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The adenovirus E4orf4 protein targets PP2A to the ACF chromatin-remodeling factor and induces cell death through regulation of SNF2h-containing complexes

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ABSTRACT

The adenovirus E4 open-reading-frame 4 (E4orf4) protein regulates the progression of viral infection and when expressed individually it induces non-classical apoptosis in transformed cells. Here we show that E4orf4 associates with the ATP-dependent chromatin-remodeling factor ACF that consists of a sucrose non fermenting-2h (SNF2h) ATPase and an Acf1 regulatory subunit. Furthermore, E4orf4 targets protein phosphatase 2A (PP2A) to this complex and to chromatin. Obstruction of SNF2h activity inhibits E4orf4-induced cell death, whereas knockdown of Acf1 results in enhanced E4orf4-induced toxicity in both mammalian and yeast cells, and Acf1 overexpression inhibits E4orf4′s ability to downregulate early adenovirus gene expression in the context of viral infection. Knockdown of the Acf1 homolog, WSTF, inhibits E4orf4-induced cell death. Based on these results we suggest that the E4orf4–PP2A complex inhibits ACF and facilitates enhanced chromatin-remodeling activities of other SNF2h-containing complexes, such as WSTF–SNF2h. The resulting switch in chromatin remodeling determines life versus death decisions and contributes to E4orf4 functions during adenovirus infection.

INTRODUCTION

The adenovirus E4 open reading frame 4 protein (E4orf4) is a multifunctional viral regulator. Within the context of the virus, E4orf4 contributes to temporal regulation of the progression of viral infection by downregulating early viral gene expression (1–4), inducing hypophosphorylation of various viral and cellular proteins (4,5), facilitating alternative splicing of adenovirus mRNAs (5), and regulating protein translation through an interaction with the mammalian target of rapamycin (mTOR) pathway (6). E4orf4 has also been shown to affect virus DNA replication, although this may be an indirect effect (7,8). When expressed individually in many cell lines, E4orf4 induces caspase-independent, non-classical apoptosis (9–12) that is preceded by G2/M arrest (13–15). At least part of the E4orf4 signaling network is highly conserved in evolution from yeast to mammalian cells (14,16–18), underscoring its importance to cell regulation. Notably, E4orf4-induced non-classical apoptosis is more efficient in oncogene-transformed cells (19), suggesting that elucidation of E4orf4 signaling may open up new cancer therapy strategies.

Studies of the mechanisms underlying E4orf4 action identified several E4orf4 partners. This group of proteins includes the B55/Bα and B56 subunits of protein phosphatase 2A (PP2A) (2,20), Src family kinases (21,22), the anaphase-promoting complex/cyclosome in the budding yeast (14), a subset of serine–arginine (SR)-rich splicing factors proteins (23) and Ynd1/Golgi UDPase (17).

PP2A is a major E4orf4 partner, and its interaction with E4orf4 was shown to contribute to all currently known functions of the viral protein (2,6,19,23–25). PP2A is composed usually of three subunits: the catalytic C subunit, a scaffolding A subunit and one of several regulatory B subunits encoded by at least four unrelated gene families: PR55/B55/B, PR61/B56/B′, B″, and B‴ [reviewed in (26)]. The various regulatory B subunits were proposed to dictate substrate specificity of the PP2A holoenzyme. Diverse PP2A complexes containing different B subunits may contribute to the various E4orf4 functions. Thus, for example, interaction with the

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PP2A-B55 subunit, but not with the PP2A-B56 subunit, contributes to E4orf4-induced cell death and cell cycle arrest in both yeast and mammalian cells (14,18,20).

