

beta-arrestin 2 oligomerization controls the Mdm2-dependent inhibition of p53.

Cédric Boularan, Mark Scott, Karima Bourougaa, Myriam Bellal, Emmanuel Esteve, Alain Thuret, Alexandre Benmerah, Marc Tramier, Maité Coppey-Moisan, Catherine Labbé-Jullié, et al.

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Cédric Boularan, Mark Scott, Karima Bourougaa, Myriam Bellal, Emmanuel Esteve, et al.. beta-arrestin 2 oligomerization controls the Mdm2-dependent inhibition of p53.. Proceedings of the National Academy of Sciences of the United States of America , National Academy of Sciences, 2007, 104 (46), pp.18061-6. 10.1073/pnas.0705550104 . inserm-00174967

HAL Id: inserm-00174967

<https://www.hal.inserm.fr/inserm-00174967>

Submitted on 14 Jan 2008

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Supporting Methods.

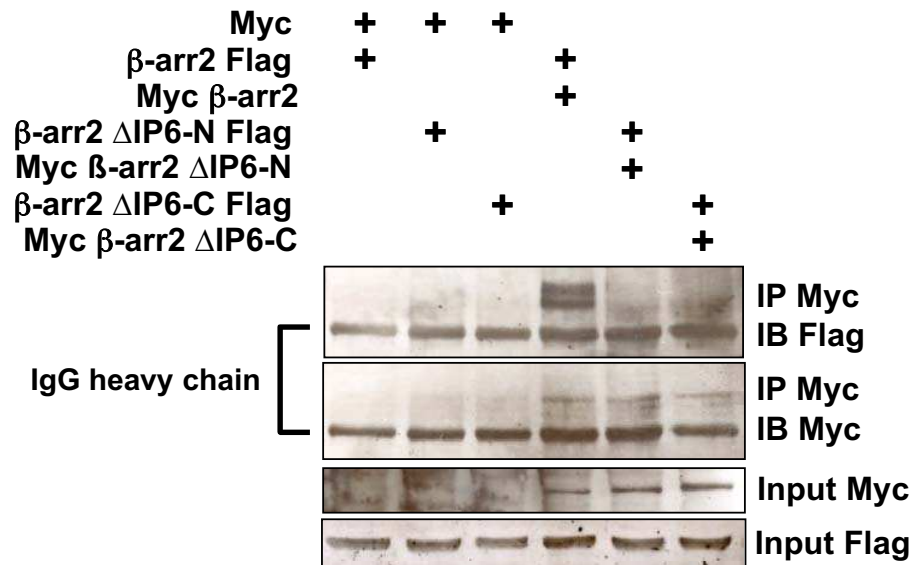
Fluorescence Lifetime Imaging Microscopy

The two-photon picosecond FLIM system combines multifocal multiphoton excitation (TriMscope, LaVisionBiotec, Bielefeld, Germany) and a fast-gated CCD camera (Picostar, LaVisionBiotec, Bielefeld, Germany) connected to an inverted microscope (IX 71, Olympus, Tokyo, Japan). A mode-locked Ti:Sa laser at 950 nm (Spectra Physics, France) is split up into 2 to 64 beams by utilizing a 50/50 beam splitter and mirrors. The set of beams is passing through a 2000 Hz scanner before illuminating the back aperture of a x60 NA 1.3 infrared water immersion objective (Olympus, Tokyo, Japan). A line of foci is then created at the focal plane, which can be scanned across the sample, making a pseudo wide-field illumination. A filter wheel of spectral filters (535AF45 for GFP) is used to select the fluorescence imaged onto a fast-gated light intensifier connected to a CCD camera (Picostar). The gate of the intensifier is triggered by an electronic signal coming from the laser and a programmable delay box was used to acquire a stack of time-gated images. For each fluorescence lifetime image, a stack of 11 time gated (1 ns gate) fluorescence intensity images at increasing interval after excitation (1 ns per step) was obtained (from 0 to 10 ns), each time gated image being acquired sequentially (from 0 to 10 ns). The acquisition time of the CCD camera was adjusted considering the level of the time integrated fluorescence signal (from 1 to 4 s). All of the instrumentation was controlled by Inspector software developed by LaVisionBiotec.

