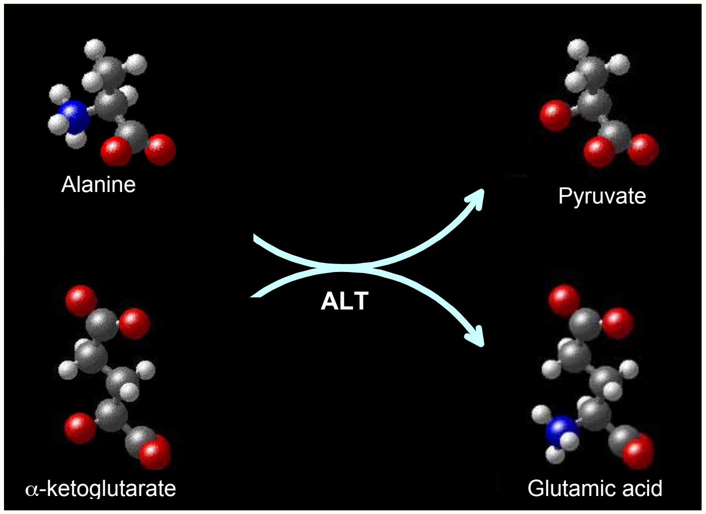


I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

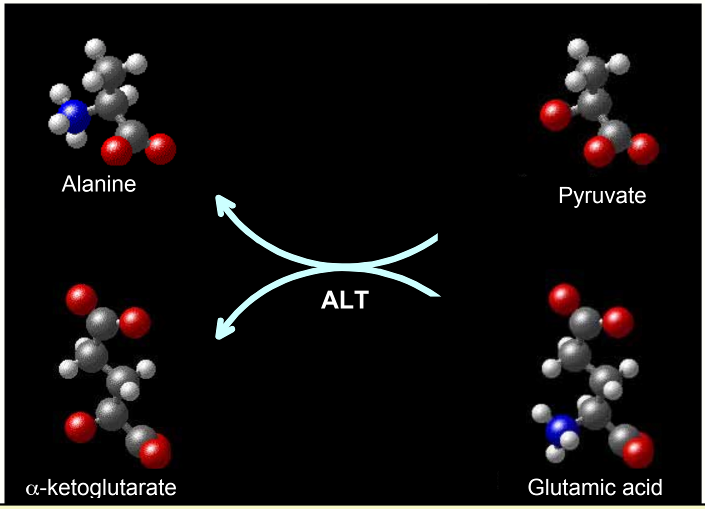
Expression of cALT1 and cALT2 in *S. cerevisiae*



Forward reaction:

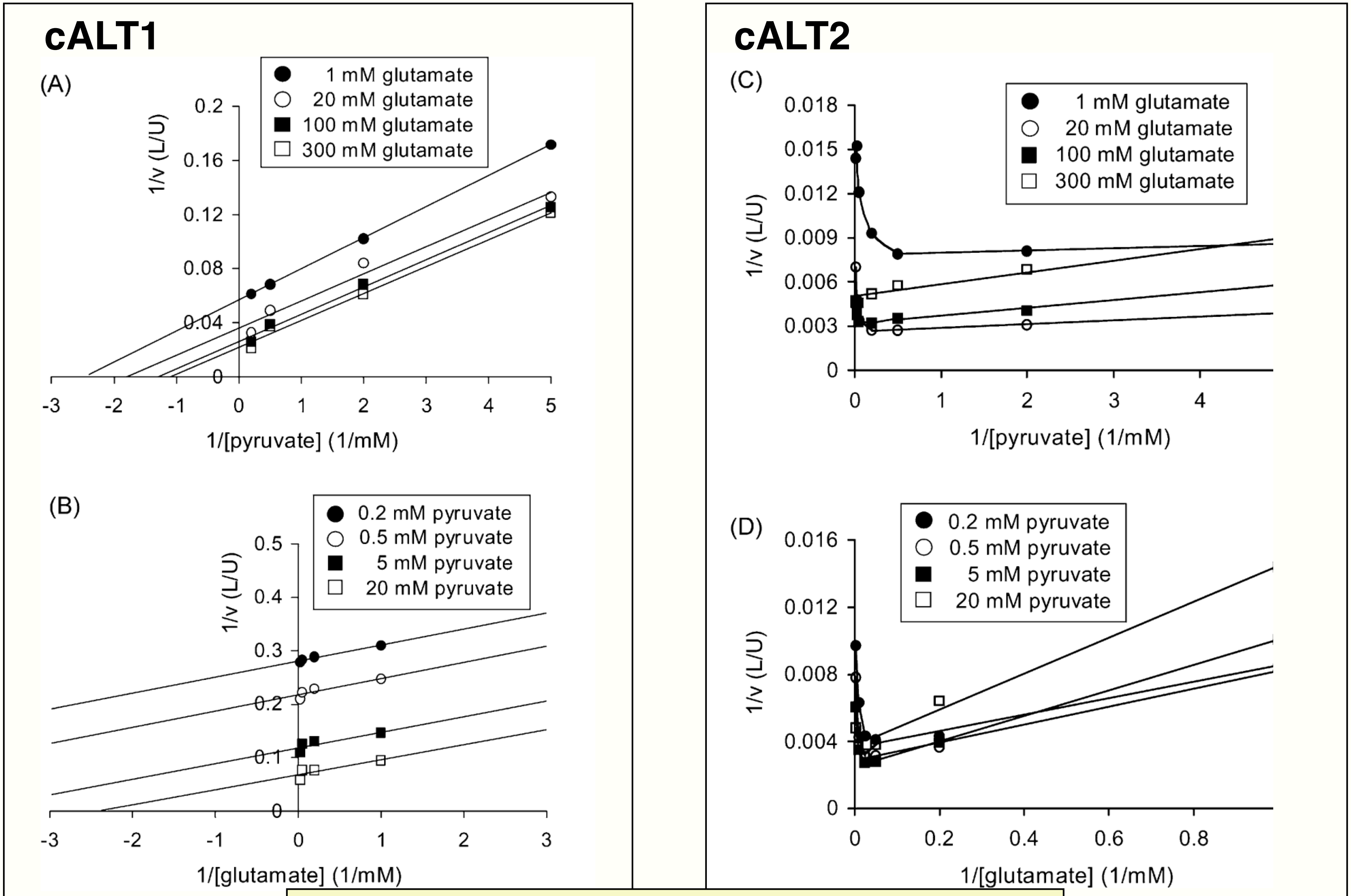


Reverse reaction:



I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

cALT1 and cALT2: Saturation kinetics for pyruvate and L-glutamate



I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

Kinetic parameters of cALT1 and cALT2

	cALT1	cALT2
Forward reaction		
K_m^{Ala} (mM)	1.82±0.33	2.21±0.70
$K_m^{2\text{-}Oxo}$ (mM)	0.048±0.006	0.051±0.006
V_{max} (μmol/min/g)	56.8±14.3	14697±4482
V_{max}/K_m^{Ala}	0.031	6.65
$V_{max}/K_m^{2\text{-}Oxo}$	1.183	288.2
$I_{50}^{Oxamate}$ (mM)	1.46±0.14	1.10±0.03
$K_i^{Oxamate}$ (mM)	9.37±0.83	2.50±0.32
$K_I^{Oxamate}$ (mM)	3.20±0.87	0.83±0.06
Reverse reaction		
K_m^{Glu} (mM)	15.91±3.99	4.53±1.55
K_m^{Pyr} (mM)	0.690±0.208	0.154±0.053
V_{max} (μmol/min/g)	11.6±2.3	20.9±0.7
V_{max}/K_m^{Glu}	0.001	0.005
V_{max}/K_m^{Pyr}	0.017	0.135
K_i^{Glu} (mM)		34.9±13.3
K_i^{Pyr} (mM)		36.5±15.9
$I_{50}^{Oxamate}$ (mM)		104.2±24.8
$K_i^{Oxamate}$ (mM)		81.5±21.5

I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

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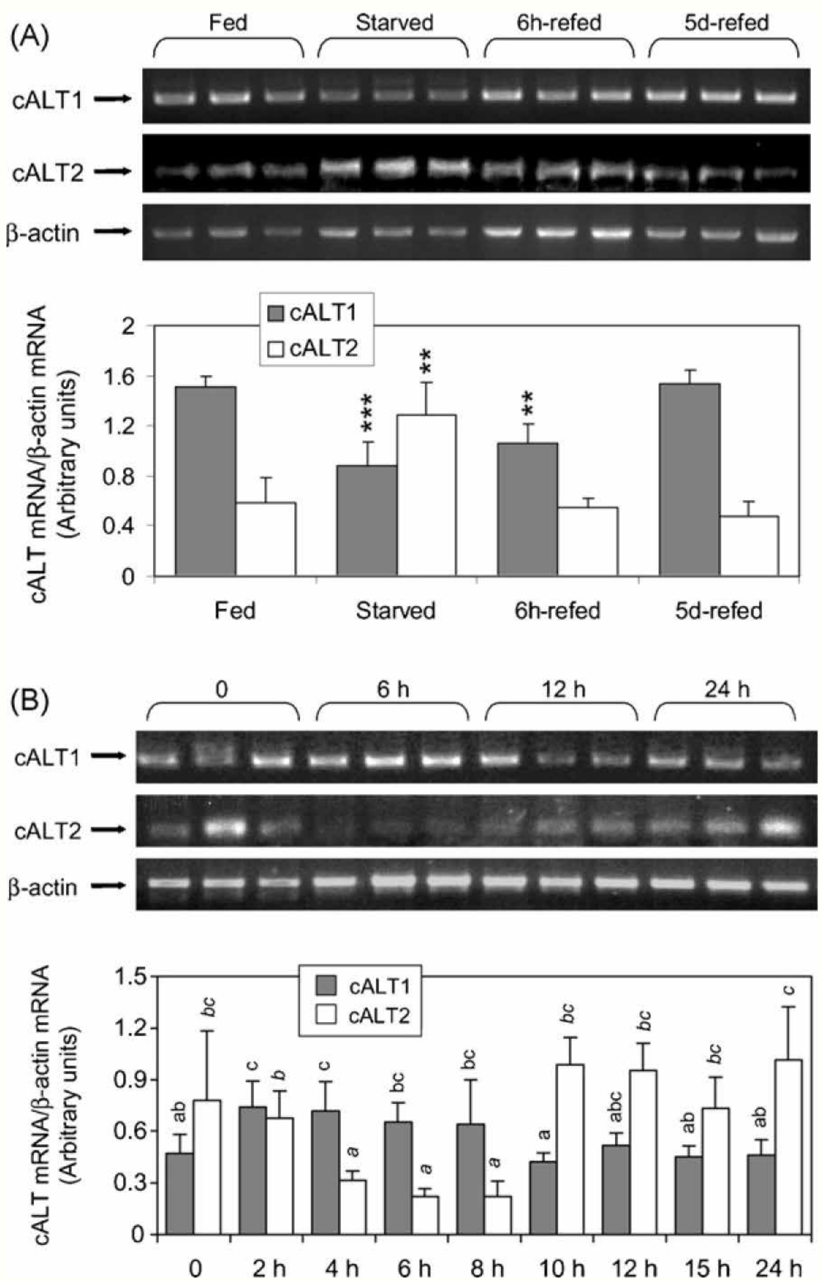
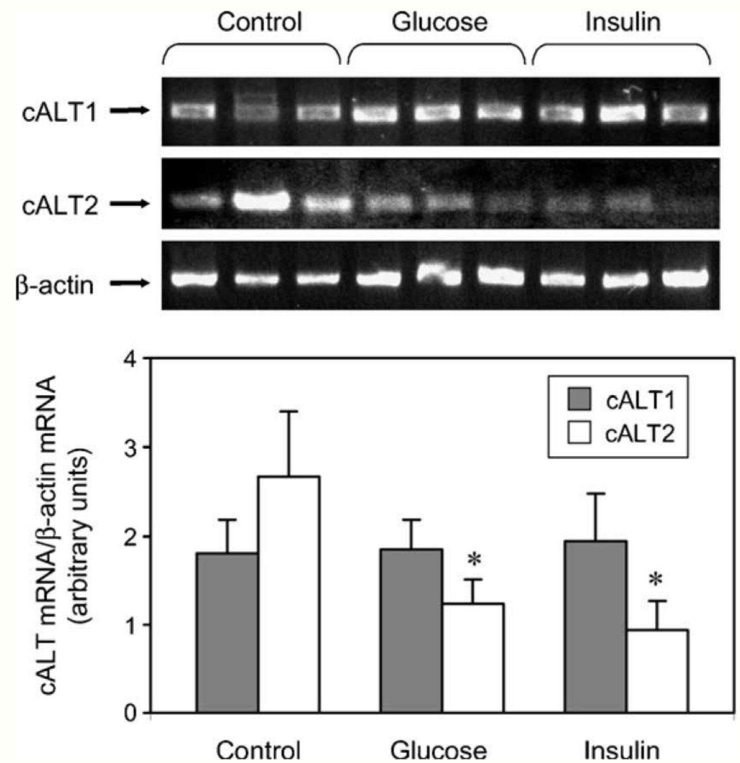
✓ **cALT2 preferably catalyses the forward reaction (conversion of L-alanine to pyruvate)**