To enable gene transcription, DNA replication, DNA repair and DNA recombination in the eukaryotic cell, numerous protein factors must obtain access to the genome that is tightly packed in chromatin. To facilitate accessibility of such factors to regulatory sequences in the DNA, cells utilize histone-modifying enzymes and ATP-dependent chromatin-remodeling complexes. ATP-dependent chromatin-remodeling complexes use the energy produced by ATP hydrolysis to disrupt contacts between DNA and histones thus facilitating repositioning or removal of nucleosomes or allowing exchange of histone variants without nucleosomal removal (27). There are currently four known families of chromatin-remodeling ATPases, including SWItch/Sucrose non fermentable (SWI/SNF), imitation switch/sucrose non fermenting (ISWI), chromo-helicase/ATPase DNA binding (CHD) and INO80. These proteins possess a similar ATPase domain but contain additional unique domains and associate with different regulatory subunits (28). Mammalian cells have two ISWI homologs, SNF2h and SNF2l that display tissue-specific expression patterns (29). SNF2h appears in at least seven different complexes, including human ATP-utilizing chromatin assembly and remodeling factor/Williams syndrome transcription factor-related chromatin remodeling factor (hACF/WCRF), chromatin-accessibility complex (CHRAC), WSTF–ISWI chromatin-remodeling complex (WICH), B-WICH, remodeling and spacing factor (RSF), nucleolar remodeling complex (NoRC) and a large complex containing cohesin and subunits of the nucleosome remodeling and deacetylase (NuRD) complex [reviewed in (30)]. In addition, ISWIs interact functionally with many important cell regulators participating in a variety of biological processes (31). The ATP-utilizing chromatin assembly and modifying factor (ACF) complex contains the SNF2h ATPase and the Acf1/Baz1A regulatory subunit, and participates in the regulation of DNA replication and in downregulation of transcription of specific genes (32–37). Acf1 and SNF2h also contribute to DNA damage repair (38). An Acf1 homolog, WSTF/Baz1B (Williams–Beurens syndrome transcription factor) participates in at least two chromatin-remodeling complexes, and one of them, WICH, contains the SNF2h catalytic subunit (39,40) and participates in replication of heterochromatin and in the cellular response to DNA damage (39,41,42).

In this study, we have examined the physical and functional interactions between E4orf4 and the ACF chromatin-remodeling factor. We show that E4orf4 targets PP2A to a complex with Acf1. Obstruction of SNF2h activity inhibits E4orf4-induced cell death, whereas Acf1 knockdown enhances it. In contrast, knockdown of another SNF2h regulatory subunit, WSTF, a component of the WICH complex, inhibits E4orf4 activity. Acf1 overexpression prevents downregulation of an early adenovirus protein by E4orf4 during viral infection. Our results suggest that E4orf4 inhibits Acf1-containing chromatin-remodeling complexes and shifts the balance of chromatin remodeling from Acf1-SNF2h to other SNF2h-containing complexes, such as WSTF–SNF2h. This shift is likely to cause alterations in chromatin that result in the induction of cell death by E4orf4 and contribute to E4orf4 functions during virus infection.

MATERIALS METHODS

Cell lines, transfections, plasmids, and yeast strains

HEK293 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). HEK293-derived cell lines containing tetracycline-inducible E4orf4 (clone 13) or an empty vector (T-REX) (24) were propagated in DMEM supplemented with 10% FCS guaranteed to be tetracycline free (BD Bioscience), 5 μg/ml blasticidine (Invitrogen) and 200 μg/ml zeocin (Invitrogen). Cell lines expressing short hairpin RNAs (shRNAs) or containing an empty vector were derived from HEK293-T-Rex cells (Invitrogen) by transfection of specific shRNAs cloned into pSuperior.neo-GFP (OligoEngine, Inc.), followed by a 2-week selection in medium containing 500 μg/ml G418 and 5 μg/ml blasticidine (Invitrogen).

The following plasmids have been used in this work: pEGFP-C1 (BD Bioscience); pCMV-E4orf4 (12); the pCMV/neo vector (43); pcDNA4/TO, pcDNA6/TR (Invitrogen); pcDNA4/TO-E4orf4 and pcDNA4/T0 expressing the R81F84A E4orf4 mutant protein (24,25); pDAD2, pDAD2-E4orf4 (14); plasmids expressing Acf1-GFP, Acf1-Flag, the dominant negative Acf1-DN-GFP, SNF2h-GFP (32), SNF2h-Flag and WSTF-Flag (44).