To analyze the FLIM data, a mean lifetime image was calculated by using time-correlated stack of images

$$\langle \tau \rangle = \frac{\sum \Delta t_i \times I_i}{\sum I_i}$$

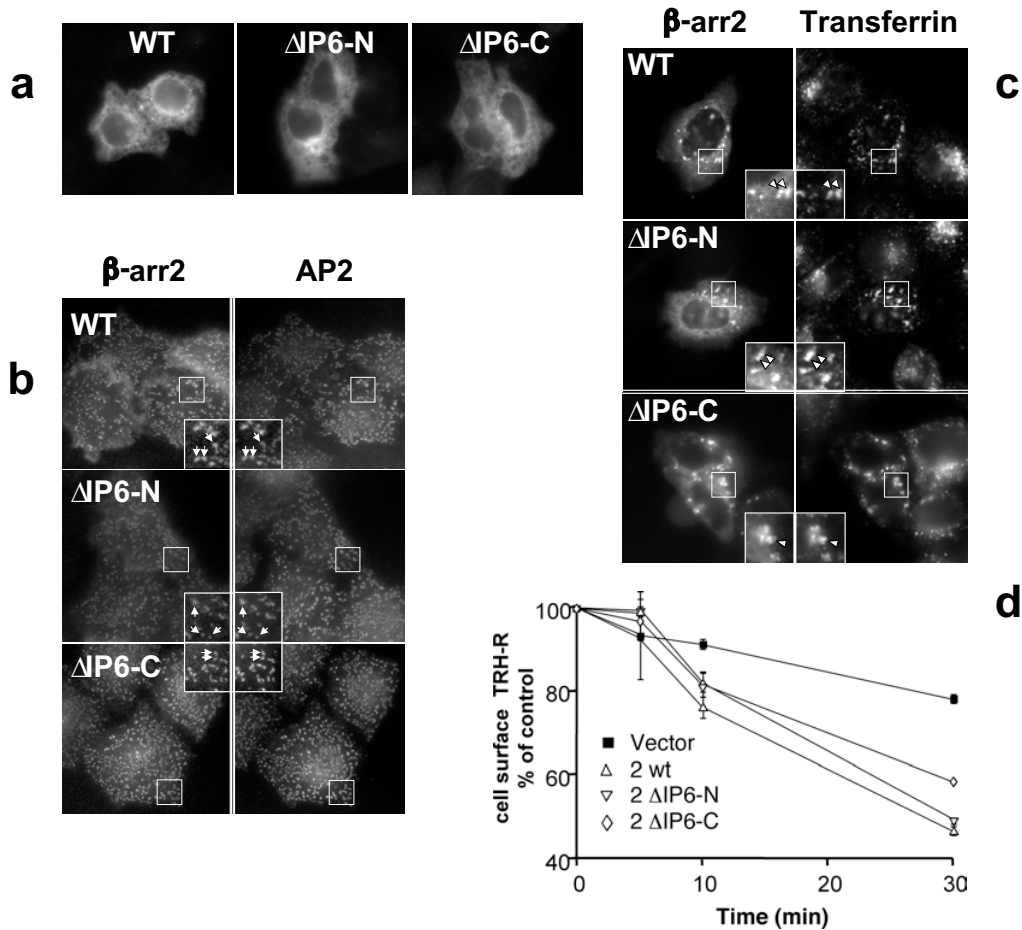
where Δt_i corresponds to the time delay after the laser pulse of the i^{th} image acquired and I_i to the pixel intensity map in the i^{th} image. Fluorescence lifetime of GFP-fused protein was recorded in the absence or in the presence of the mCherry-fused protein (acceptor). The decrease of the GFP lifetime corresponds to FRET, as presented in details (Reference 20 of the article).



