SUPPLEMENTARY DATA

A new p38 MAP kinase-regulated transcriptional co-activator that stimulates p53-dependent apoptosis

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Supplementary Materials and methods

Expression constructs

For expression in *Escherichia coli* as GST (glutathione S-transferase)-fusion proteins, human p18^{Hamlet}, p53 and cyclin G1 were amplified by PCR, cloned into the pCRII vector (Invitrogen), sequenced and then subcloned into the pGEX-KG vector as NcoI-XhoI (p18^{Hamlet}), EcoRI (p53), and BamHI-XhoI (cyclin G1) fragments. The bacterial expression constructs for GST-p38α and MBP-MKK6DD were already described (Alonso et al., 2000). For expression in mammalian cells of N-terminally Myc-tagged p38α and p18^{Hamlet}, the human cDNAs were cloned into the FTX5 vector as BamHI (p38α) and NcoI-XhoI (p18^{Hamlet}) fragments, and then subcloned into pCDNA3.1 (Invitrogen) as HindIII-XhoI fragments in both cases (HindIII partial digestion was required for p38α subcloning). For the expression of N-terminally HA-tagged cyclin G1, the cDNA was amplified by PCR, cloned into pCRII and then subcloned as an EcoRI fragment into the HA-pCDNA3.1 vector (kindly provided by Giulio Superti-Furga, Research Center for Molecular Medicine, Vienna, Austria). For the expression of GFP-tagged p18^{Hamlet} protein, the pCRII-p18^{Hamlet} vector was digested with EcoRI and p18^{Hamlet} was subcloned into the pEGFP-C2 vector (Clontech Laboratories). For the expression of YFP-tagged human cyclin G1, the pCR2.1TOPO-cyclin G1 plasmid was obtained from the German Resource Center for Genome Research (RZPD), digested with SacI ApaI and the insert was subcloned into the pEYFP vector (Clontech Laboratories), with

the correct frame being generated by mutagenesis. The construct for tetracycline-inducible $p18^{Hamlet}$ expression was generated by subcloning Myc-$p18^{Hamlet}$ as a HindIII-XhoI fragment into pCDNA4/TO vector (Invitrogen). The C-terminally deleted mutant $p18^{Hamlet(1-117)}$ was prepared by replacing the residue 118 of $p18^{Hamlet}$ for a stop codon. The HA-Ubiquitin expression construct was a kind gift from Ivan Dikic (Goethe University Frankfurt, Germany). The constructs to express truncated GST-fused p53 proteins were provided by Pedro Lazo (CIC-Universidad de Salamanca, Spain).

Generation of a $p18^{Hamlet}$ inducible system

Stable cell lines expressing inducible $p18^{Hamlet}$ were generated using the Invitrogen T-REx™ system. U2OS cells were transfected with a 6:1 ratio of the Tetracycline repressor vector pcDNA6/TR-blasticicin and the inducible expression vector pcDNA4/TO-zeocin containing Myc-$p18^{Hamlet}$. Positive clones were selected in media containing blasticidin (5 μg/ml) and zeocin (200 μg/ml). Myc-tagged $p18^{Hamlet}$ protein was induced by the addition of tetracycline (1 μg/ml).

Retroviral infections

Retroviruses were produced in HEK-293T cells by transient transfection. Culture supernatants were collected 48 h (first supernatant) and 72 h (second supernatant) post-transfection, filtered and supplemented with 4 μg/ml polybrene (Sigma). MEFs at approximately 10^6 cells per 10 cm dish were infected with 8 ml of the first supernatant and 24 h later with the second supernatant, and incubated 48 h post-infection with 1.5 μg/ml of puromycin for 4 days.

Antibodies and buffers for immunoblotting

GST-p18^{Hamlet} protein was used to generate polyclonal antibodies in rabbits, which were purified by pre-incubation of the antiserum with beads-coupled GST followed by a standard western blot affinity purification on GST-p18^{Hamlet}. Phospho-specific antibodies that recognize p18^{Hamlet} phosphorylated on Thr103, were raised in sheep against the synthetic phosphopeptide GPNYLpTACAG corresponding to residues 98-107 of human p18^{Hamlet} and were affinity purified on the same phosphopeptide.

