SUPPLEMENTARY INFORMATION:

Experimental procedures

Yeast Two-hybrid Plasmids and Yeast Strains:

We subcloned mouse LANP cDNA into the yeast two-hybrid plasmid pGBKT7 to generate the bait pGBK-LANP (Opal et al., 2003). We then transformed the bait plasmid into yeast strain AH109 (Clontech). Subsequently, we mated the transformed AH109 yeast with the Y187 yeast strain pre-transformed with a mouse brain pACT2 library (Clontech). Yeast strains that carry pGBK-LANP and a library pACT plasmid grow on a medium deficient in tryptophan and leucine (2- medium). To select for interacting clones, we performed high stringency auxotrophic selection using medium deficient in adenine and histidine (in addition to being deficient in tryptophan and leucine; 4- medium).

To delimit the region of interaction of LANP, we generated N- and C- terminally deleted LANP constructs. The C- terminally deleted LANP construct was engineered by digesting pGBK-T7 LANP with Bsm1 and Sma1 to remove DNA encoding the C-terminal acidic domain (keeping intact the LRR region) and re-ligating the back-bone. The N- terminally deleted construct was engineered by cloning a PCR-generated LANP fragment (residues 129-247) into the NdeI and SmaI sites of pGBK-T7.

The E4F construct initially isolated from a mouse brain c-DNA library encoded residues 189-773 (Accession number AAF22563). We sub-cloned full-length human E4F (Accession number AAD09139) and C terminal truncated mutations to validate and delimit the interaction with LANP. The E4F truncated constructs were generated by

taking advantage of unique restriction sites. These constructs include E4F 1-614 (SacII) and E4F 1-358 (SacI) (Sandy et al., 2000). As negative controls we tested the interactions of LANP and E4F against non-specific baits (for instance, pGBK LANP against pGADTand pGBK Lamin against pGADE4F).

Mammalian expression constructs and transfections.

Full-length mouse LANP (residues 1-247) was sub-cloned by a PCR based strategy into the mammalian expression vector pCMV-myc (Clontech). E4F was expressed with the S-tag epitope on the N terminus using the vector pCMV-s (Fernandes and Rooney, 1997). The incorporation of human ataxin-1 84Q c-DNA in the pEGFP plasmid (Clontech) has been described previously (Cummings et al., 1998). Transfections were performed using lipofectamine 2000 (Invitrogen).

RNAi duplexes and knock-down experiments using RNA interference.

Knock-down was induced using siRNA targeting LANP and E4F. The sequences of the siRNA duplexes targeting LANP were synthesized commercially (Dharmacon) and are as follows:

siRNA#SP1 (targets mouse LANP): GAACUGGAAUUCCUAAGUAUU

siRNA#3 (targets mouse LANP): AAGAGAAAUGUCCGAACCUUA

siRNA-LANP (targets human LANP): CUAAGCGAUAACAGAGUCUCA

E4F was knocked down with a proprietery pool of siRNA targeting human E4F (Dharmacon). All transfections were perfomed using lipofectamine 2000 (Invitrogen) and an siRNA concentration of 100 nM.

Immunofluorescent staining.

HeLa cells were seeded on coverslips in 6 well plates (~50,000 cells per well). Two days after plating they were stained with primary antibodies (anti E4F and anti-LANP), and fluorescently labeled secondary antibodies (Opal et al., 2003). Microscopy was performed using confocal laser-scanning microscopy (Zeiss). Images were manipulated using Adobe Photoshop 7.0.

Protein immunoprecipitations and S-tag precipitations.

HeLa cells were plated in 10 cm dishes ($2X10^6$ cells/dish) and were transfected using Effectene (Qiagen) with 2 µg of pCMV-myc LANP and pCMVs-E4F 2.5K (with empty vector (pCDNA-3) to keep the total DNA constant at 4 µg. 36 hrs post-transfection, cells were rinsed with PBS and processed for co-precipitation as follows: Cells were lysed by addition of 1.25 ml per well of lysis buffer [20 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1mM PMSF] and a cocktail of commercial protease inhibitors (Complete; Roche), passed through a $22^{1/2}$ gauge syringe, and gently mixed on a rotory shaker for 2.5 hrs at 4^oC; lysates were clarified by centrifugation at 12,000g for 30 minutes. E4F was precipitated using 100 µl of a 50% slurry of S-protein agarose beads (Novagen); LANP was precipitated using 100 µl of a 50% slurry of protein G sepharose coupled to 10 µg of 9E10 antibody (anti-myc monoclonal). The beads with bound proteins were washed six times with lysis buffer. After the last wash, 50 µl of Laemmli sample buffer was added to the beads and the samples were boiled and loaded

on 10% acrylamide SDS protein gels for western blot assays. For all precipitations, 1.5% of the input and 50% of the immunoprecipitate or S-tag precipitate is loaded on the gel.

For precipitation of endogenous LANP in N2A cells: cells were plated at a concentration of $2.5X10^6$ cells per dish and 4 μg of pCMV-s E4F (or empty DNA) were transfected prior to S-bead precipitation.