Characterization of β-arr2 IP6-binding mutants

COS7 cells were transfected with the indicated β-arr2-FLAG and Myc-β-arr2 constructs or control empty vector (1μg of each construct per 100mm dish). After immunoprecipitation with anti-Myc antibodies, immunoblots were performed with anti-Flag and anti-Myc antibodies; 10% of input is shown.

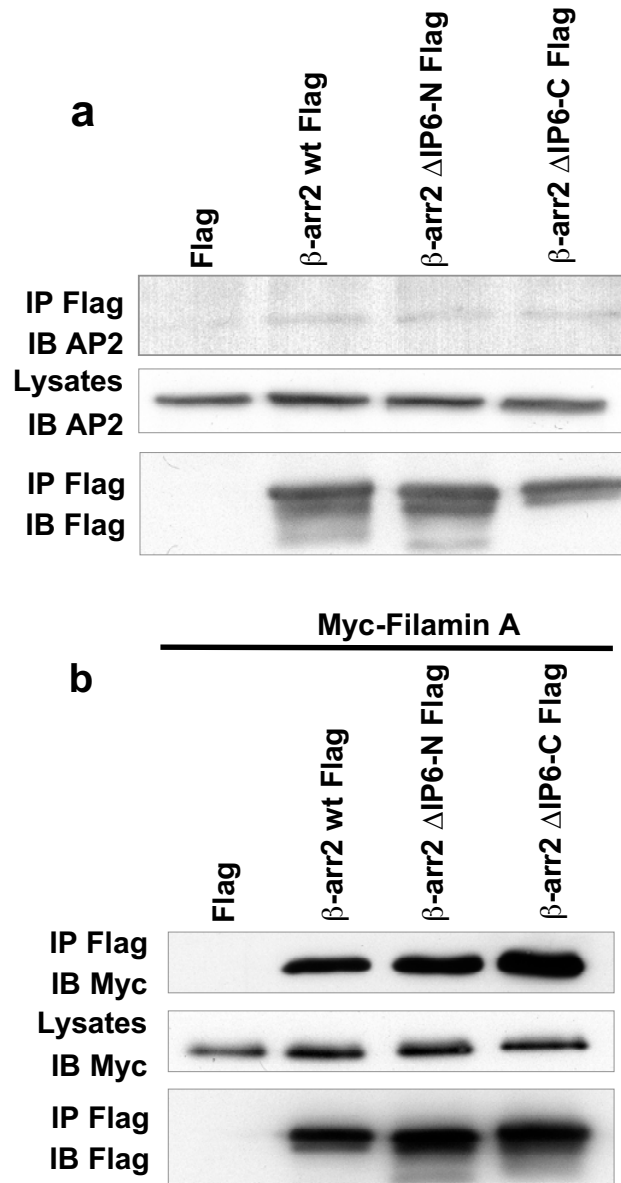
Supporting Figure 1



Functional characterization of β -arr2 IP6-binding mutants in GPCR endocytosis

a) Steady-state localization of β -arr2 constructs. HeLa cells were transiently co-transfected with β -arr2, β -arr2 Δ IP6-N or β -arr2 Δ IP6-C myc-tagged constructs, fixed 48h after transfection (4% PFA) and processed for fluorescence microscopy. **b, c)** Receptor-dependent accumulation of β -arr2 constructs in clathrin-coated pits (CCPs) and endosomes. HeLa cells were transiently co-transfected with HA-tagged angiotensin II (AngII) AT1 receptor and either β -arr2, β -arr2 Δ IP6-N or β -arr2 Δ IP6-C YFP-tagged constructs. After 2 (b) or 30 min (c) stimulation with 1 μ M AngII, cells were fixed, permeabilized, and processed for fluorescence microscopy. In the insets, arrows indicate colocalization of β -arr2 constructs and AP2 in the same CCPs (b) or colocalization of β -arr2 constructs and transferrin in the same endosomes (c). **d)** β -arr2-dependent receptor endocytosis. Endocytosis of TRH-R was studied 48 h after transient co-transfection with β -arr2, β -arr2 Δ IP6-N or β -arr2 Δ IP6-C flag-tagged constructs in COS7. Cells were incubated on ice for 1h with 3 [H]-TRH and then shifted to 37 $^{\circ}$ C for the indicated time. After chilling and extensive washing at 4 $^{\circ}$ C, surface bound 3 [H]-TRH was collected by acid-strip and internalized ligand was measured after cell lysis. For the detailed procedure see ref (10).