The following commercial antibodies were used for immunoblotting: anti-p38 (Santa Cruz), anti-phospho-p38 (Cell Signaling), anti-α-tubulin (Sigma), anti-phospho-Thr (9381, Cell Signaling), anti-Myc (9E10; Santa Cruz), anti-GFP (Clontech), anti-HA (Roche), anti-human-p53 (DO-1; Santa Cruz), anti-mouse-p53 (CM5; Novocastra), anti-phospho-p53 (Ser 15 and Ser 46; Cell Signaling), anti-cyclin G1 (H-46; Santa Cruz), anti-NOXA (Oncogene Research Products), anti-PUMA (ab 9643, Abcam Ltd), anti-Bax (P-19, Santa Cruz), anti-p21 (C-19, Santa Cruz), anti-Hdm2 (SM-p14, Abcam Ltd).

Immunoblots were developed and quantified using Alexa Fluor 680 (Molecular Probes) or Li-Cor IRDye 800 (Rockland) labeled antibodies with the Odyssey Infrared Imaging System (Li-Cor).

The agarose beads used for immunoprecipitation were anti-Myc (sc-40 AC; Santa Cruz), anti-HA (sc-7392 AC; Santa Cruz) and anti-p53 (sc-126AC; Santa Cruz). For kinase assays, immunoprecipitates were further washed in kinase buffer: 80 mM β-glycerophosphate, pH 7.5, 20 mM EGTA, 15 mM $MgCl_2$, 2.5 mM benzamidine, 1mM PMSF, 1 mM DTT, and 2 μg/ml each of aprotinin and leupeptin.

To prepare total cell lysates for immunoblotting, we used IP buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.1 mM Na Vanadate, 1 mM PMSF,

2 μM microcystin, 2.5 mM benzamidine, and 2 μg each of aprotinin, leupeptin, and pepstatin A per ml.

Quantitative Real-Time PCR

Total RNA was isolated from U2OS cells using Quiagen RNeasy kit and cDNA was synthesized with Superscritpt-II reverse transcriptase using random hexamer primers (Invitrogen) following the manufacturer indications. An Applied Biosystems 7900HT Fast Real-Time PCR System was used to determine the mRNA levels of PUMA, NOXA, BAX, Hdm2, and GAPDH using the following primers: NOXA: fw 5'-TGTCCGAGGTGCTCCAGTT-3'; rev 5'-TGAAACGTGCACCTCCTG-3'
PUMA: fw 5'-GTGCCCTCGGCAGTGTCC-3'; rev 5'-GTACTGTGCGTTGAGGTC-3'
BAX: fw 5'-CACCAGCTCTGAGCAGATC-3'; rev 5'-GCTGCCACTCGGAAAAAG-3'
HDM2: fw 5'-GTGCTGTAACCACCTCAC-3'; rev 5'-GCTCTTTCACAGAGAAGC-3'
GAPDH: fw 5'-GACCCCTTCATTGACCTCAAC-3'; rev 5'-GAGGGGCCATCCACAGTCTTC-3'. Data analysis was done by normalizing to GAPDH mRNA levels.

Northern blot

Total RNA was isolated with the RNeasy kit (Qiagen), fractioned in formaldehyde agarose gels and blotted onto nylon membranes. The full-length p18^{Hamlet} cDNA was used as a probe, which was prepared by using the Ready-to-go kit (Amersham-Pharmacia).

ChIP analysis

ChIP analysis was performed exactly as described (Espinosa et al., 2003). After immunoprecipitation and reversal of the cross-linking, DNA was purified and used as

template for PCR reactions that were performed in the exponential range of amplification that varied from 30 to 35 cycles. Amplification products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide. All PCR products were in the size range of 200-250 bp. Primers used for PCR of HDM2, GAPDH, and PUMA were previously described (Koutsodontis et al., 2005; Zeng et al., 2002). For NOXA promoter amplification we used the following set of primers: NxA fw (58° C) 5'-TTTTCTGGGCTTGTTTACCC-3'; NxA rev 5'-TACAAAACGAGGTGGGAGGA-3'.

Immunofluorescence and confocal microscopy

Cells were rinsed in PBS, fixed in 4% paraformaldehyde for 30 min and washed again with PBS. Non-specific sites were blocked by incubation in PBS containing 1% BSA and 0.5% Triton-X100 for 1 h at RT. Cells were then washed 4 times in PBS and incubated with the following primary antibodies: affinity-purified polyclonal anti-$p18^{Hamlet}$ (1:200), monoclonal anti-HA (1:500) and monoclonal anti-Myc (1:500). After four washes with PBS, cells were incubated with Alexa Fluor 488 and 594 (1:500) secondary antibodies (Molecular Probes), stained with DAPI (0.1 μg/ml) for 10 min, and mounted in mowiol. The samples were examined using both fluorescence and confocal microscopy (Leica Microsystems).

