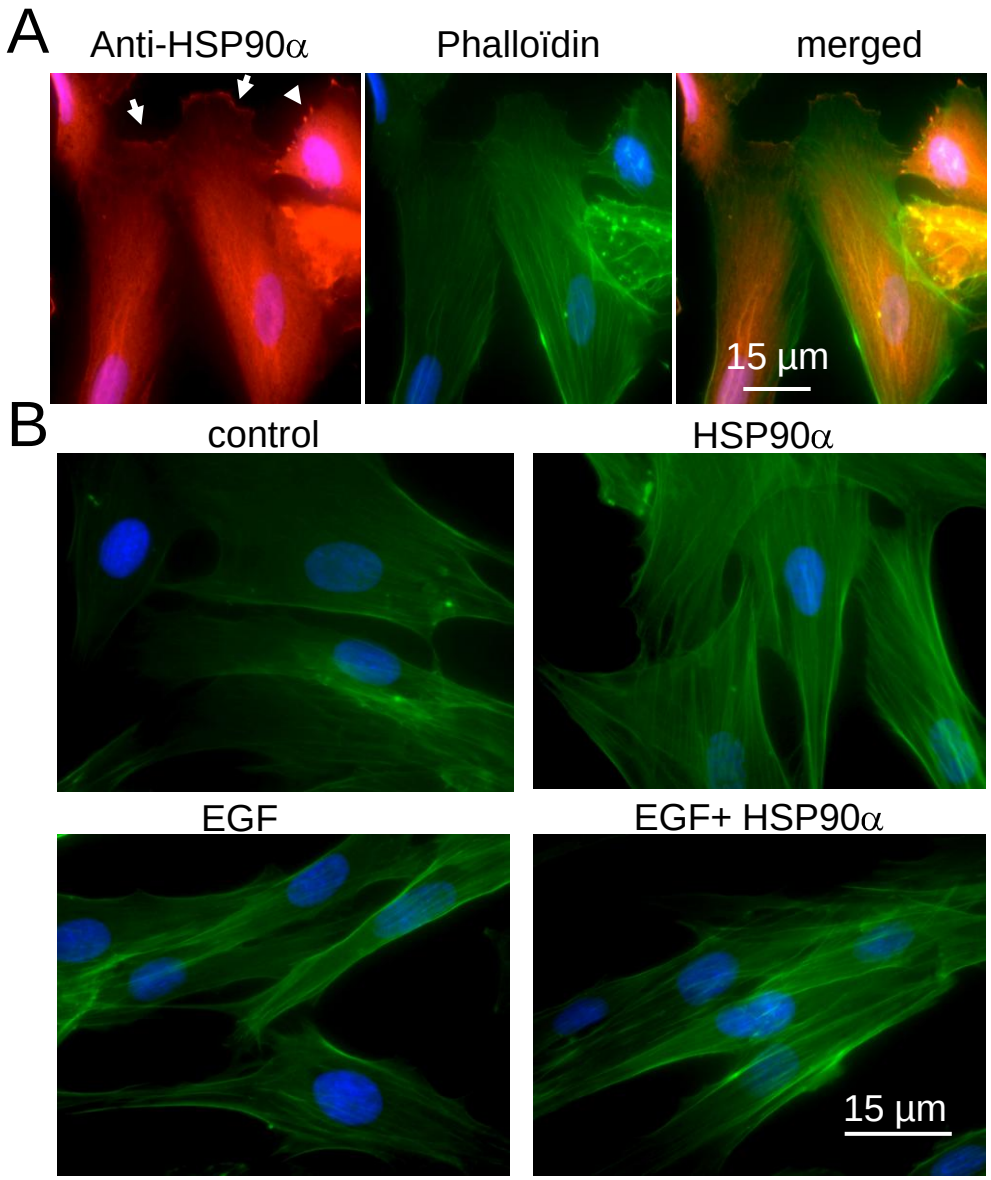



**Figure S1.** *Phalloidin staining of F-actin in unstimulated and stimulated U87 cells with HSP90a and/or EGF for 15 min (nuclei staining with DAPI).* A. Immunofluorescence detection of HSP90 isoforms a in permeabilized U87 cells. Note the co-localization of HSP90a isoform with polymerized F-actin bundles at the leading edge of migrating cells (n=3). B. Upon staining with phalloidin, EGF rapidly increased F-actin content (as estimated from Alexa Fluor 488-conjugated phalloidin fluorescence intensity) and to rearrange the actin filaments, which was further amplified in the presence of HSP90a. The mean intensity of labelled area (per 10 µm2) was: 42.3 7.1 in control, 65.4 9.3 with HSP90a, 105.9 4.0 with EGF, and 146.2 11.1 with HSP90a plus EGF (mean SD; n=7).