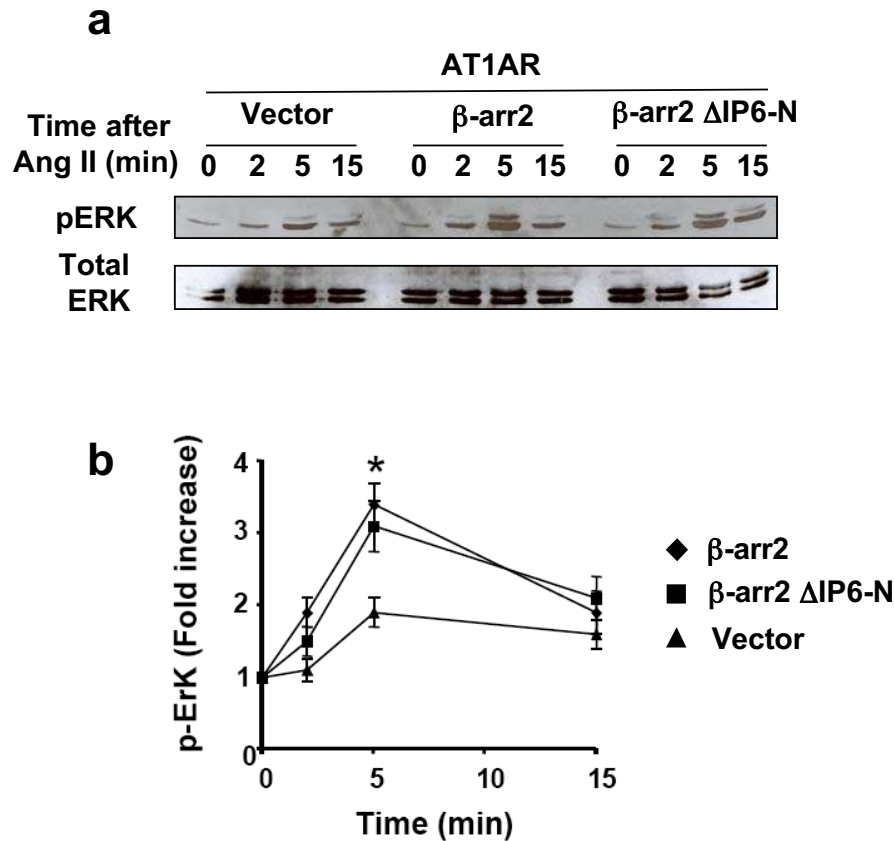
Supporting Figure 2



IP6 binding mutants of β -arr2 retain the interaction with AP2 and Filamin A

COS7 cells were transfected with the indicated β -arr2 constructs alone (a) or in combination with Myc-Filamin A domains 22-24 (b) ($1\mu\text{g}$ of each construct per 100mm dish). After immunoprecipitation with anti-Flag antibodies, endogenous AP2 or transfected Filamin A were revealed respectively with anti-AP2 (M300, Santa Cruz) or anti-Myc (AbCam) antibodies; 10% of input is shown.