Ubiquitination assay

To detect $p18^{Hamlet}$ ubiquitination, empty vector or the Myc-$p18^{Hamlet}$-encoding plasmid (4 μg) were co-transfected with HA-Ubiquitin (6 μg). 16 h after transfection, cells were treated with 25 μM MG132 for 5 h. Cells were harvested and used for immunoprecipitation with anti-Myc antibody coupled to beads. Ubiquitinated $p18^{Hamlet}$ forms were visualized with $p18^{Hamlet}$ and HA antibodies.

Pull-down and kinase assays

Recombinant GST-fusion proteins were expressed in *E. coli* BL21 DE3 and purified using standard protocols. For GST pull-down assays, ^{35}S-labelled proteins were generated using the TNT Coupled System (Promega, Madison, WI) and incubated with GST-proteins in IP buffer for 2 h at 4° C. Beads were washed four times with IP buffer and proteins were analyzed by SDS-PAGE followed by autoradiography. p38 MAPK activity assays using as substrates GST-ATF2 (residues 19-96) or recombinant wild type and mutant GST-p18^{Hamlet} proteins were carried out as described (Alonso et al., 2000).

Supplementary References

Alonso, G., Ambrosino, C., Jones, M. and Nebreda, A.R. (2000) Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. *J Biol Chem*, **275**, 40641-40648.

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Zeng, S.X., Dai, M.S., Keller, D.M. and Lu, H. (2002) SSRP1 functions as a co-activator of the transcriptional activator p63. *Embo J*, **21**, 5487-5497.

Supplementary Figure Legends

Figure 1. $p18^{Hamlet}$ phosphorylation by and binding to p38 MAPK family members. *Left*: GST-pull down assay with GST or GST-$p18^{Hamlet}$ and ^{35}S-labelled p38 MAPKs (α, β, γ, and δ) and MKK6. *Right*: HEK-293 cells were transfected with Myc-tagged $p18^{Hamlet}$ alone or together with the four Myc-tagged p38 MAPKs. 48 h after transfection, $p18^{Hamlet}$ and the p38 MAPKs were immunoprecipitated with Myc antibodies and subjected to a kinase assay in the presence of ^{32}P-γ-ATP and MBP-MKK6DD protein. Total cell lysates and Myc IPs were analyzed by Western blotting.

Figure 2. Binding to and phosphorylation by p38α MAPK of $p18^{Hamlet}$ proteins.
Left: In vitro pull down assay with recombinant GST-$p18^{Hamlet}$ mutants incubated with in vitro translated ^{35}S-labelled p38α. *Right*: Kinase assay was performed with active p38α and the indicated GST-$p18^{Hamlet}$ proteins (1 μg).

Figure 3. Expression of $p18^{Hamlet}$ mRNA in human tissues as determined by semi-quantitative RT-PCR using primers that recognize the full-length $p18^{Hamlet}$ coding sequence. β-actin was amplified as a control.

Figure 4. MG132-induced $p18^{Hamlet}$ accumulation depends on p38 MAPK activity. MEFs were either untreated or treated with the proteasome inhibitor MG132 (25 μM) in the presence or absence of the p38 MAPK inhibitor SB203580 (10 μM) for 2 h. Total lysates were analyzed by Western blotting with the indicated antibodies.

Figure 5. U2OS cells were co-transfected with MKK6DD (600 ng), $p18^{Hamlet-T103A}$ (1 μg) and increased amounts of p38α as indicated. Total lysates were analyzed by Western blotting with the indicated antibodies.

Figure 6. Tetracycline-induced $p18^{Hamlet}$ expression in U2OS cells. Cells were treated with 1 μg/ml of tetracycline for the indicated times and $p18^{Hamlet}$ levels were evaluated by Western blotting.

Figure 7. Knockdown of $p18^{Hamlet}$ by siRNA. U2OS cells expressing tetracycline-inducible $p18^{Hamlet}$ (upper panel) and MCF7 cells (lower panel) were transfected with $p18^{Hamlet}$ siRNA or lamin A siRNA (as a control). After 48 h, U2OS were treated with tetracycline for 16 h. Levels of $p18^{Hamlet}$ were evaluated by Western blotting three days after transfection. *Right*: MCF7 cells were treated with cisplatin (10 μg/ml) for 16 h and apoptosis was quantified by measuring DNA fragmentation in a colorimetric assay. Means ± standard deviations of two independent experiments performed in triplicates are represented.