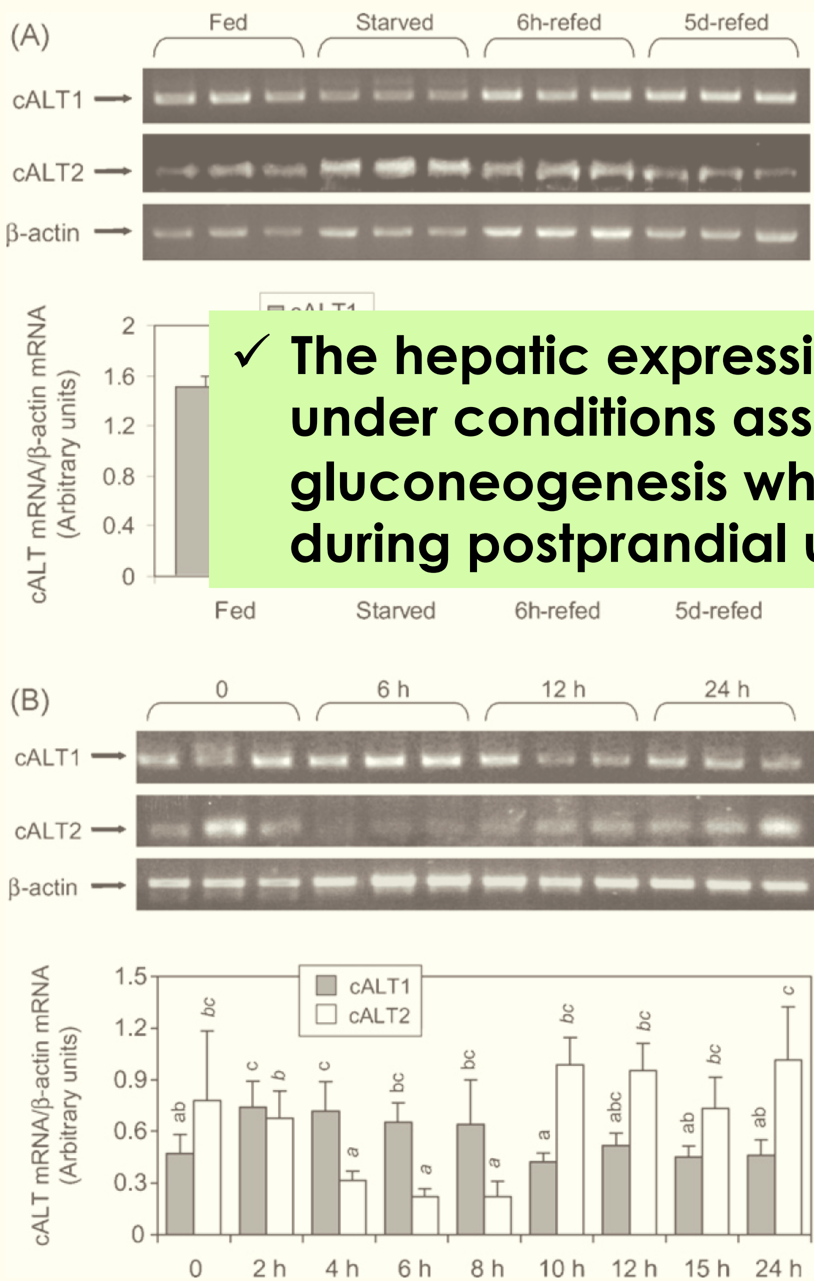
I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

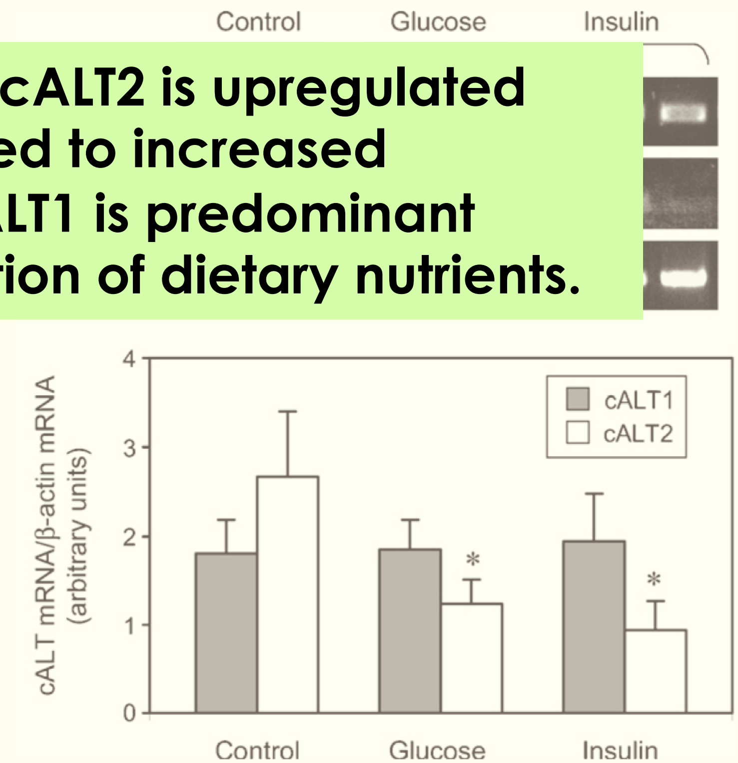
Nutritional regulation of cALT1 and cALT2 expression





I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

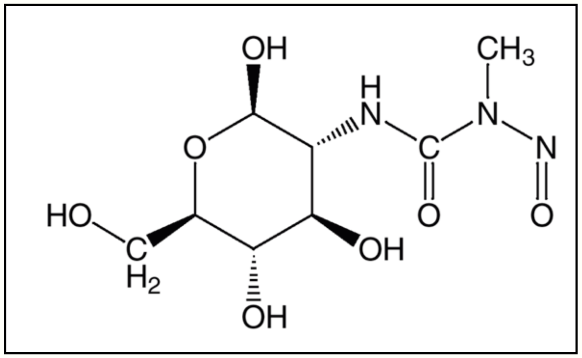




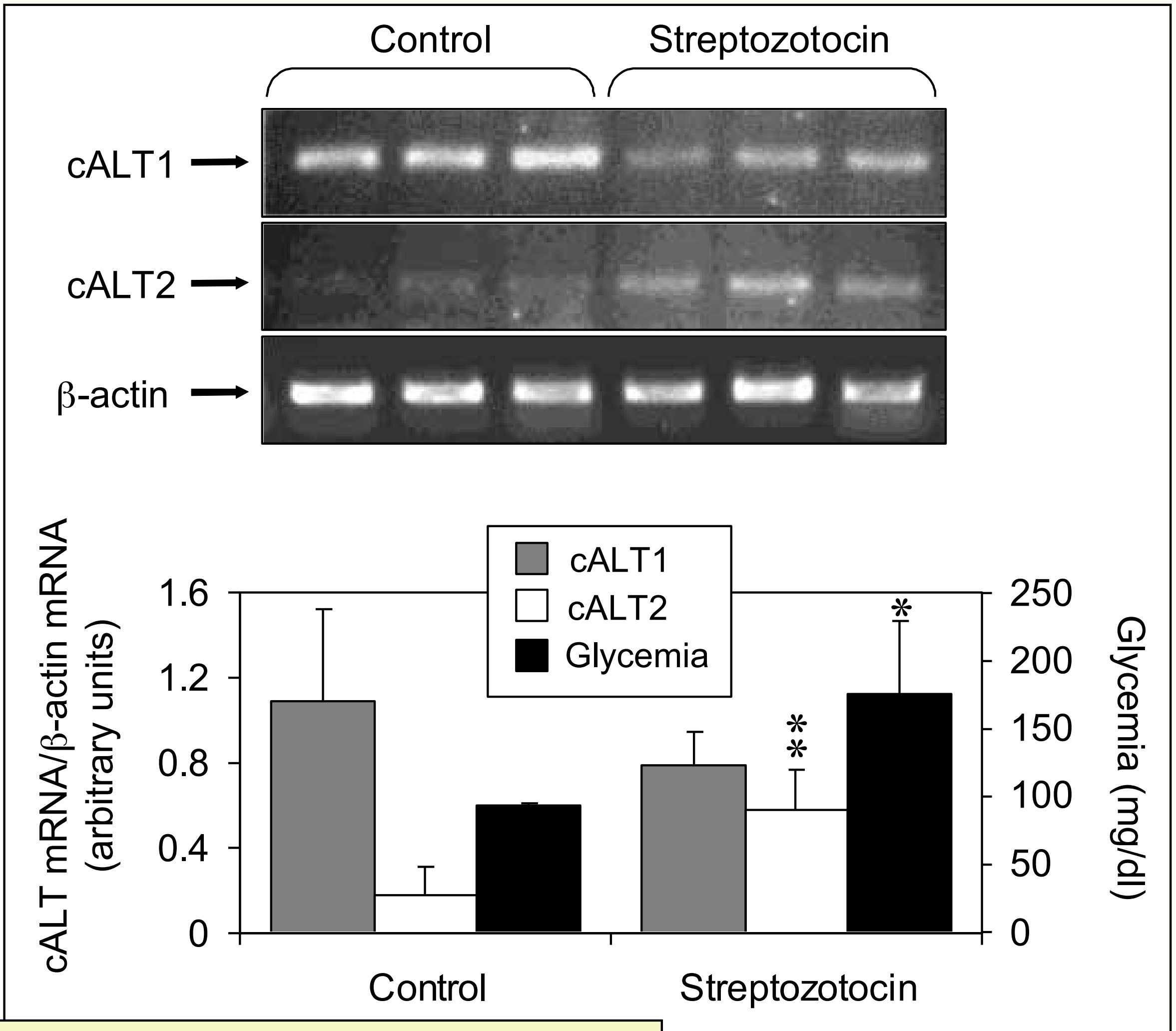
- ✓ **The hepatic expression of cALT2 is upregulated under conditions associated to increased gluconeogenesis while cALT1 is predominant during postprandial utilization of dietary nutrients.**