Specific shRNAs for Acf1, SNF2h and WSTF were sub-cloned into pSuperior.neo-GFP (OligoEngine, Inc) according to the manufacturer's protocol. The following primers were used: Acf1 shRNA forward primer: 5′-GAT CCC CGC GAT GAA GAA GAA GGT CAA ACT CGA GTT TGA CCT TCT TCT TCA TCG CTT TTT A-3′; Acf1 shRNA reverse primer: 5′-AGC TTA AAA AGC GAT GAA GAA GAA GGT CAA ACT CGA GTT TGA CCT TCT TCT TCA TCG CGG G-3′; SNF2h shRNA forward primer: 5′-GAT CCC CCG ACT GCT GAT GTA GTA ATT TCT CGA GAA ATT ACT ACA TCA GCA GTC GTT TTT A-3′; SNF2h shRNA reverse primer: 5′-AGC TTA AAA ACG ACT GCT GAT GTA GTA ATT TCT CGA GAA ATT ACT ACA TCA GCA GTC GGG G-3′; WSTF shRNA forward primer: 5′-GAT CCC CGA ACA GGA AGT TGC TGA GCT TCA AGA GAG CTC AGC AAC TTC CTG TTC TTT TTA-3′; WSTF shRNA reverse primer: 5′-AGC TTA AAA AGA ACA GGA AGT TGC TGA GCT CTC TTG AAG CTC AGC AAC TTC CTG TTC GGG-3′.

Mutants resistant to knockdown by shRNAs were generated from Acf1-GFP, SNF2h-Flag and WSTF-Flag using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's protocols.

A catalytically inactive SNF2h mutant was prepared by exchanging amino acid Lys211 in the nucleotide-binding motif with Arg (K211R) using the QuikChange

mutagenesis kit (Stratagene). This mutation abrogates ATP hydrolysis (45,46).

Yeast strains used in this study were congenic to W303-1A, except that a weak *rad5* mutation in the original W303 strain was repaired. The strains included WT (W1588-4C) and *itc1Δ*, previously described (47).

Viral mutants and infections

Adenoviral mutants *dl366**, lacking the complete E4 region, and *dl366*+E4orf4*, lacking all E4 open reading frames (ORFs) except E4orf4, were previously described (48). All virus infections were performed at a multiplicity of 5 ffu (fluorescent forming units) in medium supplemented with 2% FCS at 37°C for 2 h.

Immunoprecipitations and western blot analysis

For immunoprecipitation experiments, nuclear extracts were prepared according to Dignam *et al.* (49). Nuclear proteins (0.5 mg) were incubated for 2 h on ice with polyclonal antibodies against Acf1, which were covalently bound to protein A sepharose beads (Pharmacia). Covalent antibody binding was achieved using dimethyl pimelimidate, as described elsewhere (50). Alternatively, the nuclear extract was incubated with an antibody against E4orf4 or antibodies against GFP for 1 h and consequently with protein A sepharose beads (Pharmacia) for an additional 1 h. Finally, the samples were washed three times with buffer C [20 mM Hepes–KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% v/v glycerol, 1/10 volume of complete protease inhibitor cocktail (Roche)], boiled in SDS loading buffer, and separated by SDS–PAGE. Immunoprecipitated proteins and input lysates were analyzed by western blots using specific antibodies.

To determine the levels of protein expression in the cells, whole cell extracts were prepared in lysis buffer [50 mM Tris–HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40 and a 1/10 volume of complete protease inhibitor cocktail (Roche)]. Proteins were analyzed by western blots using the indicated antibodies.

Antibodies specific for the following proteins were used in this work: E4orf4 (12), E2A-72 kDa (DBP, clone B6) (51), PP2A-B (24), PP2A-C (BD Transduction Laboratories), GFP (MBL, Woburn, MA, USA), SNF2h and Acf1 (Bethyl), α-Tubulin and Flag (Sigma), HA (Covance). Polyclonal antibodies against Acf1 and SNF2h were a kind gift of P.D. Varga-Weisz (Babraham Institute, Cambridge, England, UK).