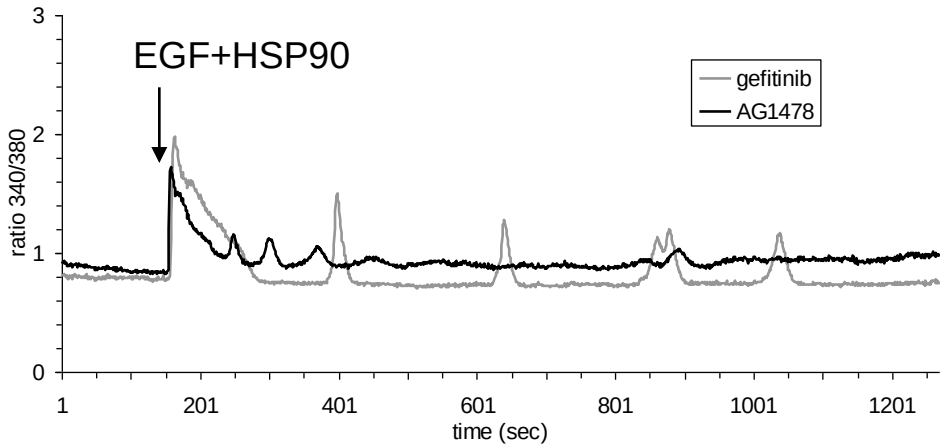



**Figure S2.** *Contribution of EGFR/ErbB1 to HSP90a-induced Ca2+ oscillations in the presence of EGF*. Superimposed traces obtained from cells preincubated for 10 min with the EGFR/ErbB2 inhibitor gefitinib (10 µM) or with the EGFR/ErbB1 inhibitor tyrphostin (10 µM AG1478) (n=10). These results suggested that HSP90a could promote calcium oscillations through EGFR/ErbB1.

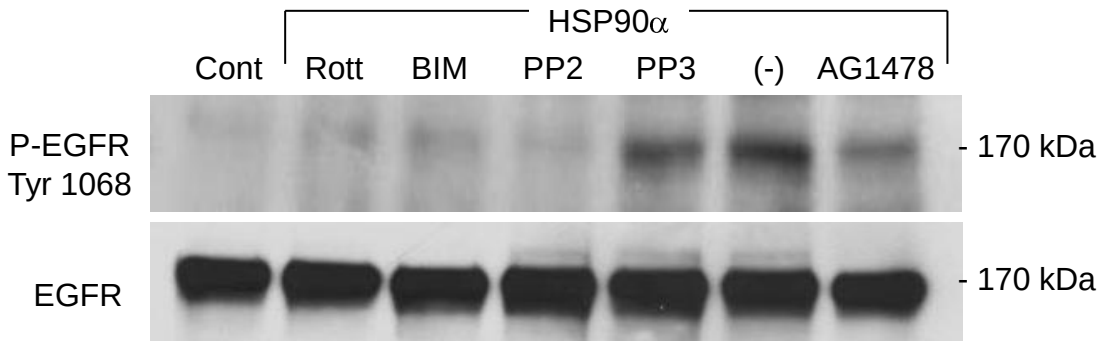



**Figure S3.** *Effect of BIM (PKC inhibitor), rottlerin (PKC-d inhibitor) or PP2 (c-Src inhibitor) on EGFR Tyr1068 activation by HSP90a.* Serum-starved U87 cells were incubated with inhibitors (10 µM) for 60 min then stimulated with HSP90a (6µg/ml) for 15 min. Cell extracts were immunoblotted for EGFR Tyr-1068. Blots were stripped and re-probed for total EGFR (n=2). The inactive isomer PP3 did not have any effect and the EGFR inhibitor, AG1478, exerted a partial inhibition. These results suggest that HSP90a transactivates EGFR by activating the PKCd/c-Src pathway in glioblastomas.

