Supplemental material and methods:

Animals

Birds were housed on long days (16L:8D) and received food and water *ad libitum*. They were killed by rapid decapitation. The five largest follicles and the rest of the ovary were collected and most of the yolk was removed from the follicles by squeezing them between sheets of absorbent paper. Aromatase was measured in the 4th and 5th follicles in the egg laying sequence (F4 and F5; F1 would be the biggest follicle or egg ready to be laid) and in the rest of the ovary. The tissue was frozen on dry ice. These samples were later homogenized in KTH (KCl 150 mM, Tris 10 mM and HEPES pH 7.2) at 0°C and stored at -80°C until used.

Phosphorylation sites

Consensus phosphorylation sites in the human aromatase sequence were analyzed using public domain softwares (NETPHOS 2.0 PREDICTION server at http://www.cbs.dtu.dk/services/NetPhos/). Based on a deduced amino acid sequence (in the present case, the human aromatase), this program identifies all serine, threonine and tyrosine residues in the protein that could potentially be phosphorylated providing for each residue a phosphorylation score ranging from 0 to 1.0 indicative of the probability that the residue can be phosphorylated. A score equal or larger to 0.5 is considered to predict a likely phosphorylation consensus site (1). In a second step, an associated prediction algorithm found on the same web server (http://www.cbs.dtu.dk/services/NetPhosK/) was used to research the type(s) of kinase(s) that could catalyze the addition of a phosphate group based on a set of primary structure consensus sequences in proximity of the target residue (see results in Table 1)

Aromatase mutants

Based on the bio-informatic analysis described in the previous paragraph, PCR-based site-directed mutagenesis was performed using the primers described in supplementary table 1 to obtain 6 different mutants, where a Serine (S118, S247, S267, S497) or a Threonine (T462, T493), or a combination of

several amino acids, were mutated to an Alanine (A; see Table 1). Briefly, 25 ng of plasmid were subjected to PCR with *Pfu* DNA polymerase (Promega) for 12 cycles (30s at 95°C, 60°C at 50°C, 10 min at 72°C). The PCR products were then digested with *DpnI*, which only digests the parental methylated plasmid and transformed into DH5α E.coli. Plasmid DNA was then extracted with Plasmid Midi Kit QIAGEN and mutations were confirmed by sequencing (V. Dhennin, Genotranscriptomics Platform, GIGA, University of Liège) before transfection.

Insert supplementary Table 1 here

Transfection:

15 µL of transfection reagent was added to serum-free DMEM, incubated for 20 min at RT, added with 2.5 µg of plasmid, vortexed and incubated for another 20 min before addition to the cells. After 24 hours, medium was changed to DMEM and 10% FBS containing 10 µg/µL Blasticidin S Hydrochloride (BioChemika, or geneticine G418, Sigma) for selection of stable transfectants.

ATP incorporation

Cell lysate (100 µg protein) from HEK293 cells containing the pRC/CMV-AROM/c-myc, was incubated in control condition (KTH) or in phosphorylating conditions (KTH, 10 mM $MgCl_2$, 2mM $CaCl_2$) for 15 min at 37°C in the presence of 100 µCi [γ-32P]- ATP (3000 Ci/mmol 10mCi/ml, PerkinElmer). The reaction was stopped by transferring the samples in an ice-water bath. A mix of proteinase and phosphatase inhibitor was then quickly added to all samples (Complete™ proteinase inhibitor, EDTA-free Roche; Okadoic acid (100 nM), Sodium Vanadate (2 mM), Sodium fluoride (2 mM), EDTA 10 mM). Samples were then incubated overnight at 4°C with 20 µl rabbit anti-human c-myc IgG-agarose conjugate (Alpha Diagnostic international) to purify aromatase. Beads were then washed and the immunoprecipitated protein was eluted using loading buffer (SDS 4% β–mercaptoethanol 10%, Tris-HCl 135 mM pH 6.8, Glycerol 20%). Electrophoresis was then performed on 8% SDS-PAGE and proteins were fixed in gel (methanol 60%, acetic acid 10%).

Protein concentration and aromatase Western blots

Total protein concentration from lysed cell cultures was determined with Coomassie Plus™ protein assay reagent following manufacturer's instructions (ThermoScientific) prior to analysis by Western blot. Total proteins or proteins purified on mouse anti-human c-myc IgG (Clone E10) -agarose conjugate (Alpha Diagnostic International) were then denatured in loading buffer (SDS 4%, β-Mercaptoethanol 10%, Tris-HCl 135 mM pH 6.8, Glycerol 20%) and separated by electrophoresis on SDS-PAGE 8%. Transfer was performed on PDVF membrane (Hybond, GE Healthcare Life Sciences). Membranes were blocked for 1 hr at room temperature in blocking buffer (5% non-fat dry milk for anti-aromatase or 5% Bovine serum albumin for anti-serine) in Tris-buffered saline pH 7.6 containing 0.1% Tween 20), and incubated overnight at 4°C in blocking buffer containing a rabbit anti-human aromatase antibody [1/1000, (2, 3)] or rabbit anti-serine antibody (1/1000, StressMark). Membranes were rinsed, incubated with Goat anti-rabbit HRP (AbCam, 1/3000, 1 hr at room temperature), rinsed again, dried and subjected to SuperSignal West Pico Chemiluminescent substrate for 5 min (ThermoScientific). Membranes were then stripped, extensively rinsed, blocked for 1 hr, incubated for 2 hrs at room temperature with a monoclonal β-actin antibody (1:10,000; Sigma, clone AC-74), incubated with Goat anti-mouse HRP (AbCam, 1/5000, 1 hr at room temperature) and the β-actin bands were subsequently visualized as described above. Optical density of the bands were measured by image analysis (ImageJ, Wayne Rasband, NIH, Bethesda, MD, USA) on images obtained from an ImageQuant LAS4000 analyzer (GE Healthcare Life Sciences)

References:

1. **Blom N, Gammeltoft S, Brunak S** 1999 Sequence and structure-based prediction of eukariotic protein phosphorylation sites. J Mol Biol 294:1351-1362
2. **Harada N** 1988 Novel properties of human placental aromatase as cytochrome P-450: purification and characterization of a unique form of aromatase. J Biochem 103:106-113
3. **Balthazart J, Foidart A, Harada N** 1990 Immunocytochemical localization of aromatase in the brain. Brain Res 514:327-333

Mutation	Primer
S118A	5’ CAGCTCTCGATTCGGC***GCC***AAACTTGGGCTGCAGTGC 3’ ***AGC***
S247A	5’ CAAAAAGTATGAGAAGT***GCT***GTCAAGGATTTGAAAGATGC 3’ ***TCT***
S267A	5’ GCAGAAAAAAGACGCAGGATT***GCC***ACAGAAGAGAAACTG 3’ ***TCC***
T462A	5’ GACGATTCCACGTGAAG***GCA***TTGCAAGGACAGTGTG 3’ ***ACA***
T493A	5’ GCTGGAAATGATCTTT***GCC***CCAAGAAACTCAGACAGG 3’ ***ACC***
S497A	5’ GATCTTTACCCCAAGAAAC***GCA***GACAGGTGTCTGGAAC 3’ ***TCA***

Supplemental Table 1 : Sequences of forward primers used in site-directed mutagenesis. The original codon is in bold and underlined [Serine (S) or Threonine (T)] while the new codon is in bold only [alanine (A)]. The number between the two amino acids in the first column represents the localization of the targeted amino acid.