I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

cA
LT1 and cALT2 expression in streptozotocin-induced diabetic *Sparus aurata*



✓ **Streptozotocin causes β-cell necrosis and is widely used to generate diabetic animal models.**



I.G. Anemaet et al (2008) *Int J Biochem & Cell Biol* 40: 2833-2844

Specific objective 2

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- **Specific objectives:**

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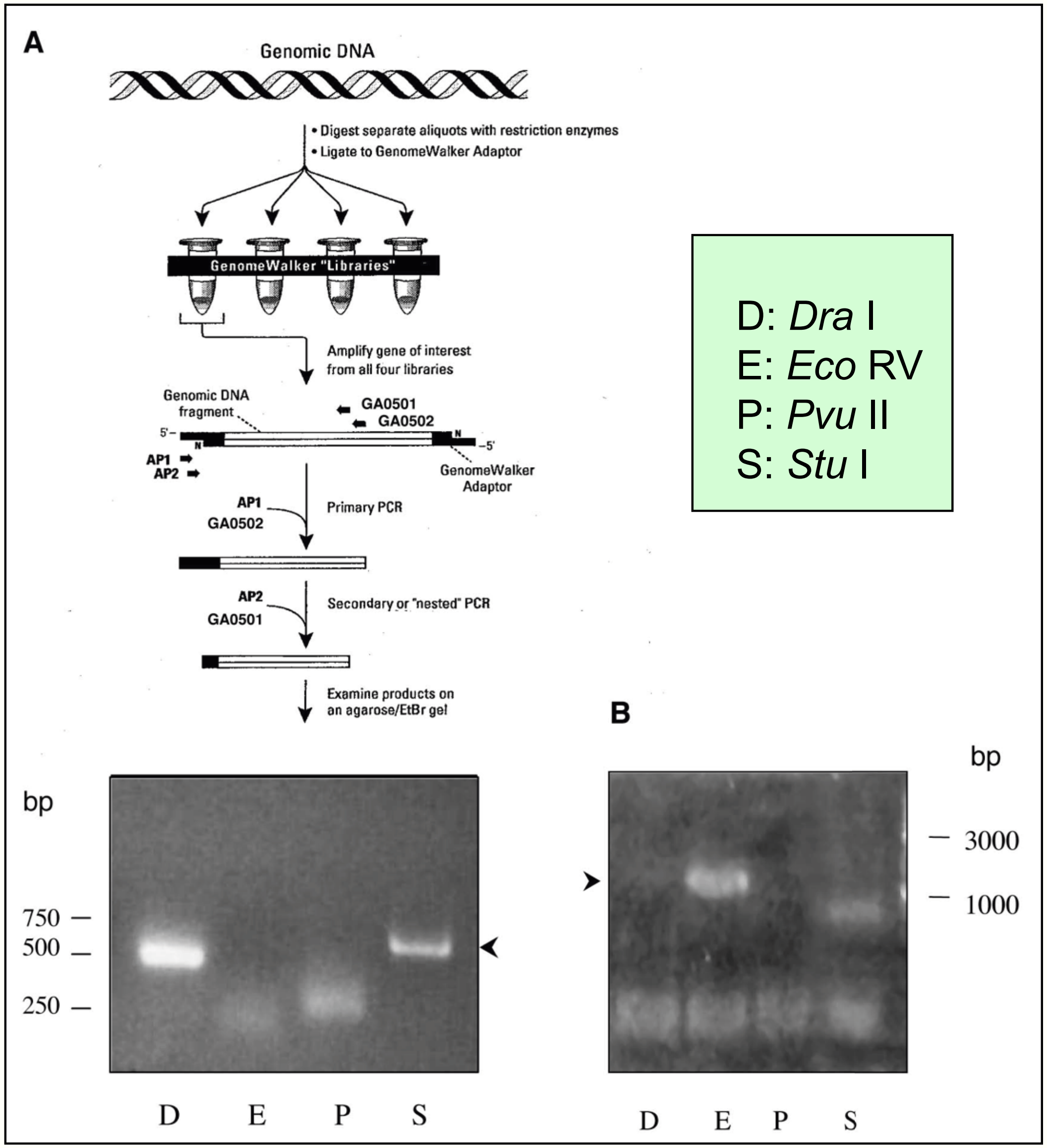
② Characterization of ALT gene promoters.

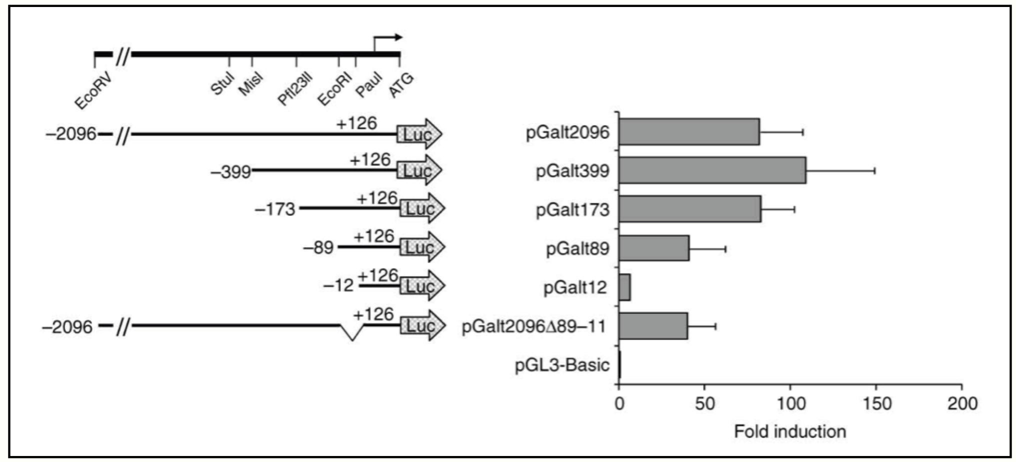
③ Identification of proteins that may interact with ALT isoforms and eventually regulate the enzyme activity.

④ Effect of ALT inhibition on the intermediary metabolism of *Sparus aurata*.

Cloning and characterization of *Sparus aurata* cALT gene promoter (SBL cells)

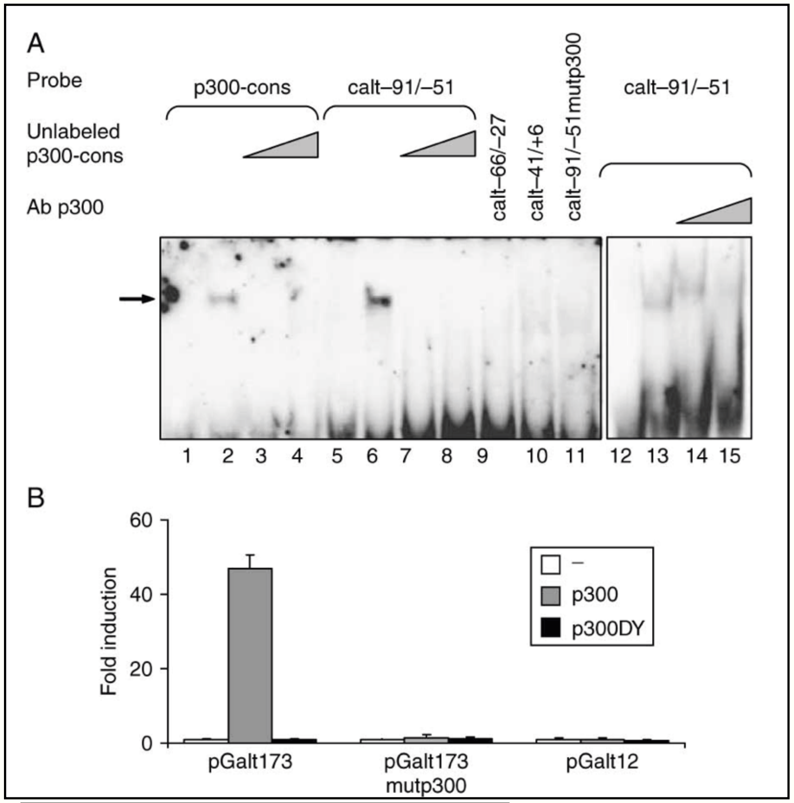


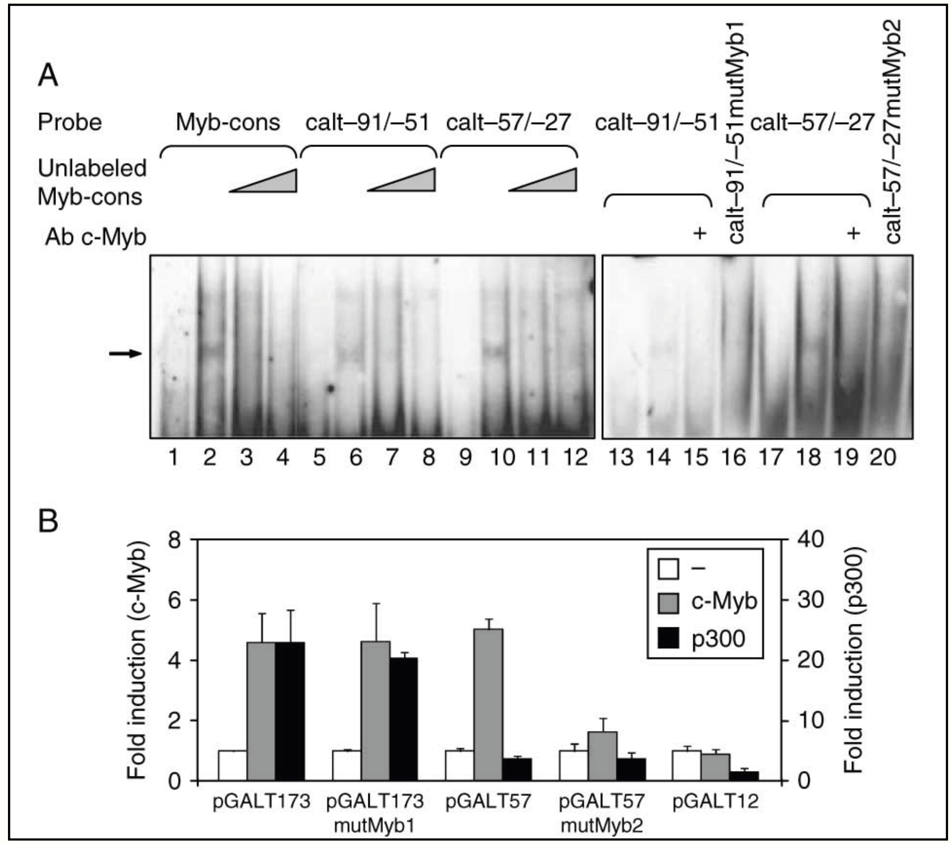
D: *Dra* I
E: *Eco* RV
P: *Pvu* II
S: *Stu* I