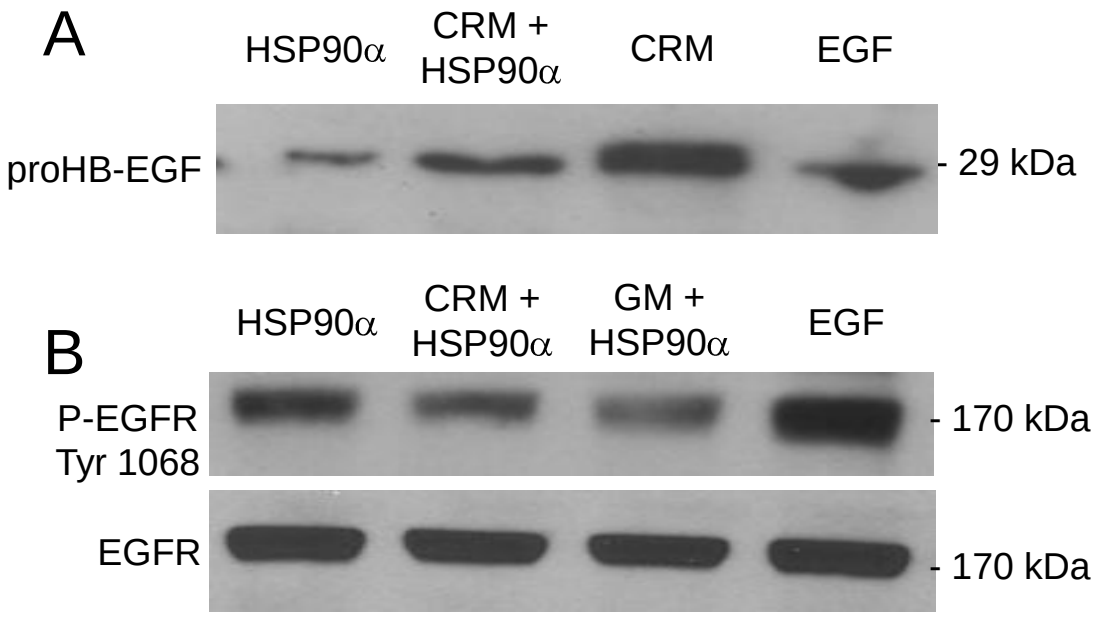



**Figure S4.** *Contribution of proHB-EGF shedding and MMPs in HSP90a-induced tyrosine phosphorylation of EGFR*. Serum-starved U87 cells were pretreated with CRM197 (CRM 10μM) for 30 min or with GM6001 (GM 20 μM) for 1 hour before their stimulation with HSP90a (6 μg/ml) or EGF 10 ng/ml for 15 min. Cell extracts were immunoblotted with anti-proHB-EGF (A) or with anti-phospho-(Tyr1068) EGFR and anti-EGFR (B). HSP90a treatment stimulated tyrosine phosphorylation of EGFR and significantly decreased the total proHB-EGF levels in cells, which were attenuated by treatment with CRM197, a non-toxic mutant of diphtheria toxin that binds specifically to HB-EGF. To examine whether HSP90a-induced proHB-EGF shedding is dependent on activation of matrix metalloproteinases, we used galardin (GM6001), a broad-spectrum MMP inhibitor. GM6001 reduced HSP90a-induced tyrosine phosphorylation of EGFR. Shown are representative blots of two independent experiments.