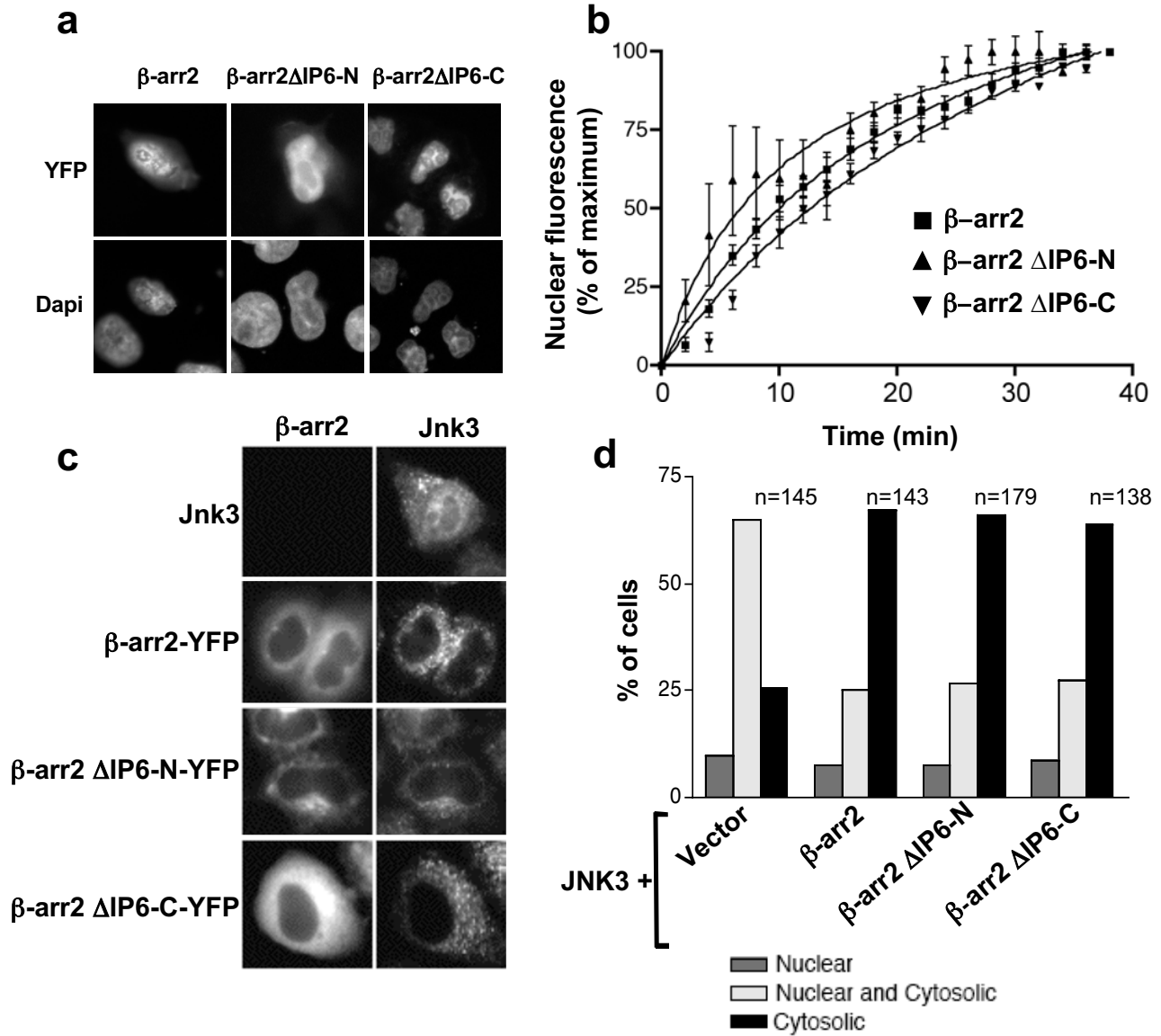
Supporting Figure 3



ERK-scaffolding properties of β -arr2 IP6-binding mutants

a) HeLa cells were transfected with plasmids encoding the HA-tagged angiotensin II (AngII) AT1 receptor (AT1AR) and β -arr2 Flag or β arr2 Δ IP6-N Flag or empty vector. After overnight serum starvation, cells were left untreated or stimulated with 1 μ M AngII for 2, 5 or 15 min at 37°C. Cell lysates were resolved by SDS-PAGE and phosphorylation of ERK1/2 was assessed by immunoblotting using an anti-phospho-ERK1/2 antibody. Total ERK1/2 expression was measured by probing the immunoblots with anti-ERK1/2 antibodies. **b)** Gels were scanned and phospho- and total-ERK1/2 levels determined using the NIH Image J software. The ratio of phosphorylated over total ERK1/2 was calculated and normalized as fold increase over unstimulated cells. Data represent the mean \pm SEM of 3 independent experiments (* p <0.05). Comparable results were obtained in cells transfected with the β arr2 Δ IP6-C Flag construct (not shown).