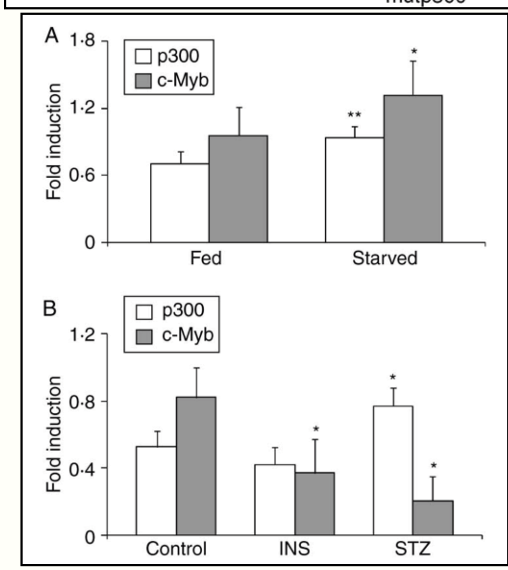


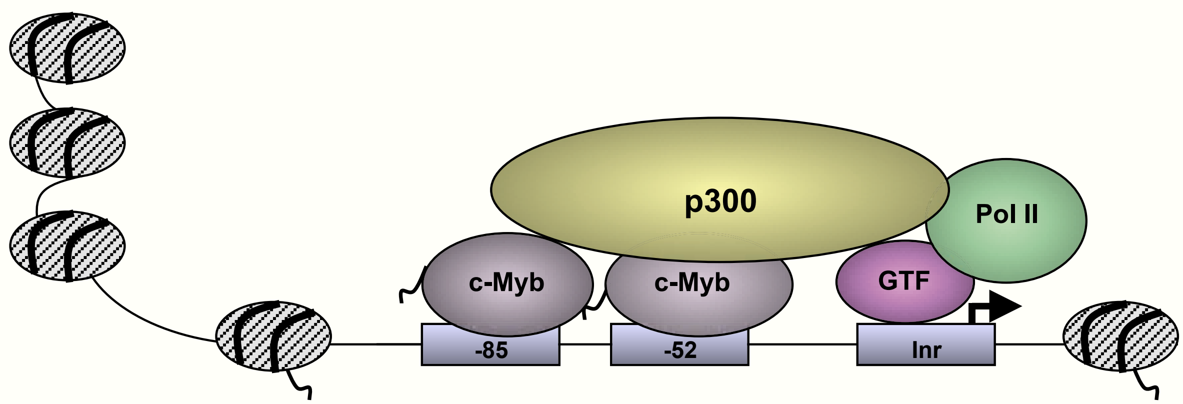
I.G. Anemaet et al (2010) *J Mol Endocrinol* 45: 119-132

p300 and c-Myb transactivate the cALT gene promoter



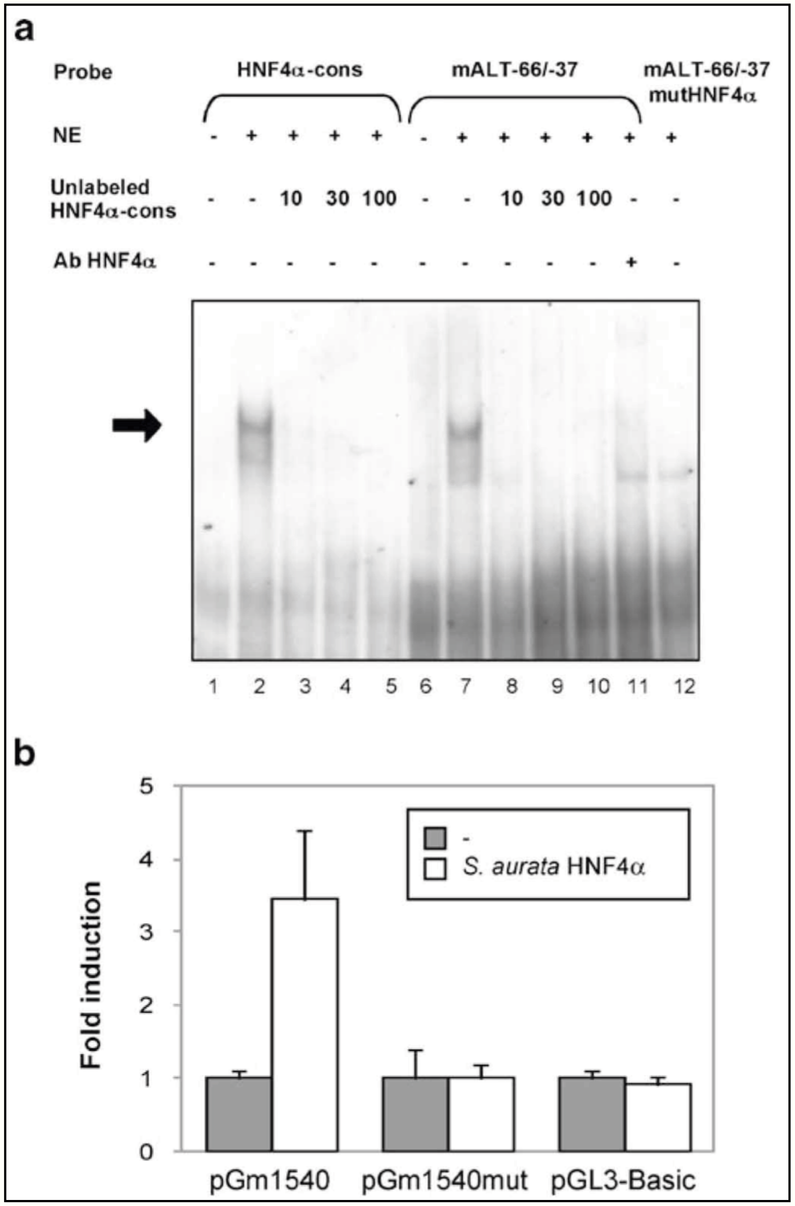


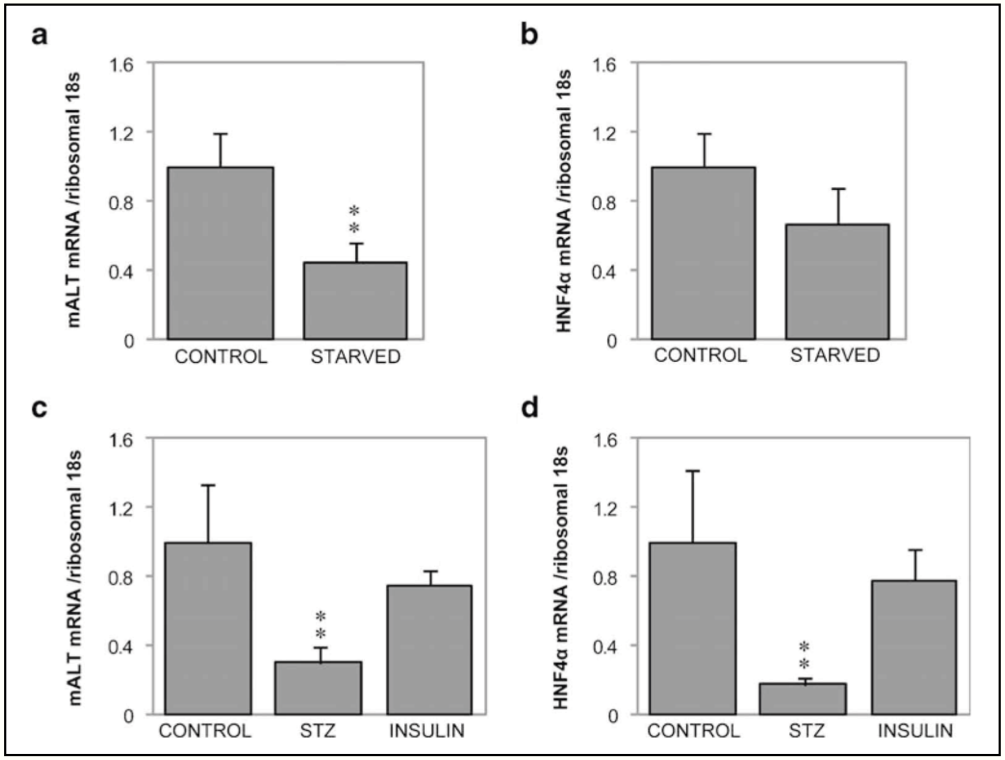




I.G. Anemaet et al (2010) *J Mol Endocrinol* 45: 119-132

HNF4α transactivates the mALT gene





M.C. Salgado et al (2012) *Mar Biotechnol* 14: 46-62

Specific objective 3

- **Global objective:**
  - ✓ **To increase the current knowledge on the regulation of the expression of ALT isozymes in *Sparus aurata* to enable a biotechnological action to spare protein and improve the use of dietary carbohydrates.**
- **Specific objectives:**