Figure 8. The specificity of $p18^{Hamlet}$ antibody was tested by immunofluorescence of tetracycline inducible $p18^{Hamlet}$-expressing U2OS cells treated with siRNA control or siRNA $p18^{Hamlet}$.

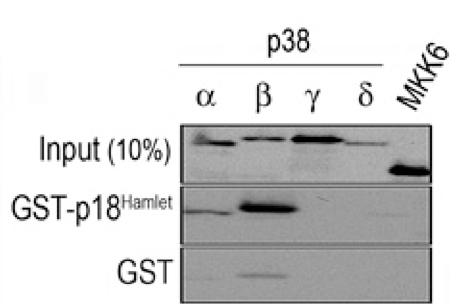
Figure 9. GST pull-down assays were performed by incubation of ^{35}S-labelled $p18^{Hamlet}$ proteins with GST, GST-p53 full-length, GST-$p53^{90-290}$, or GST-$p53^{290-390}$, as indicated. Input represents 10% of the ^{35}S-labelled proteins. Localization of transfected GFP-$p18^{Hamlet}$ proteins in U2OS cells is shown.

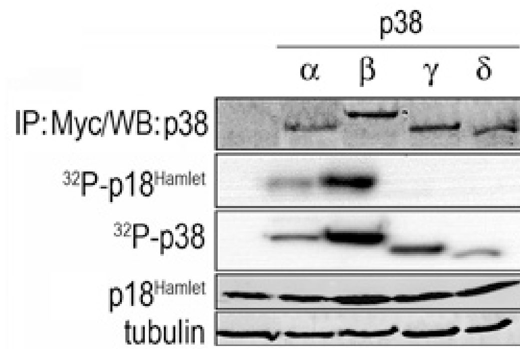
Figure 10. SAOS cells were transfected with Hdm2-luc and Bax-luc reporter constructs alone or in combination with 10 ng of p53 expression vector. Luciferase activity was analyzed 16 h later and normalized to Renilla.

Figure 11. SAOS cells were transfected with $p18^{Hamlet}$ or empty vector (control) either alone or in combination with 10 ng of p53 expression vector, together with reporter constructs containing the wt or mutated PUMA 4xBS2 minimal promoter, as indicated. Luciferase activity was analyzed after 16 h and was normalized to Renilla activity.

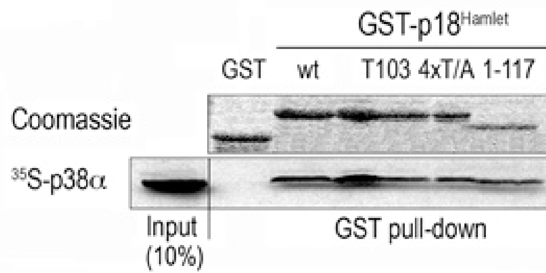
Figure 12. U2OS were transfected with GFP or the indicated GFP-tagged $p18^{Hamlet}$ proteins (upper panel) or with GFP together with the indicated Myc-tagged $p18^{Hamlet}$ proteins (lower panel). Protein level expression was analyzed by Western blotting 48 h after transfection.

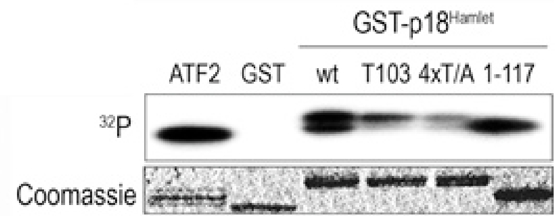
Figure 13. U2OS cells were transfected with Myc-tagged $p18^{Hamlet}$ expression vector and stained with $p18^{Hamlet}$ antibodies or DAPI. Two different nuclear fields are shown.