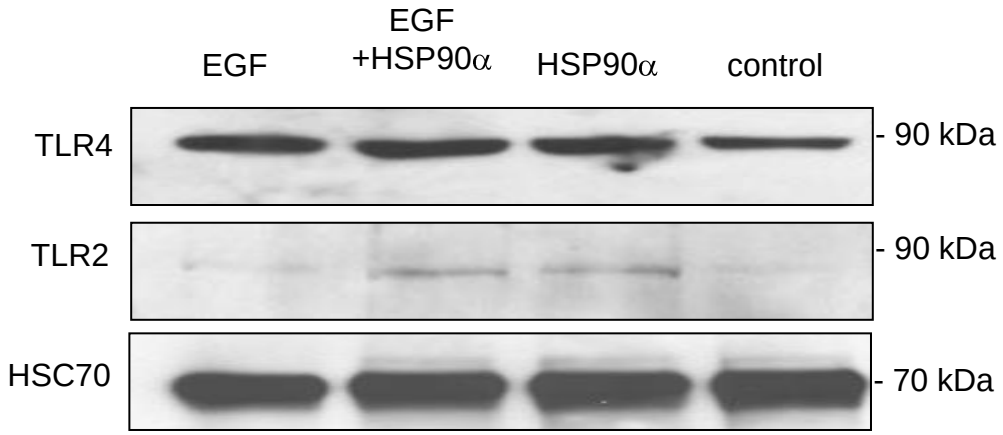



**Figure S5.** *Cell stimulation with EGF and/or HSP90a did not affect the TLR expression*. Serum-starved U87 cells were stimulated with HSP90a (6 µg/ml) and EGF (10 ng/ml) for 6 hours. Cell extracts were immunoblotted for TLR-4 and TLR-2 (n=3). One representative image is shown. A slight but not significant increase in TLR-2 expresssion could be observed in the presence of HSP90a while no variation was detected in TLR-4 content in glioblastoma cells.