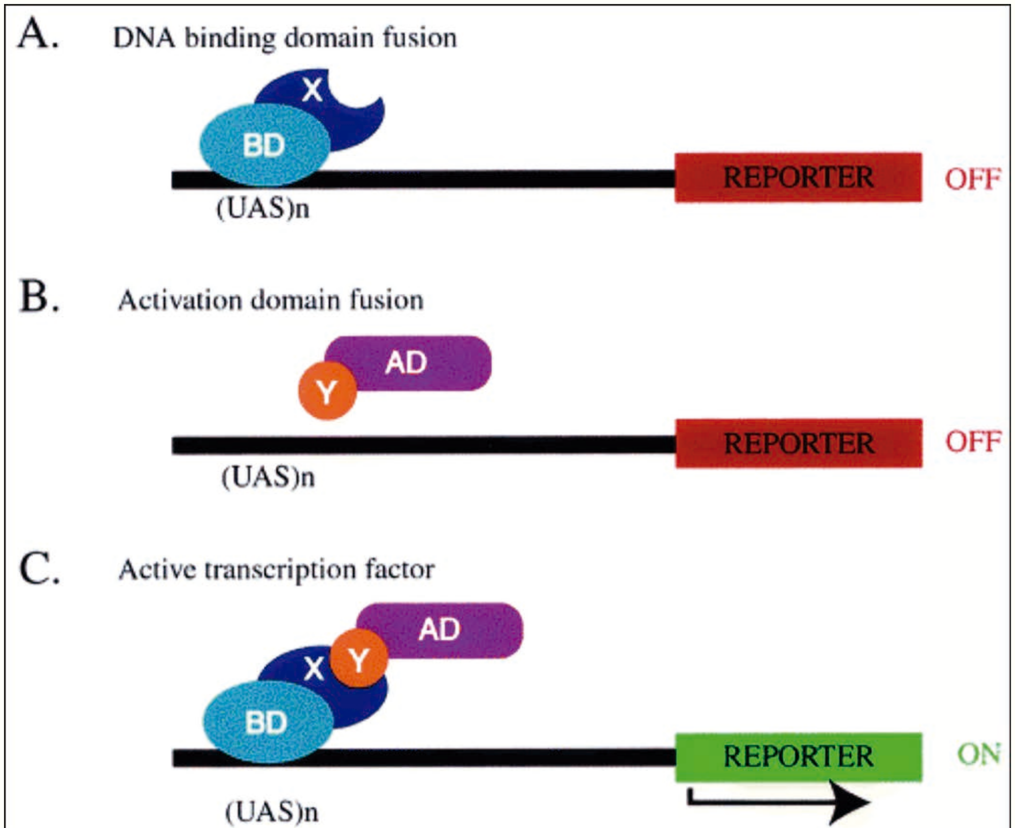
① Cloning and molecular and kinetic characterization of cytosolic and mitochondrial isoforms of ALT from *Sparus aurata*.

② Characterization of ALT gene promoters.

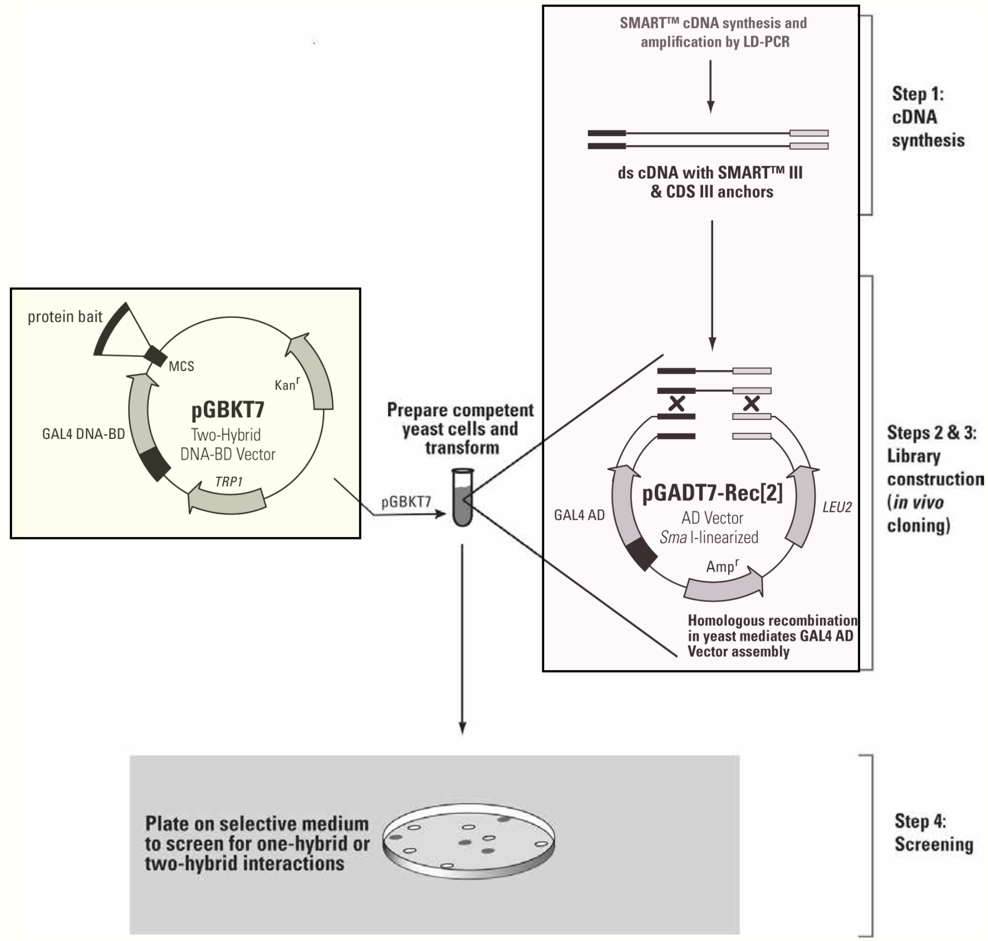
③ Identification of proteins that may interact with ALT isoforms and eventually regulate the enzyme activity.

④ Effect of ALT inhibition on the intermediary metabolism of *Sparus aurata*.

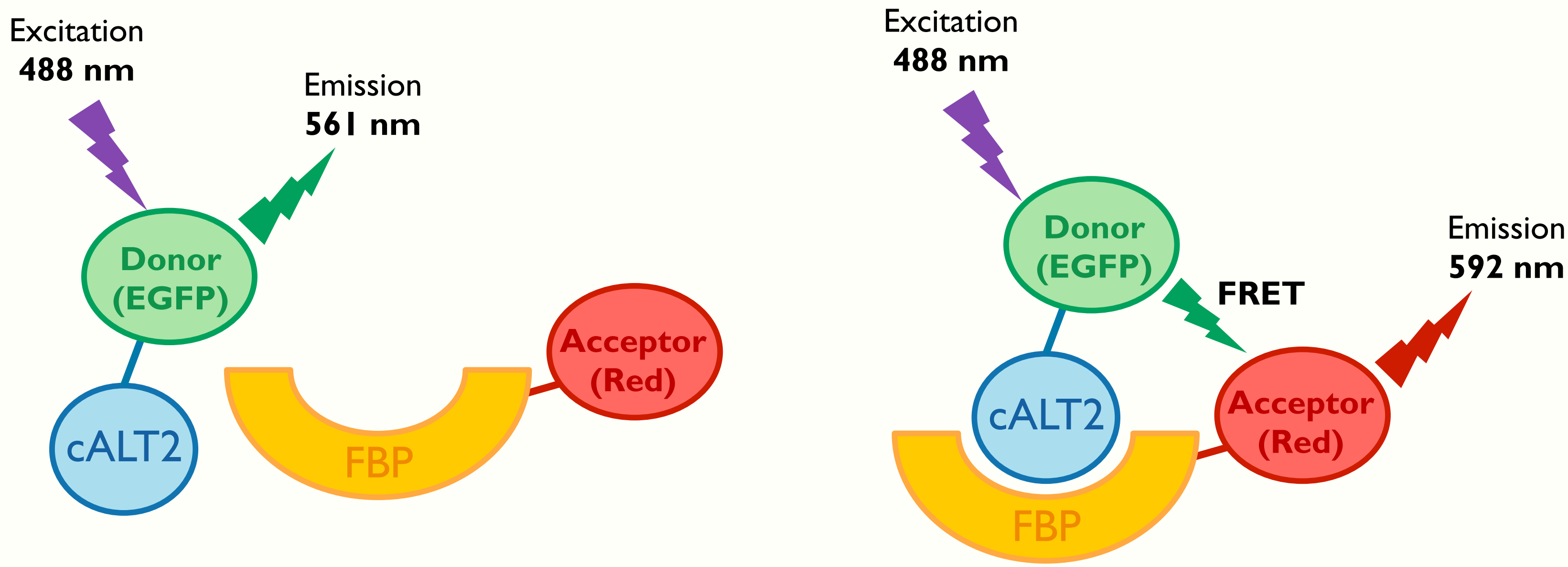
Yeast two-hybrid screening: searching for cALT-interacting proteins