Cell death assays

Cells were transfected with the indicated plasmids. Twenty-four hours post transfection, the cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, washed again, incubated with 0.5% Triton X-100 for 5 min and then blocked with 5% BSA (Sigma) for 0.5 h. After staining with E4orf4 antibodies (1:500) or with a mixture of antibodies [polyclonal anti- E4orf4 (1:1000) and monoclonal anti-Flag (Sigma), 1:1000] for 2 h at 37°C, cells were washed three times for 5 min each time with PBS and incubated for 40 min with Cy3-conjugated anti-mouse antibodies [dilution 1:1000; (Jackson)] or with a mixture of antibodies [fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies (dilution 1:200; Jackson)] and Cy3-conjugated anti-rabbit antibodies [dilution 1:5000; (Jackson)]. The nuclei were stained by 0.1 μg/ml DAPI (Sigma). Finally, the cells were washed three times for 5 min each time with PBS and a cover slide was mounted on the plates using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA). Fluorescent cells were visualized by a Zeiss Axioskop microscope at a 400-fold magnification. The fraction of E4orf4-expressing cells with condensed, abnormal or fragmented nuclei was determined in each experiment by counting 200 transfected nuclei. The average of two or three experiments, each containing duplicate plates was calculated. Statistical significance of the results was determined by a Student's *t*-test.

In another assay, the cells were transfected with the appropriate plasmids and cell monolayers were visualized by phase microscopy and photographed, using the Zeiss Axioskop at a 400-fold magnification.

Chromatin fractionation by salt extraction

HEK293T cells were transfected with an empty vector, with a plasmid expressing E4orf4, or with a plasmid expressing the R81F84A mutant. The cells were harvested 24 h later, washed twice in PBS, resuspended in Chromatin buffer A [110 mM K-acetate, 15 mM Na-acetate, 2 mM Mg-acetate, 0.5 mM EDTA, 0.5 mM EGTA, 20 mM HEPES (pH 7.3), 0.05 mg/ml digitonin and proteinase inhibitors], and rotated for 30 min at 4°C. Nuclei were isolated by centrifugation, resuspended in chromatin buffer B [0.5 mM EGTA, 1 mM HEPES (pH 7.5) and 0.5% NP40] and rocked for 10 min at 4°C. The samples were loaded on a sucrose cushion composed of 0.1 M sucrose and 0.5 mM Tris–HCl (pH 8.5) and centrifuged at 3350 *g* for 20 min. The chromatin pellet was resuspended in chromatin extraction buffer [0.5 mM $MgCl_2$, 0.3 M sucrose, 20 mM HEPES (pH 7.5)] and was subjected to subsequent extractions with increasing salt concentrations (150, 250 and 500 mM NaCl) in the same buffer. Proteins were chromatographed on SDS–PAGE and a western blot was sequentially stained with the indicated antibodies and subjected to densitometry. The relative band intensity for each individual extraction step out of the sum of intensities of all extraction steps for each protein was calculated and the ratio between control (defined as 1) and WT E4orf4 values was determined.

Image acquisition and processing

Plated cells were photographed by an Axiocam camera linked to a Zeiss Axioskop at a 400-fold magnification. Yeast colonies and gels were scanned with an Epson Photo 4990 scanner. Images were processed using Adobe PhotoShop 5.0 or 7.0.

RESULTS

E4orf4 associates specifically with Acf1 in mammalian cells

E4orf4 participates in temporal control of adenovirus infection and induces non-classical apoptosis in transformed cells. Hypothesizing that insight into mechanisms of E4orf4 action might be gained by the identification of its partner proteins, we used the Ras-recruitment system in yeast (52) to detect new E4orf4 partners. One E4orf4 interacting protein that was identified by this protocol was Acf1, also known as Baz1A or WCRF180, that acts as a regulatory subunit of the ACF and CHRAC ATP-dependent chromatin-remodeling complexes (32,53). To confirm that E4orf4 associates with Acf1 in mammalian cells, co-immunoprecipitation experiments were carried out. A plasmid expressing E4orf4 was transfected into HEK293T cells and nuclear extracts that were prepared 24 h post-transfection were immunoprecipitated with Acf1-specific antibodies or with pre-immune serum. Figure 1A demonstrates that E4orf4 was precipitated by the Acf1-specific antibodies above background levels associated with the non-specific serum. To further confirm the specificity of the E4orf4–Acf1 interaction, a reciprocal immunoprecipitation was carried out. Cells were transfected with plasmids expressing E4orf4 and GFP-tagged Acf1, previously shown to retain Acf1 functions (32), and E4orf4 was immunoprecipitated from nuclear extracts. As shown in Figure 1B, Acf1-GFP was precipitated in the presence, but not in the absence, of E4orf4, indicating specific association between the two proteins. Endogenous SNF2h was also present in the E4orf4 immune complexes (Figure 1B and C), and a similar experiment revealed that the endogenous Acf1 protein also co-precipitated with E4orf4 (Figure 1C). When an inducible E4orf4 construct was used to