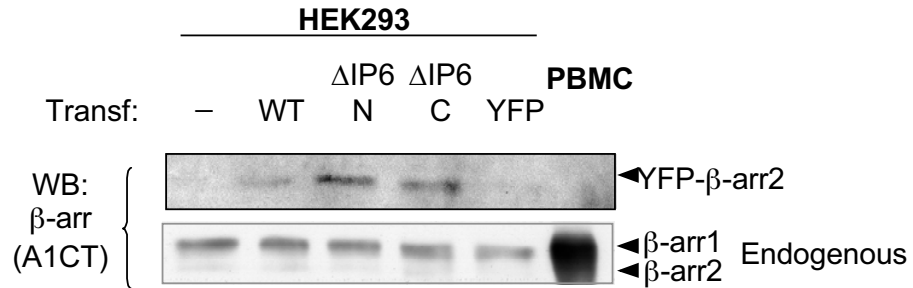
Supporting Figure 4



Nucleo-cytoplasmic shuttling of β -arr2 IP6-binding mutants and JNK3 redistribution

a) Nuclear accumulation of β arr2-YFP, β arr2 Δ IP6-N-YFP and β -arr2 Δ IP6-C-YFP 40min after LMB treatment in HeLa cells. **b)** Kinetics of the nuclear accumulation of β -arr2-YFP, β -arr2 Δ IP6-N-YFP and β -arr2 Δ IP6-C-YFP after LMB addition. **c)** HeLa cells were transiently co-transfected with β -arr2, β -arr2 Δ IP6-N or β -arr2 Δ IP6-C YFP-tagged constructs and FLAG-JNK3. Cells were fixed, permeabilized, and processed for fluorescence microscopy. The mouse anti-flag M2 monoclonal antibody was revealed using a secondary Cy3-labeled donkey anti-mouse antibody. **d)** For quantitation, cells were classified respective to their predominantly nuclear, cytosolic or diffuse localization of JNK3. Results are expressed as the percentage of cells in each category. Similar results were obtained in Cos7 cells (data not shown).

Supporting Figure 5



Estimation of exogenous β -arr in BRET experiments

Western blots comparing the amount of BRET acceptors expressed in cells displaying half-maximal BRET values in Fig. 2c, compared to the endogenous β -arrs present in HEK293 and peripheral blood mononuclear cells (PBMC). Equivalent amounts of protein lysates were analyzed using A1CT antibody. To observe transfected BRET acceptors film was exposed for a longer period of time compared to native β -arrs indicating that the level of expression was at most equivalent to endogenous β -arr levels.

	Sub G1		G1		S		G2M	
	basal	+p53	basal	+p53	basal	+p53	basal	+p53
βarr2 wt	0,72	0,94	69,49	67,95	16,67	12,48	11,28	19,34
βarr2 ΔIP6-N	0,98	1,08	68,78	69,31	17,40	15,82	8,36	12,35
βarr2 ΔIP6-C	1,65	0,83	69,52	71,27	16,92	13,46	11,27	13,50
βarr2 ΔNES	2,21	0,90	69,72	69,25	17,20	15,86	10,10	13,00

Cell distribution (%) in various phases of the cell cycle

H1299 cells were transfected with the indicated β-arr2 YFP-tagged constructs alone or in combination with p53 (0,5 μg of each construct). The total amount of transfected DNA was kept constant by adding empty vector. 48 h post transfection, H1299 cells nuclei were stained with propidium iodide (50 μg/ml) and cell cycle phases were analyzed with a LSR flow cytometer (Becton-Dickinson).