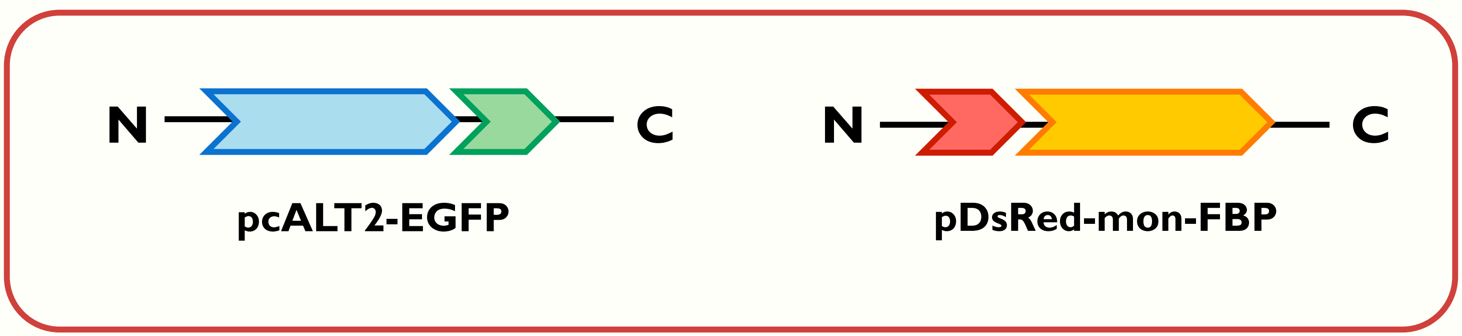


D. Stephens & G. Banting (2000) *Traffic* 1, 763-768

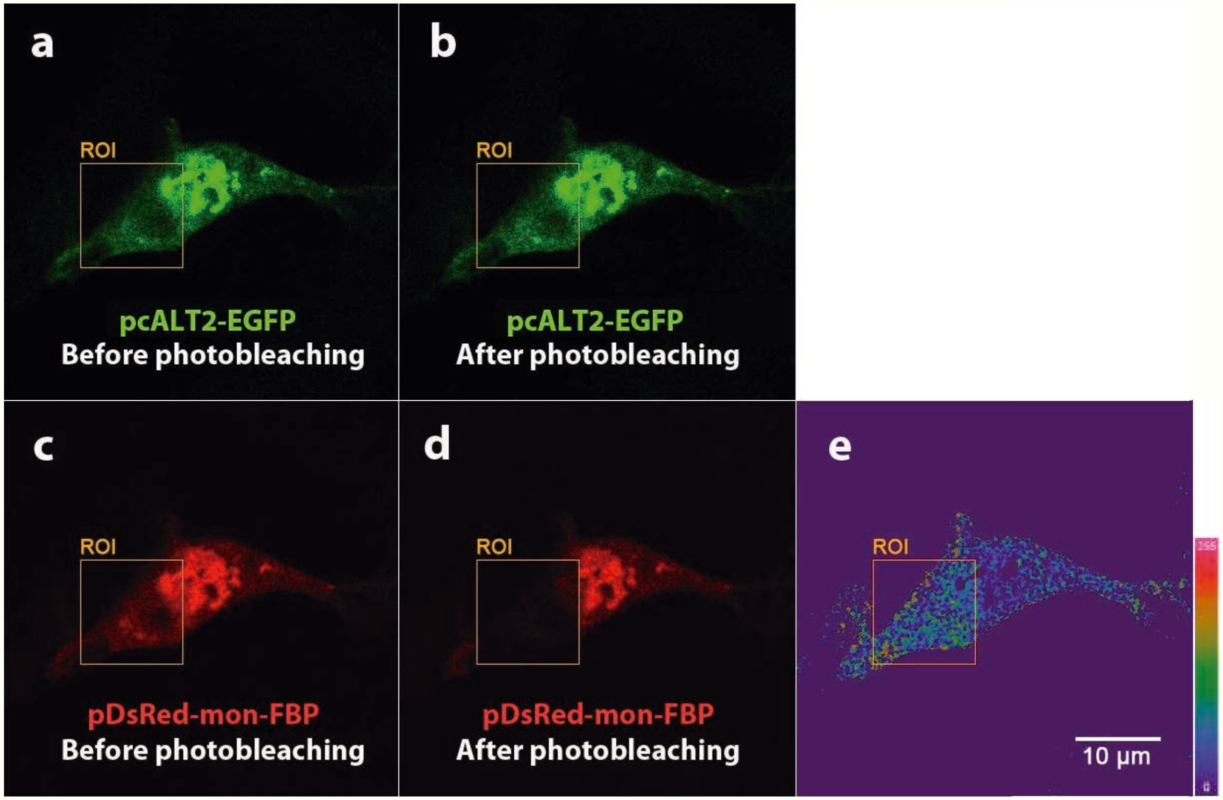


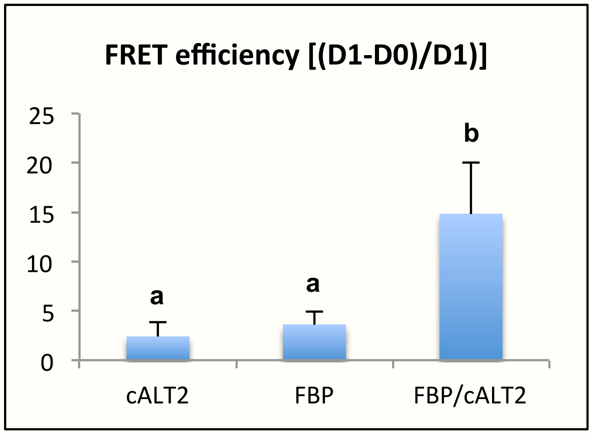
Förster Resonance Energy Transfer (FRET) assay: confirming protein-protein interaction

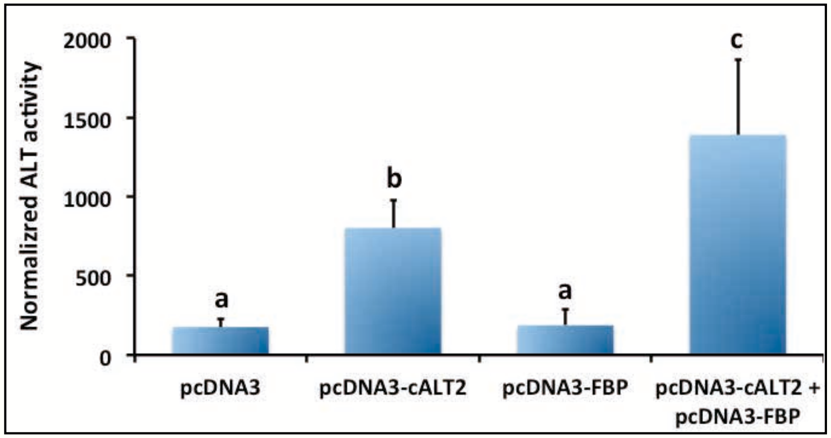




F-lectin (FBP) regulates cALT2 activity through protein-protein interaction







Specific objective 4

- **Global objective:**
  - ✓ **To increase the current knowledge on the regulation of the expression of ALT isozymes in *Sparus aurata* to enable a biotechnological action to spare protein and improve the use of dietary carbohydrates.**
- **Specific objectives:**

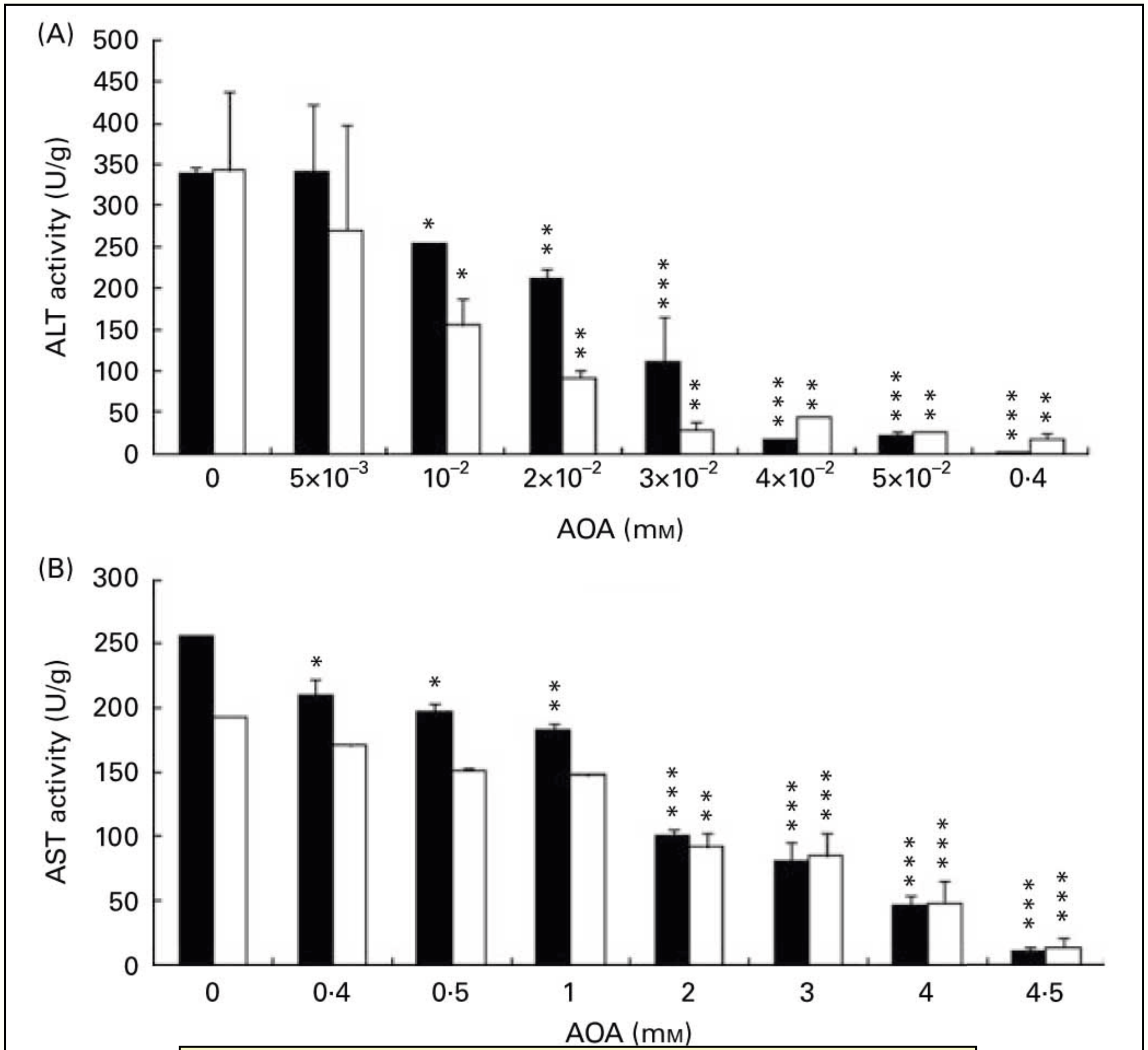
① Cloning and molecular and kinetic characterization of cytosolic and mitochondrial isoforms of ALT from *Sparus aurata*.

② Characterization of ALT gene promoters.

③ Identification of proteins that may interact with ALT isoforms and eventually regulate the enzyme activity.

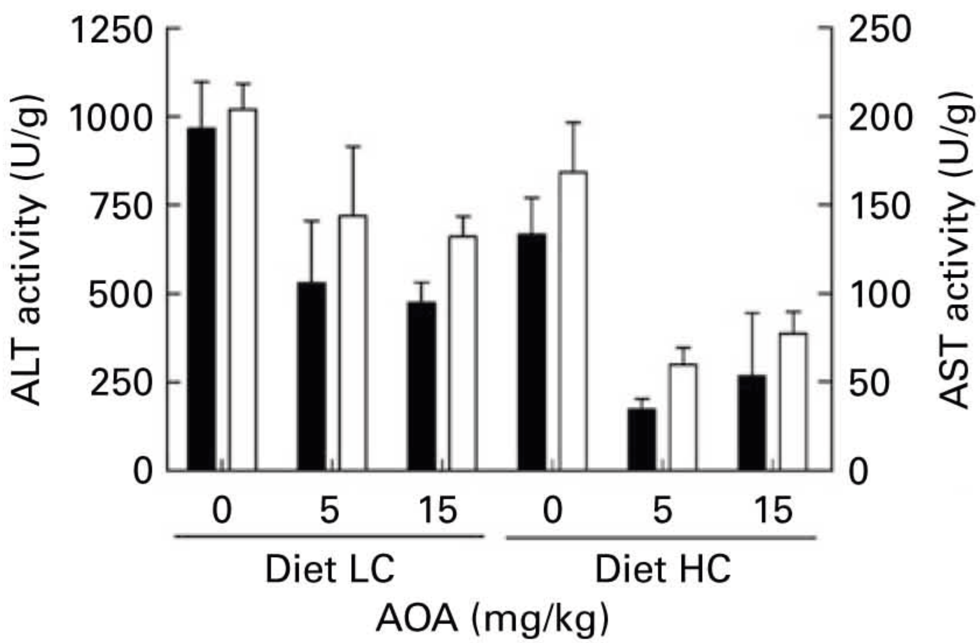
④ **Effect of ALT inhibition on the intermediary metabolism of *Sparus aurata*.**