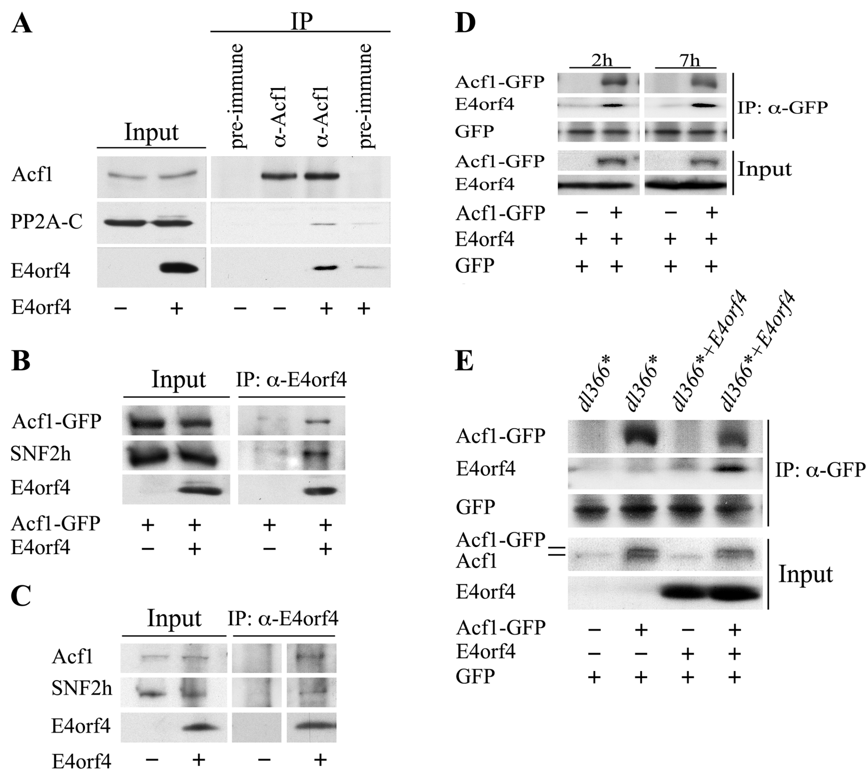

Figure 1. E4orf4 associates with Acf1 and SNF2h in mammalian cells. (**A**) HEK293T cells were transfected with a plasmid expressing E4orf4 or with an empty vector. Nuclear extracts were prepared 24 h later and subjected to immunoprecipitation (IP) with Acf1-specific antibodies or with pre-immune serum. The western blot shows the presence of Acf1, the PP2A-C subunit and E4orf4 in the immune complexes and in input lysates. The amount of proteins in the input represents 10% of the amount of proteins used for IP. (**B**) HEK293T cells were transfected with a plasmid expressing Acf1-GFP, together with a plasmid expressing E4orf4 or the empty vector. Nuclear extracts were prepared as in (A) and subjected to IP with E4orf4-specific antibodies. The western blot showing input lysates and immune complexes (IP: αE4orf4) was stained sequentially with antibodies to E4orf4, GFP and SNF2h. The amount of proteins in the input represents 10% of the amount of proteins used for IP. (**C**) HEK293T cells were transfected with a plasmid expressing E4orf4 or an empty vector and were processed as in (B). Western blots were stained sequentially with antibodies to Acf1, SNF2h and E4orf4. (**D**) HEK293T cells were transfected with plasmids expressing Acf1-GFP or the corresponding empty vector, together with a plasmid expressing E4orf4 from a doxycycline-inducible promoter and a plasmid expressing GFP, which served as a transfection efficiency and loading control. Nuclear extracts were prepared at the indicated times after E4orf4 induction and subjected to IP with GFP-specific antibodies. The western blot showing input lysates and immune complexes (IP: α-GFP) was probed sequentially with antibodies to E4orf4 and GFP. The amount of proteins in the input represents 10% of the amount of proteins used for IP. (**E**) HEK293T cells were transfected with an empty vector or a plasmid expressing Acf1-GFP and with a plasmid expressing GFP, which served as a transfection efficiency and loading control. The cells were subsequently infected with *dl366** or *dl366*+E4orf4* adenoviruses. Cells were harvested 24 h after infection and equal amounts of nuclear extracts were subjected to IP with GFP-specific antibodies. The western blot showing input lysates and immune complexes (IP: αGFP) was probed sequentially with antibodies to E4orf4, Acf1 and GFP.