Cuadrado et al. Supplementary Figure 1





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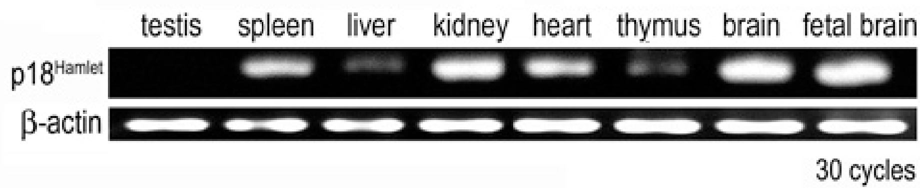

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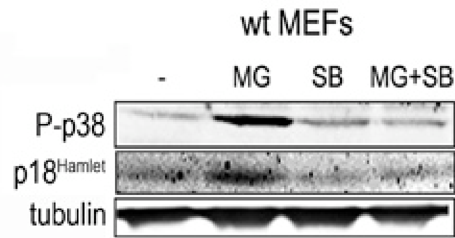

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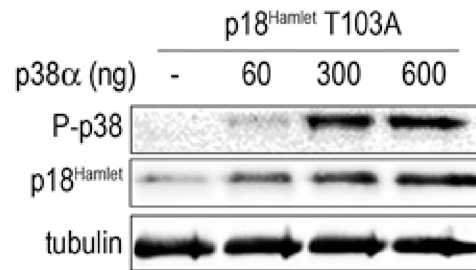

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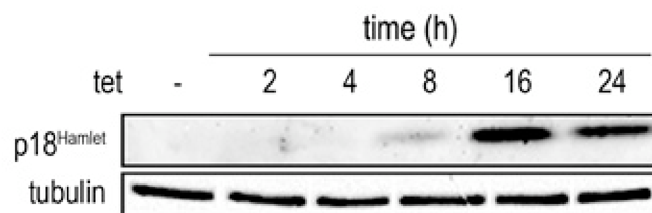

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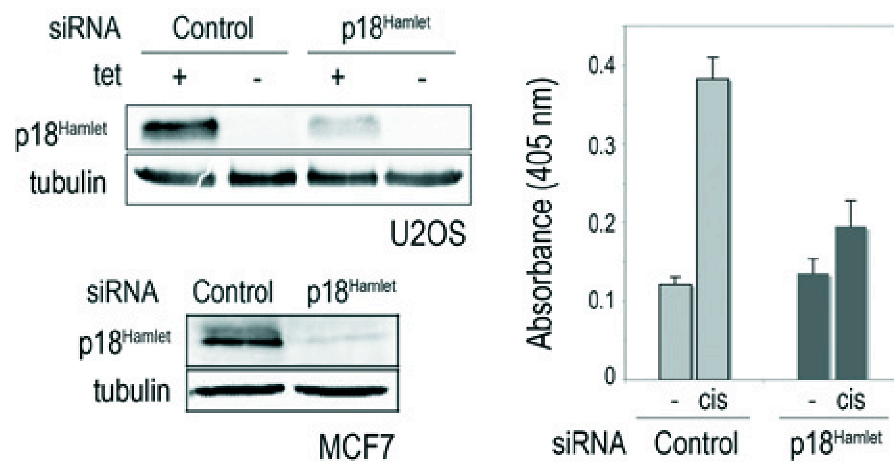

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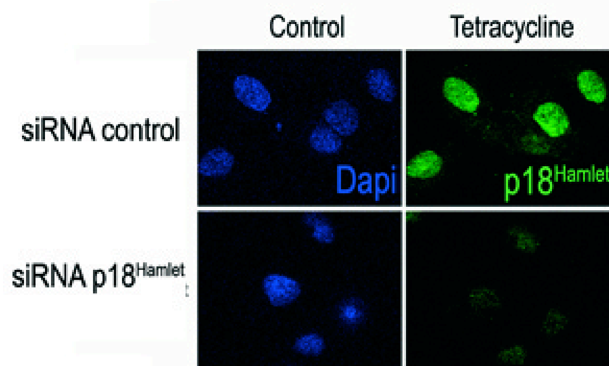

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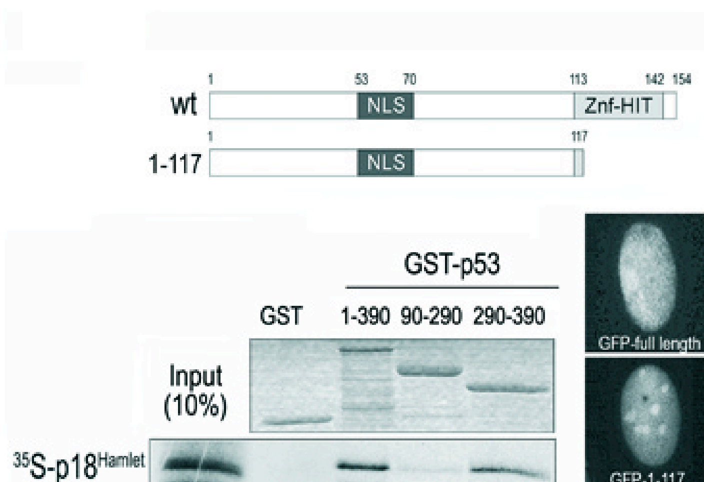