Amino-oxyacetate (AOA) inhibits cALT activity *in vitro*



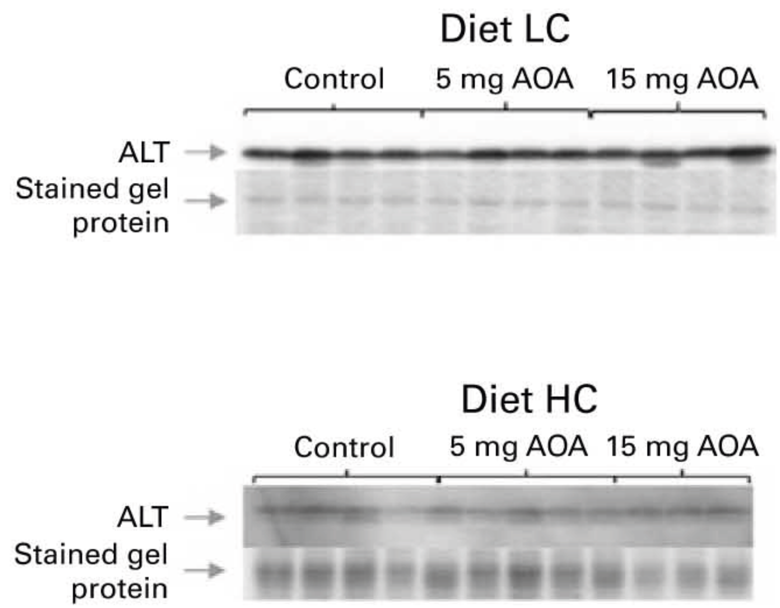
J.D. González et al (2012) *Br J Nutr* 107: 1747-1756

Amino-oxyacetate (AOA) inhibits cALT activity *in vivo*



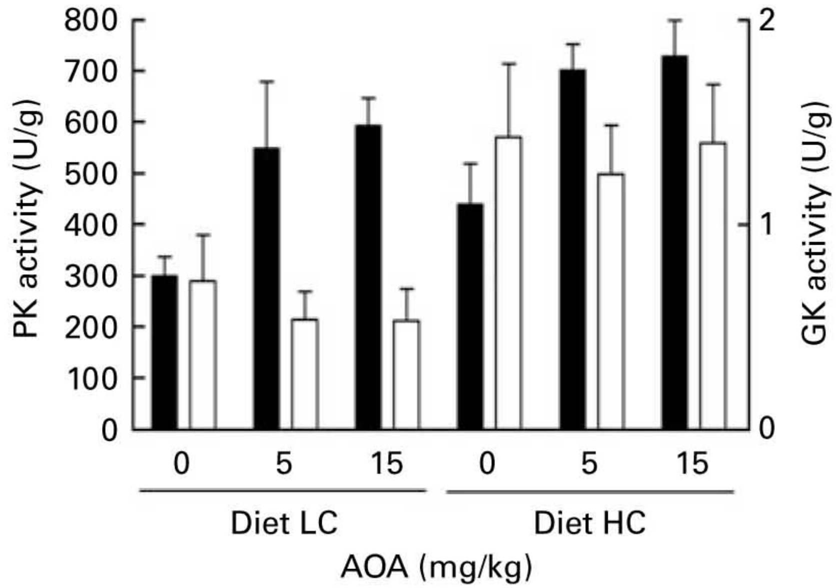
Two-way ANOVA

Dependent variable	Interaction	Diet	AOA	AOA 0	AOA 5	AOA 15
ALT	NS	***	***	b	a	a
AST	*	***	***	b	a	a



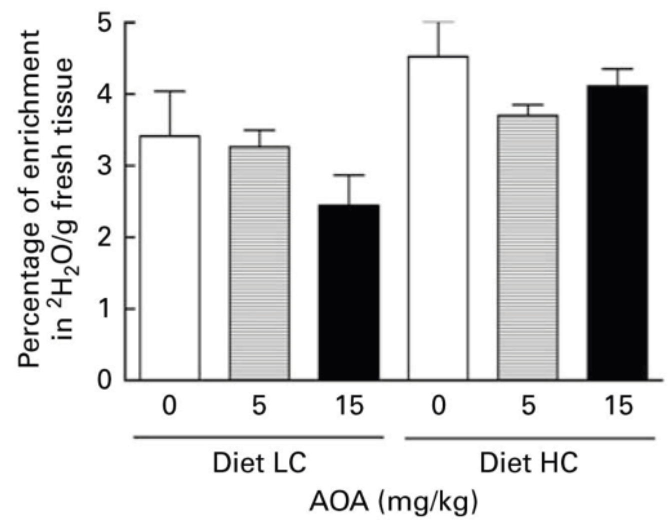
J.D. González et al (2012) *Br J Nutr* 107: 1747-1756

Effect of long-term exposure to AOA on the intermediary metabolism of *Sparus aurata*



Two-way ANOVA

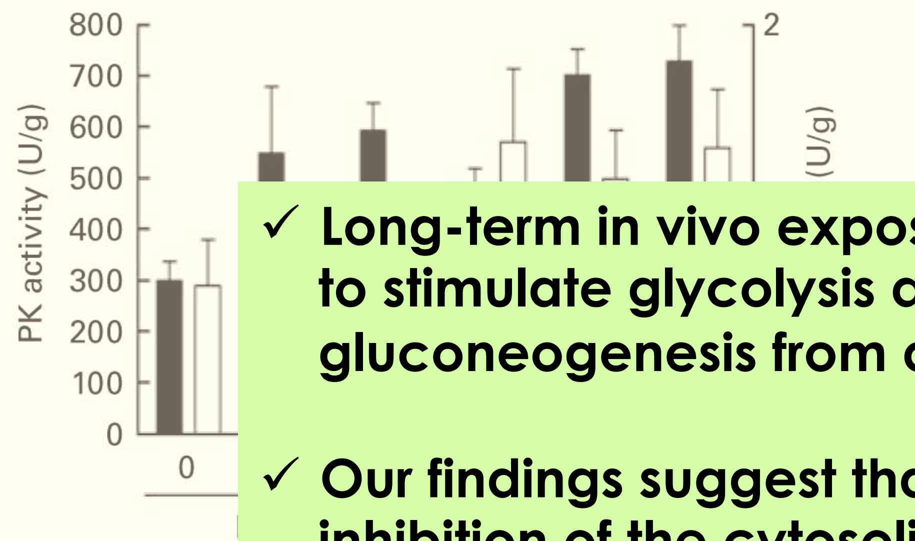
Dependent variable	Interaction	Diet	AOA	AOA 0	AOA 5	AOA 15
PK	NS	***	***	a	b	b
GK	NS	**	NS	–	–	–
FBPase	*	***	NS	–	–	–
PFK-1	NS	***	NS	–	–	–
G6PDH	NS	NS	NS	–	–	–
6PGDH	NS	**	NS	–	–	–



Two-way ANOVA

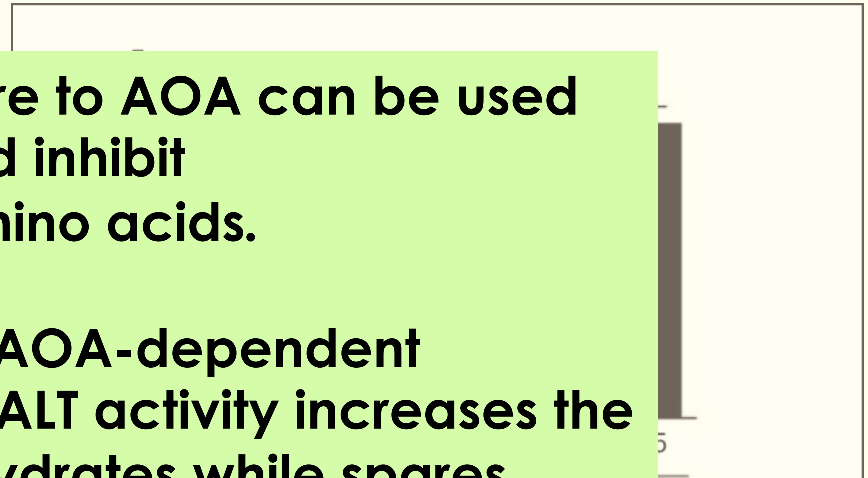
Dependent variable	Interaction	Diet	AOA	AOA 0	AOA 5	AOA 15
Alanine	NS	***	*	b	a,b	a

J.D. González et al (2012) *Br J Nutr* 107: 1747-1756



Two-way ANOV

Dependent variable	Interaction	Diet	AOA	0	5	15
PK	NS	***	***	a	b	b
GK	NS	**	NS	–	–	–
FBPase	*	***	NS	–	–	–
PFK-1	NS	***	NS	–	–	–
G6PDH	NS	NS	NS	–	–	–
6PGDH	NS	**	NS	–	–	–

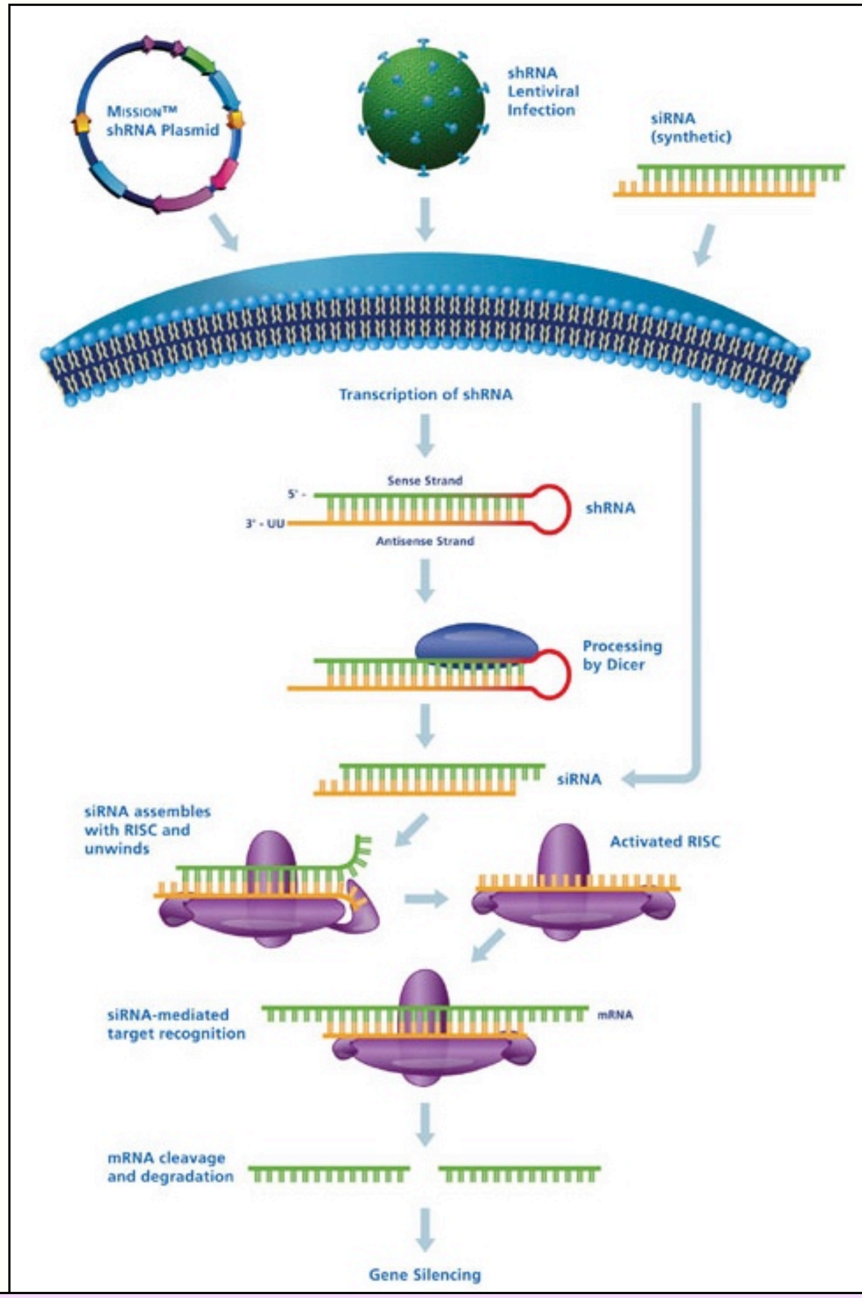


Dependent variable	Interaction	Diet	AOA	AOA 0	AOA 5	AOA 15
Alanine	NS	***	*	b	a,b	a