Chromatin immunoprecipitations (ChIP) and S-tag precipitations.

Chromatin precipitations were performed essentially as described (Christova and Oelgeschlager, 2002) with modifications. Briefly, 24 hrs after transfection with the indicated constructs, $4X10^6$ cells were cross-linked in 1% formaldehyde for 10 minutes at room temperature (with gentle shaking). The reaction was stopped by adding glycine to a 125 mM final concentration. Cells were scraped, washed in ice-cold PBS, and lysed in 400 μl lysis buffer (50 mM Hepes-KOH pH 7.9, 10 mM EDTA pH 8.0, 1% SDS with protease inhibitors). Lysates were sonicated till cross linked DNA was sheared to average size of 0.5 kb (approximately 25 pulses with 10 seconds/pulse at 40% amplitude). Samples were centrifuged at 4 degrees for 10 min at 16,000g, and supernatants with cross-linked DNA were diluted 1:10 in immunoprecipitation (IP) buffer (10 mM Hepes-KOH pH 7.9, 1% Triton X-100, 150 mM NaCl and protease inhibitors). Chromatin samples were precleared with 200 ul of a 50% slurry of Protein A Agarose/Salmon Sperm DNA for 1 hr at 4 ^{0}C.

The pre-cleared lysates were then divided into 2 ml aliquots and subjected to precipitation with the indicated beads (S-beads: 100 μl of S-beads; myc beads: 100 μl of protein G-sepharose beads with 10μg of anti-myc antibody (9E10); and control beads:

100 µl of protein G-sepharose beads with 10 µg normal mouse IgG). An aliquot of 100 µl of cell lysate was taken from diluted precleared samples prior to immunoprecipitations, and processed and treated as the immunoprecipitated sample. This served as the input control for the PCR component of the chromatin precipitations.

Immunoprecipitates were washed three times with 1 ml of each: (i) IP buffer, (ii) wash buffer (10mM Tris-HCl pH 8.0, 0.25 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA pH 8.0), and (iii) TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Pellets were resuspended in 500 µl of TE buffer and digested with 50 µg/ml RNaseA at 37ºC (30 min). SDS was added to 0.125% final, and associated proteins were digested away with 250 µg/ml proteinase K (incubating for 1 hr at 37ºC). Formaldehyde crosslinks were reversed by adding NaCl to a final concentration of 200 mM and by incubating at 65 ºC for 4 hrs. DNA was extracted once with an equal volume of phenol-chlorophorm and once with chlorophorm alone. 10 µg of glycogen was added to the extracted DNA. The extracted DNA was then precipitated with ethanol, and resuspended in 30 µl TE. 1µl of resuspended DNA was used as a template for the PCR component of the chromatin precipitation.

PCR was performed using primers that target the adenovirus E4 promoter sub-cloned into pGL-E4. PCR primers:

E4F forward primer: CGACACGGCACCAGCTCAATC

E4F reverse primer: TTAGGGCGGAGTAACTTGTAT

Semi-quantititative PCR was performed using the Fermentas 2X PCR mix. PCR products were analysed by electrophoresis on 2% agarose gels. Quantitative real time PCR was performed using the Applied Biosystems SYBR green PCR master mix and

protocols. Copy numbers for real time PCR were calculated using standard curve calibrated with the pGL-E4 reporter over five orders of magnitude by linear regression according to the formula: $CN = 10^{-[(TC - b)/m]}$, where CN is the template copy number and TC is the observed threshold crossing point (Cvetanovic and Ucker, 2004). The derived constants are m = -1.4335 and b = 35.735. Finally, the fold enrichment of the PCR product was calculated by normalizing the data to chromatin immunoprecipitations performed on base-line conditions (reporter construct transfected alone).

Antibodies.

To detect LANP, we either used antibody 3118 that we previously generated and characterized (Opal et al., 2003), or used a commercial antibody sc-5652 (Santacruz). Anti-E4F-N term is a rabbit polyclonal antisera raised against a GST fusion that contained human E4F amino terminal residues 1-84 (Fernandes and Rooney, 1997). The c-myc epitope was detected using the mouse monoclonal antibody, 9E10 (Sigma). All antibodies were used at dilutions of 1:1000 for western blots and 1:200 for immunofluorescent staining.

Luciferase Assays.

Luciferase reporter construct pGL-E4 contains a firefly luciferase cassette downstream of the wild type adenovirus E4 promoter that has two E4F binding sites and an ATF binding site. The mutant version (pGL-E4mut10 that we have referred to here as pGL-E4mut) contains only the ATF binding site (Fernandes and Rooney, 1997; Nicholas and Nevins, 1991).