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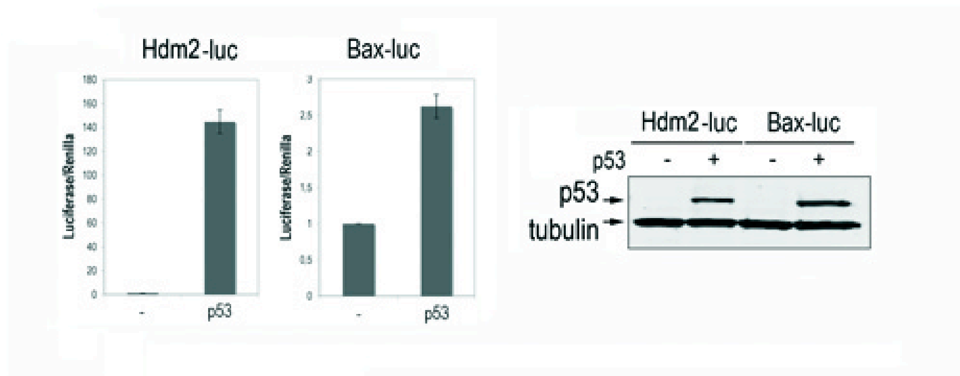

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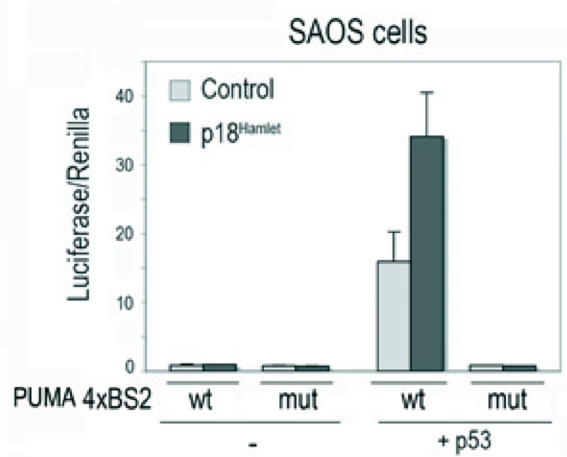

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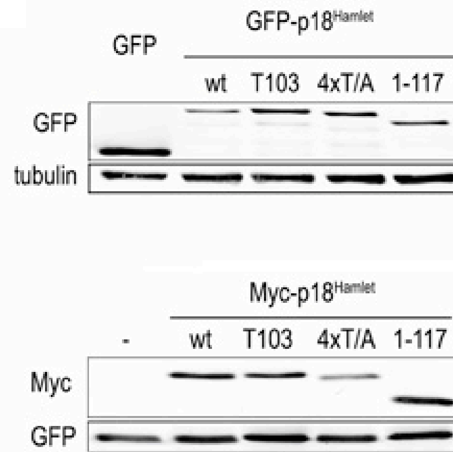

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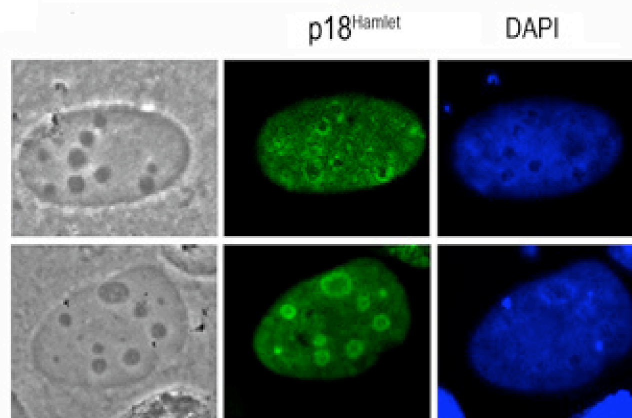

Cuadrado et al. Supplementary Figure 13