examine the association between Acf1-GFP and E4orf4 at various times after induction of E4orf4, the interaction with Acf1 could be detected as early as 2 h post-induction (Figure 1D), whereas apoptotic morphologies were detected starting at 6 h after induction (24). These results suggest that the association between E4orf4 and Acf1 is an early event in E4orf4-expressing cells that precedes induction of cell death by the viral protein. To inquire whether E4orf4 associates with Acf1 during adenovirus infection, HEK293T cells were transfected with an empty vector or a plasmid expressing Acf1-GFP and with a plasmid expressing GFP, which served as a transfection efficiency and loading control. The cells were infected 24 h later with either one of two adenovirus mutants: *dl366** that lacks the complete E4 region of adenovirus or a *dl366*+E4orf4* mutant devoid of all the E4 ORFs except E4orf4 (48). We used these mutants rather than WT adenovirus to eliminate possible complications that may be introduced by other E4 proteins that affect chromatin response to viral infection (54). Nuclear extracts were prepared 24 h post-infection and subjected to immunoprecipitation with antibodies to GFP. Figure 1E demonstrates that E4orf4 and Acf1 are in complex during virus infection. It should be noted that although it appears that Acf1-GFP (but not native GFP) was precipitated less efficiently by the GFP-specific antibodies in the presence of E4orf4 (Figures 1E and 4D), this altered efficiency was not detected when endogenous Acf1 was immunoprecipitated with Acf1-specific antibodies (Figure 1A and data not shown), and thus may not be significant. In sum, our results indicate that E4orf4 and Acf1 form a specific complex in cells both under conditions of individual expression of E4orf4 or during virus infection.

E4orf4 targets PP2A to Acf1 and to chromatin

PP2A has been reported to be involved in all known E4orf4 functions. We thus examined next whether PP2A is present in the Acf1–E4orf4 protein complex. Results presented in Figure 1A, show the presence of low but significant levels of PP2A-C in immune complexes precipitated with Acf1-specific antibodies but not with pre-immune serum. Moreover, PP2A-C was co-precipitated with Acf1 in the presence of E4orf4, but not in its absence. These results suggest that E4orf4 recruits PP2A to a complex containing Acf1. To confirm this inference, we tested whether the interaction between E4orf4 and PP2A was required for PP2A recruitment to Acf1. To this end we used the R81F84A E4orf4 mutant that lacks the ability to interact with an active PP2A (25). Results shown in Figure 2A demonstrate that the R81F84A mutant maintained an ability to interact with Acf1-Flag similarly to the WT E4orf4 protein. To test whether this mutant was able to recruit PP2A to Acf1, HEK293 cells were transfected with plasmids expressing WT E4orf4 or the R81F84A mutant. Nuclear extracts of transfected cells were subjected to immunoprecipitation with Acf1-specific antibodies, and western blots were stained with antibodies to PP2A-C, SNF2h and Acf1. As shown in Figure 2B, PP2A-C co-precipitated with Acf1 in the presence of WT E4orf4, but not in the