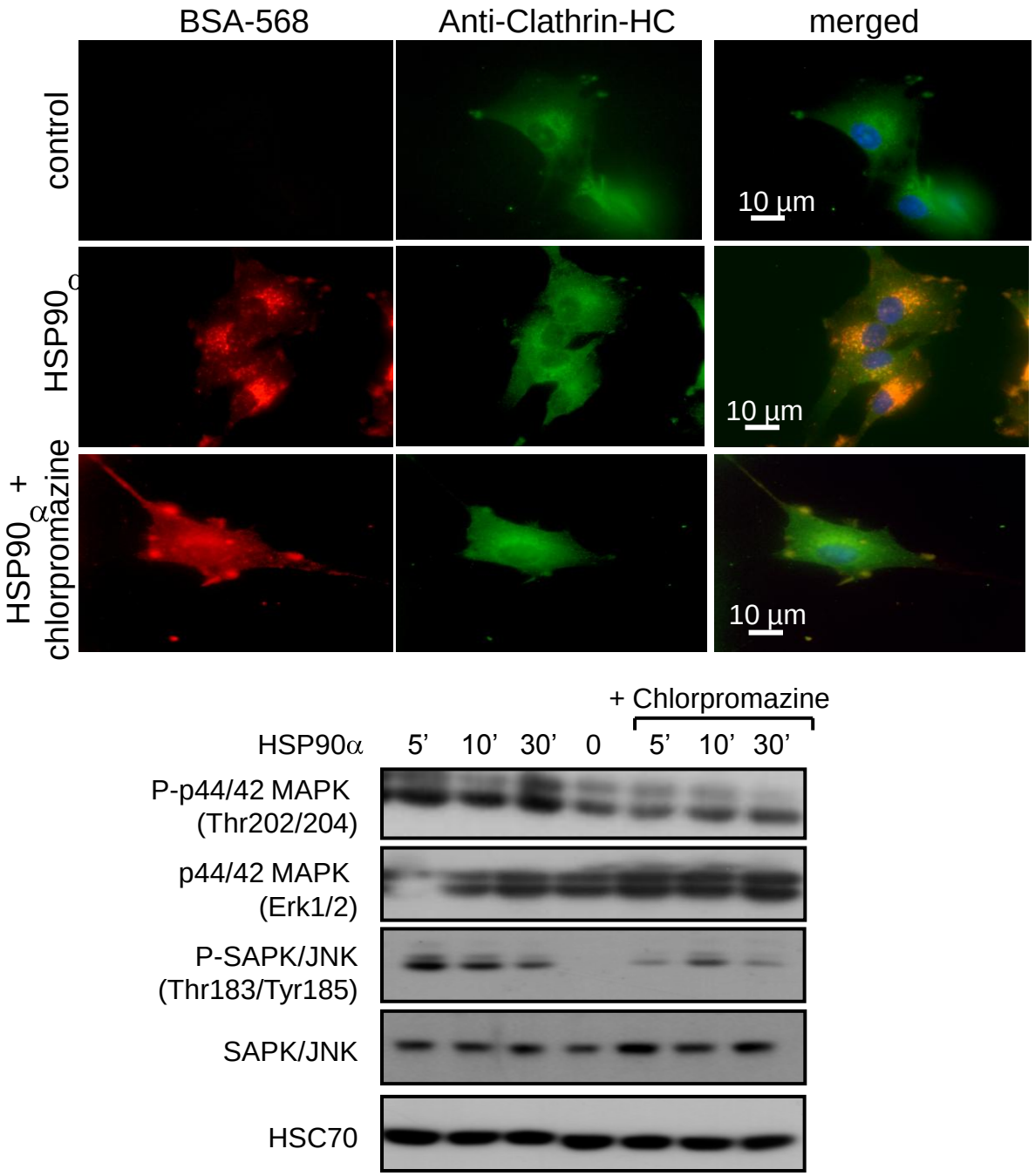
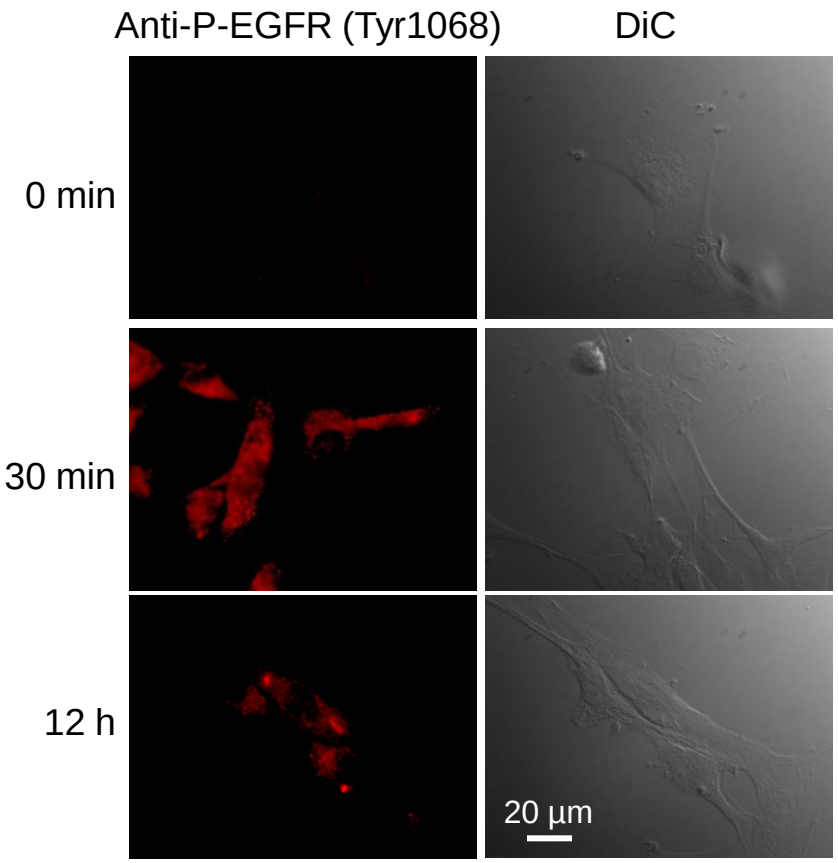



**Figure S6.** *Chlorpromazine significantly blocks endocytosis and the sustained EGFR signalling*. The cationic amphiphilic drug chlorpromazine inhibits assembly of clathrin adapter protein AP2 on clathrin-coated pits, and blocks clathrin-mediated endocytosis at dose ranging from3 to 30 µg/ml. Serum-starved U87 cells were incubated for 30 min in HBSS containing 6µg/ml HSP90a plus 5 µM BSA-alexa568 (red), then fixed and labelled with a polyclonal anti-clathrin-alexa488 (green). Clearly internalization of BSA-alexa568 was significantly inhibited in cell pre-treated with 10µM chlorpromazine for 30 min. Note the presence of BSA-alexa568 at the plasma membrane of treated cells as compared to control (no HSP90a addition) indicating the blockage of endosomes at the cell periphery. *Merged*, co-localization of HSP90a-induced BSA labelled endosomes and clathrin-HC in untreated cells (yellow; n=3; observation made with a widefield Leica microscope). Western blot analysis was performed on whole-cell lysates using the indicated antibodies in untreated and treated cells. Chlorpromazine reduced drastically the HSP90a-mediated phosphorylation of both p44/42 MAPK and SAPK/JNK (n=2). Thus, clathrin-mediated internalization is essential for a sustained HSP90a-mediated EGFR signalling.