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega); pRL-CMV (a CMV IE promoter-driven renilla luciferase reporter construct; Promega) was included in all transfections as an internal control for experimental variability and normalization. Cells were plated in 24 well plates at 1.5-4 X 10^4 cells/well and transfected 24 hrs later using Effectene Transfection Reagent, as per the manufacturer's instructions (Qiagen). Each transfection contained a total of 250ng DNA, which included 50ng of pGL-E4, 0.2ng of pRL-CMV and 0-150ng of pCMVs-E4F, pCMV-myc LANP vector, pEGFP ataxin-1 84Q, or a combination of the above (as indicated in the figures). Empty pCMV vector was used to bring the total to 250 ng DNA. RNAi duplexes were transfected at 100 nM. In each independent experiment, all transfections were performed at least in duplicate and each transfection lysate was assayed in duplicate. Results were calculated as the ratio of firefly luciferase activity/renilla luciferase activity and were normalized to the relative basal activity generated by control samples. Luciferase assays were performed in CV-1, Cos7, T98G glioblastoma, and N2A neuroblastoma cells.

For each transfection, pGL-E4 firefly luciferase activity was calculated relative to renilla luciferase activity expressed from the internal control plasmid pRL-CMV and normalized to the relative basal activity of pGL-E4 alone. In several experiments, repression was plotted as a percentage inhibition (calculated relative to E4F-induced inhibition in the control sample). The experiments were perfomed multiple times (with each independent experiment performed in duplicate or triplicate). Thus, data in Figure 3 (A) are from 7 independent experiments, data in 3 (B) are from 3 independent

experiments. Data in figures 4 and 5 is representative of 4 independent experiments in each case. Error bars signify standard deviations.

To quantify the levels of E4F and LANP we lysed the cells in a nuclear extraction buffer (NuPer; Pierce)—that enriches for endogenous E4F— and loaded 60 μg of extract for quantitative western blots. The levels of overexpression and knock-down of indicated proteins was estimated using quantitative densitometry employing the Chemidoc XRS Imaging system and QuantityOne software (Biorad).

Supplementary Figure Legends

Supplemental Figure S1.

LANP recruitment to the E4 promoter is E4F dependent as demonstrated by testing LANP occupancy of the pGL-E4 promoter in the presence or absence of overexpressed E4F.

(**A**) Lysates of HeLa cells expressing pGL-E4, myc-tagged LANP, and S-tagged E4F, were precipitated with sepharose beads bound to myc or IgG as indicated. PCR using primers that amplify E4 promoter was employed to detect precipitated DNA.

(**B**) Real time PCR to quantify the amount of pGL-E4 immunoprecipitated with LANP. The level of enrichment over base-line is statistically significant in the presence of overexpressed s-tagged E4F (histogram 3 versus 1; $p<0.05$); but not when myc-tagged LANP is expressed alone (histogram 2 versus 1). Error bars = standard deviations.

Supplemental Figure S2.

(**A**) Endogenous E4F is required for LANP mediated repression. Testing LANP repression on the E4 promoter in the context of depletion of endogenous E4F. HeLa cells were transfected with pGL-E4 promoter and siRNA targeting E4F (or control siRNA) in the presence or absence of LANP. Expressed in terms of percentage inhibiton, we show that LANP causes significantly less repression when E4F is depleted ($p<0.05$). Inset: shows the raw data presented in the form of relative luciferase activity. Error bars = standard deviation.

(**B**) Western blot shows the level of overexpression of LANP (approximately 2 fold) and depletion of E4F (greater than 60%) to allow comparison with the functional luciferase studies. Note that myc-tagged LANP runs slightly heavier than wild type LANP on SDS protein gels. Western blot against actin serves as a loading control.

Supplementary References

Christova, R. and Oelgeschlager, T. (2002) Association of human TFIID-promoter complexes with silenced mitotic chromatin in vivo. *Nat Cell Biol*, 4, 79-82.

Cummings, C.J., Mancini, M.A., Antalffy, B., DeFranco, D.B., Orr, H.T. and Zoghbi, H.Y. (1998) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat. Genet.*, 19, 148-154.

Cvetanovic, M. and Ucker, D.S. (2004) Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *J Immunol*, 172, 880-889.

Fernandes, E.R. and Rooney, R.J. (1997) The adenovirus E1A-regulated transcription factor E4F is generated from the human homolog of nuclear factor phiAP3. *Mol Cell Biol*, 17, 1890-1903.

Nicholas, J. and Nevins, J.R. (1991) Distinct DNA targets for trans-activation by HTLV-1 tax and adenovirus E1A. *Virology*, 182, 156-167.

Opal, P., Garcia, J.J., Propst, F., Matilla, A., Orr, H.T. and Zoghbi, H.Y. (2003) Mapmodulin/leucine-rich acidic nuclear protein binds the light chain of microtubule-associated protein 1B and modulates neuritogenesis. *J Biol Chem*, 278, 34691-34699.

Sandy, P., Gostissa, M., Fogal, V., Cecco, L.D., Szalay, K., Rooney, R.J., Schneider, C. and Del Sal, G. (2000) p53 is involved in the p120E4F-mediated growth arrest. *Oncogene*, 19, 188-199.