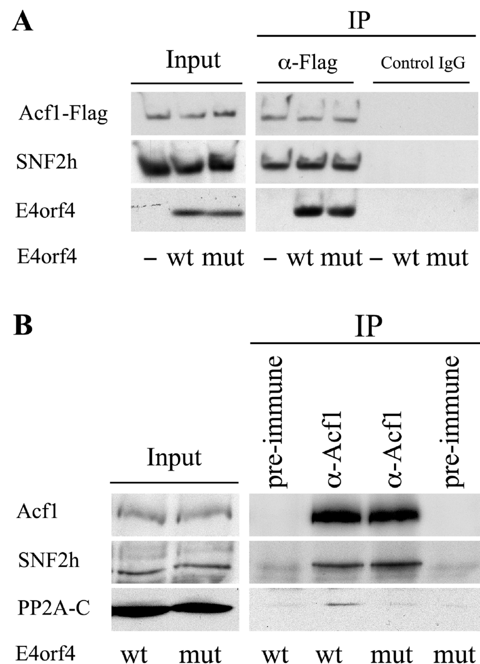

Figure 2. E4orf4 targets PP2A to an Acf1-containing complex. (**A**) HEK293 cells were transfected with a plasmid expressing Acf1-Flag together with a plasmid expressing WT E4orf4 (wt), the E4orf4 R81F84A mutant that cannot bind PP2A (mut) or the corresponding empty vector (–). Nuclear extracts were prepared 24 h later and were subjected to immunoprecipitation (IP) with Flag-specific antibodies or with anti-HA antibodies, serving as control IgG. The western blot showing input lysates and immune complexes (IP-αFlag or control IgG) was stained sequentially with antibodies to E4orf4, SNF2h and the Flag tag. (**B**) HEK293 cells were transfected with plasmids expressing WT E4orf4 (wt) or the E4orf4 R81F84A mutant (mut). Nuclear extracts were prepared as in (A) and subjected to IP with Acf1-specific antibodies or with pre-immune serum. A western blot showing input lysates and immune complexes was stained sequentially with antibodies to the PP2A-C subunit, SNF2h and Acf1. The amount of proteins in the input represents 10% of the amount of proteins used for IP in both (A) and (B).

presence of the E4orf4 mutant that was incapable of interacting with PP2A, whereas SNF2h co-precipitated with Acf1 in the presence of both E4orf4 proteins. These results are consistent with the conclusion that E4orf4 recruits PP2A to a complex with Acf1.

Since Acf1 is a component of a chromatin-associated chromatin-remodeling protein complex, and as E4orf4 appears to recruit PP2A to the Acf1-containing complex, we inquired whether E4orf4 increased the chromatin-bound levels of PP2A. Since E4orf4 chromatin targets have not been identified yet, chromatin fractionation experiments were carried out to address this question rather than chromatin immunoprecipitation assays. Chromatin was extracted from nuclei of HEK293T cells that were transfected with an E4orf4-expressing plasmid or with an empty vector, and chromatin proteins were successively extracted with increasing salt concentrations and subjected to western blot analysis. Densitometer-quantified intensities of the protein bands in blots of control and E4orf4-expressing fractions were normalized to the sum of the protein intensities in all fractions of the same sample and ratios between the normalized values for