| 0 min | 10 min | 30 min | 1 h | 6 h | 14 h | 22 h | 24 h |
|---|---|---|---|---|---|---|---|
| 5.85<br>0.006 | 26<br>0.027 | 60.50<br>0.007 | 30.50<br>0.021 | 71.70<br>0.024 | 33.50<br>0.049 | 19.45<br>0.008 | 1.35<br>0.002 |

**Figure S7.** *Internalized EGFR by HSP90a is not targeted for degradation.* Several routes of internalization of EGFR are now described involving diverse plasma membrane proteins, although the major mechanism of internalization of EGFR is clathrin-mediated endocytosis, whereby the receptor is removed from the surface via clathrin-coated pits and then routed to be recycled to the membrane, degraded or addressed to the nucleus. In order to see whether EGFR remained active, we examined the cell expression of the phosphorylated receptor (P-EGFR Tyr1068) after several hours of cell exposure to 6 µg/ml HSP90a using immunofluorescence microscopy (upper panels) and flow cytometry analysis (table). In control conditions, no labelling was observed. The percentage of P-EGFR expression remained low. After 30 min of cell stimulation, a strong labelling and a high level of expression were observed in accordance with our previous results. Although the P-EGFR expression tended to decrease in the following hour, it remained high until 6h. After 12h, a slight labelling was found around the nucleus and the total cell expression started to decrease to be very low after 24h of cell treatment. Thus EGFR is not targeted to degradation within the first 12 hours of cell exposure to HSP90a. This is in agreement with previous reports showing that the clathrin-internalization is essential for sustained EGFR signalling but dispensable for degradation (Sigismund S et al, Developmental cell 15, 209-219, 2008).