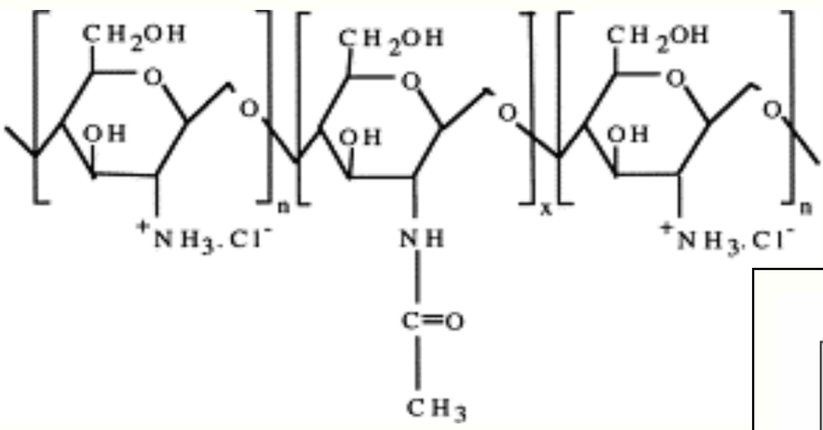
- ✓ **Long-term in vivo exposure to AOA can be used to stimulate glycolysis and inhibit gluconeogenesis from amino acids.**
- ✓ **Our findings suggest that AOA-dependent inhibition of the cytosolic ALT activity increases the use of the dietary carbohydrates while spares protein.**

J.D. González et al (2012) *Br J Nutr* 107: 1747-1756

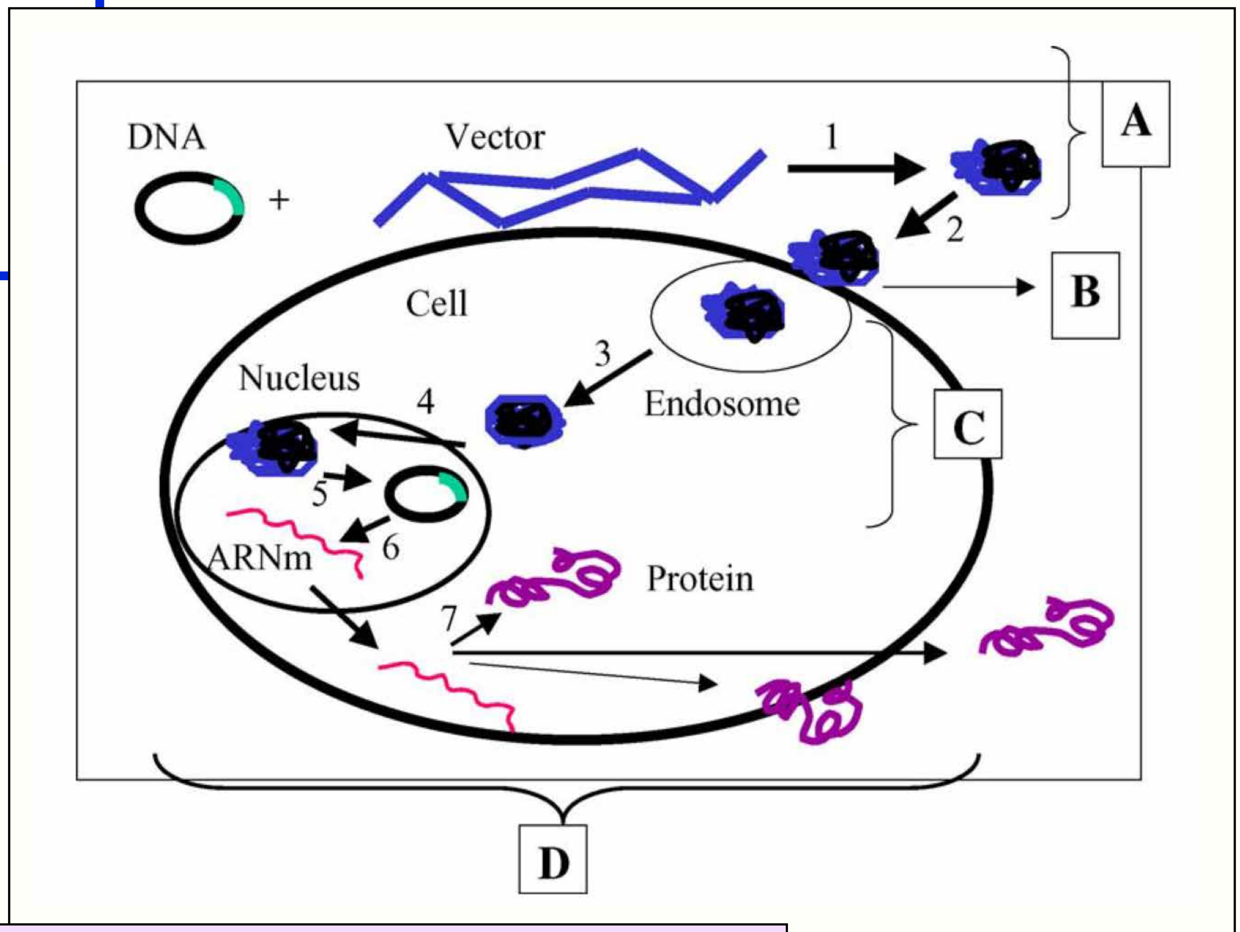
Silencing *Sparus aurata* cALT expression: shRNA



Chitosan as a non-viral vector for nucleic acids delivery



Chitosan



S. Mansouri et al. (2004) *Eur J Pharm Biopharm* 57, 1-8

Conclusions

- ✓ We isolated three ALT isozymes from *Sparus aurata*: two cytosolic isoforms resulting from alternative splicing of the cALT gene (cALT1 and cALT2) and a mitochondrial enzyme (mALT). Upregulation of cALT2 occurs under gluconeogenic conditions in the liver, whereas cALT1 is associated to postprandial utilization of dietary nutrients.
- ✓ Distinct transcription factors enhance the activity of cALT (p300 and c-Myb) and mALT (HNF4α) gene promoters.
- ✓ F-lectin stimulates cALT2 activity through a mechanism involving protein-protein interaction.
- ✓ Long-term inhibition of cALT isoforms increases glycolysis and decrease the renewal of alanine.

Future perspectives

- **Ultimate aim:**
  - ✓ **To improve the use of dietary carbohydrates by fish in culture and spare protein.**

- **Projects on course:**
  - ✓ **Effect of knocking-down the expression of cALT isoforms on the *Sparus aurata* intermediary metabolism and feed conversion efficiency.**
  - ✓ **To explore the possibility of a multifactorial action on several genes to perform a more robust protein sparing effect, we are performing transcriptomic and microarray analysis to identify other candidate genes as potential biomarkers to optimize the use of dietary nutrients in *Sparus aurata*.**
  - ✓ **Characterization of human ALT promoters (*M.C. Salgado et al.*, 2014, *BBA-Gene Regul. Mech.* 1839: 288-296).**

Nutrition, metabolism and functional genomics in fish

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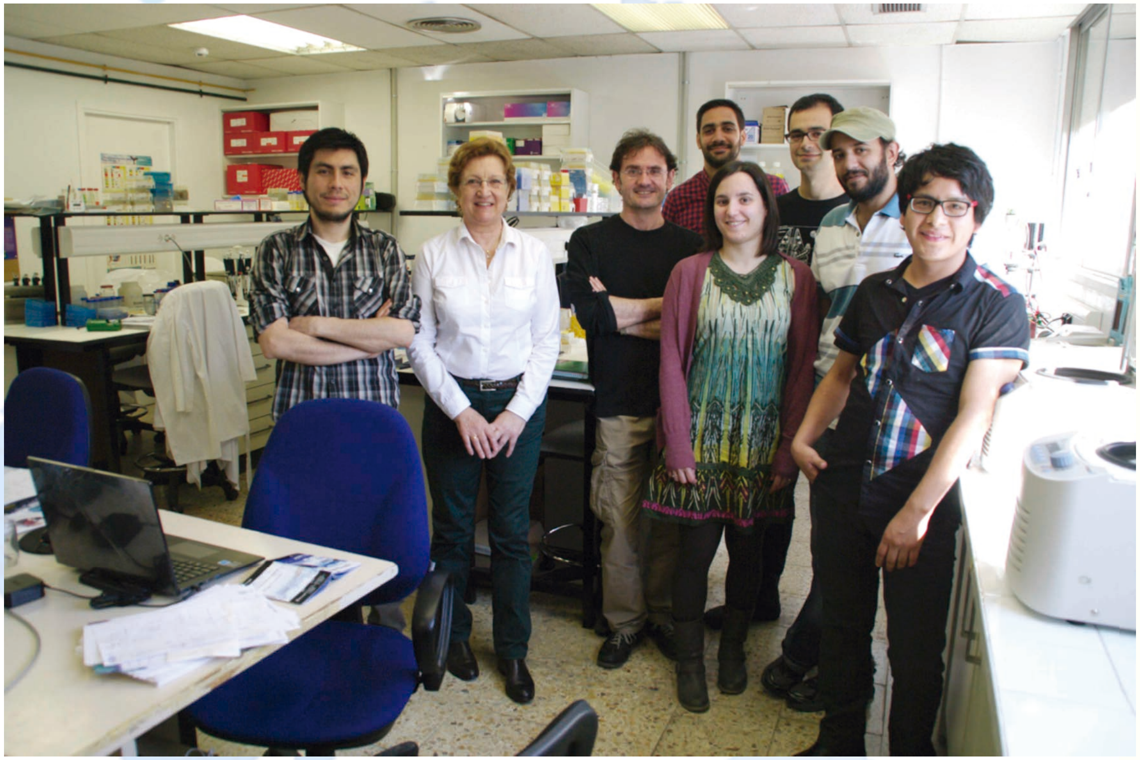

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Montserrat Miñarro (Universitat de Barcelona)

Gràcies per la vostra atenció!