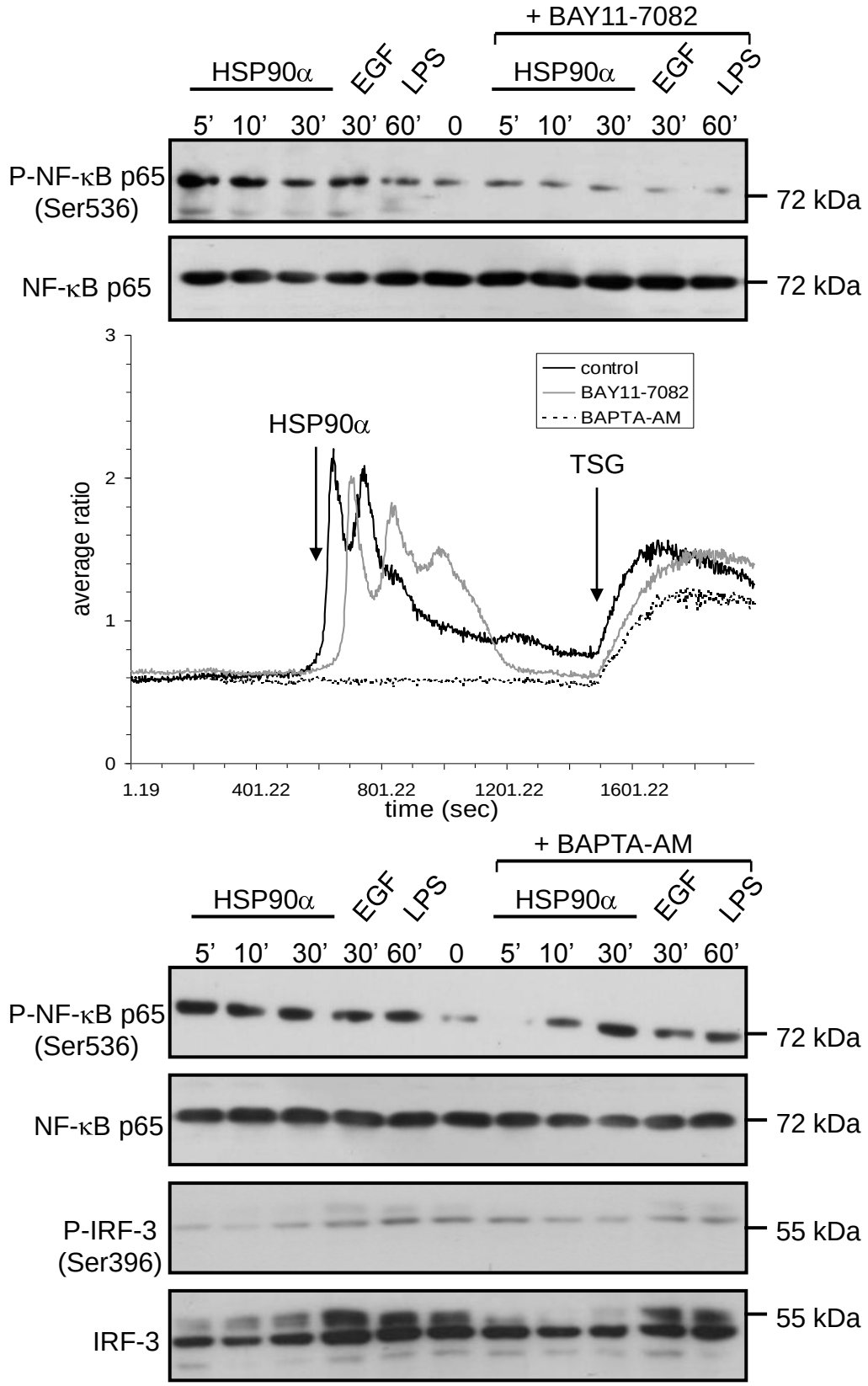

+ BAY11-7082
HSP90α
EGF
LPS
HSP90α
EGF
LPS
5’ 10’ 30’ 30’ 60’ 0 5’ 10’ 30’ 30’ 60’
P-NF-κB p65 (Ser536)
72 kDa
NF-κB p65
72 kDa
control
BAY11-7082
BAPTA-AM
HSP90α
TSG
average ratio
0
1
2
3
1.19
401.22
801.22
1201.22
1601.22
time (sec)
+ BAPTA-AM
HSP90α
EGF
LPS
HSP90α
EGF
LPS
5’ 10’ 30’ 30’ 60’ 0 5’ 10’ 30’ 30’ 60’
P-NF-κB p65 (Ser536)
72 kDa
NF-κB p65
72 kDa
P-IRF-3 (Ser396)
55 kDa
IRF-3
55 kDa

**Figure S8.** *Intracellular calcium is required for NFkB p65 phosphorylation.* To determine whether calcium-regulated signalling is required for or is dependent on the phosphorylation of NFkB and/or IRF-3, we firstly examined the effects of the IKBa/NFkB-p65 phosphorylation inhibitor, BAY11-7082 on HSP90a-induced [Ca2+]i increase. Serum-starved U87 cells were pre-treated with 10 µM BAY11-7082 for 30 min before their stimulation with 6µg/ml HSP90a, 10 ng/ml EGF or 1 µg/ml LPS. Western blot analysis was performed on whole-cell lysates using the indicated antibodies in untreated and treated cells. Clearly BAY11-7082 reduced drastically the phosphorylation of NFkB mediated by HSP90a and specific agonists (n=2). In untreated and BAY11-7082-treated cells, HSP90a always induced a transient increase in [Ca2+]i within the first 5 min of cell exposure (average ratio of 10 cells). No inhibitory effect was observed. We next examined whether the cell-permeable calcium chelator BAPTA-AM (acetomethyl ester form) had an effect on the HSP90a-induced phosphorylation of NFkB or IRF-3. Cell treatment for 30 min with 100 µM BAPTA-AM totally suppressed the HSP90a-induced [Ca2+]i increase, and significantly attenuated NFkB p65 phosphorylation (no detectable effect could be observed on IRF-3 phosphorylation). These results demonstrate that the effects of HSP90a on NFkB p65 phosphorylation are mediated at least in part by calcium.