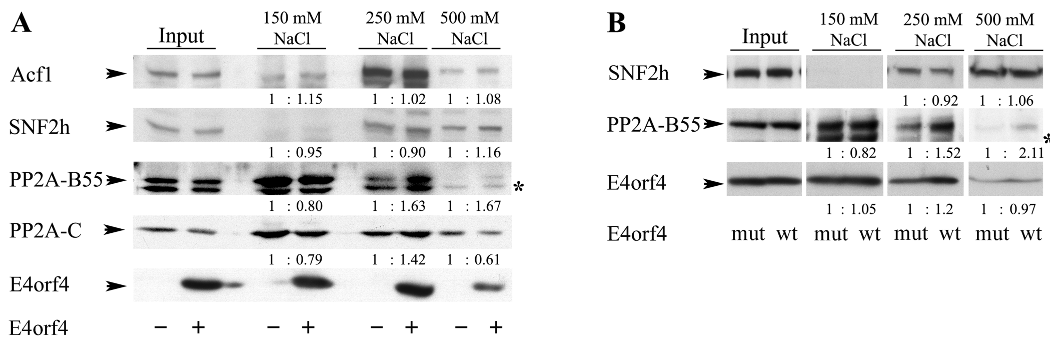

Figure 3. A subpopulation of PP2A binds more tightly to chromatin in the presence of E4orf4. HEK293T cells were transfected with an empty vector (−) or a plasmid expressing E4orf4 (+) (**A**), or were transfected with plasmids expressing either WT E4orf4 (wt) or the R81F84A mutant (mut) (**B**). Twenty-four hours later a chromatin fraction was prepared from nuclei and subjected to extraction with increasing salt concentrations. Proteins were chromatographed on SDS–PAGE and western blots were sequentially stained with antibodies to Acf1, SNF2h, PP2A-B55, PP2A-C and E4orf4 in A or stained with antibodies to SNF2h, PP2A-B55 and E4orf4 in (B). Arrowheads mark the relevant protein bands and the asterisk marks a non-specific band. The stained western blot was subjected to densitometry. The relative band intensity for each individual extraction step out of the sum of intensities of all extraction steps for each sample was calculated and the ratio between control (defined as 1) and E4orf4 values is shown below the bands. The western blots shown here represent three independent experiments with similar results.

control and E4orf4-expressing fractions were calculated. As seen in Figure 3A, equal amounts of Acf1, SNF2h, and the PP2A-B55 and PP2A-C subunits were present in input nuclear extracts from control and E4orf4-expressing cells. The majority of Acf1, which was reported previously to interact exclusively with SNF2h (32), was extracted from chromatin by 250 mM NaCl, and a minor amount was extracted by 150 and 500 mM salt. Most of SNF2h, which participates in several different chromatin-remodeling complexes, was extracted at salt concentrations ≥250 mM. However, no significant changes were detected in the quantities of Acf1 and SNF2h proteins extracted at various salt concentrations from chromatin of control or E4orf4-expressing cells. In contrast, slightly less PP2A-B55 subunit was extracted from chromatin of E4orf4-expressing cells at 150 mM NaCl, compared with control cell chromatin, whereas more PP2A-B55 subunit was extracted from chromatin of E4orf4-expressing cells at 250 and 500 mM NaCl compared with control cell chromatin. Furthermore, the PP2A-B55 subunit was enriched in higher salt fractions in the presence of the WT E4orf4 protein, compared with its extraction in the presence of the R81F84A mutant incapable of binding this subunit (Figure 3B). The PP2A-C subunit behaved similarly to the PP2A-B55 subunit at 150 and 250 mM NaCl concentrations, but was not enriched in chromatin proteins of E4orf4-expressing cells extracted at 500 mM NaCl (Figure 3A). The difference between the behavior of the B55 and C subunits of PP2A in this assay may be due to the fact that the C subunit participates in several PP2A heterotrimeric and heterodimeric complexes (55,56), some of which may bind chromatin in an E4orf4-independent manner. The results indicate that a subpopulation of PP2A complexes containing the B55 subunit is more tightly bound to chromatin in the presence of WT E4orf4, allowing it to be extracted from chromatin only at higher salt concentrations (250 mM, 500 mM NaCl). The finding that WT E4orf4, but not the R81F84A mutant, alters the chromatin binding properties of PP2A is consistent with recruitment of PP2A to chromatin-bound Acf1-containing protein complexes by means of an interaction with E4orf4.

The contribution of Acf1 and SNF2h to E4orf4-induced cell death

We next undertook to determine whether the interaction between E4orf4 and Acf1 contributes to E4orf4-induced cell death. It has been previously shown that the most typical morphologies associated with E4orf4-induced cell death include membrane blebbing, nuclear condensation and cell detachment, whereas morphologies associated with classical apoptosis such as DNA fragmentation, caspase activation, phosphatidylserine externalization or mitochondrial changes do not always accompany E4orf4-induced cell death (9,10,15). We thus measured E4orf4-induced cell death by assaying nuclear condensation and fragmentation, or cell loss. First, we tested whether an Acf1-DN mutant, which is unable to bind the SNF2h ATPase and acts in a dominant negative fashion (32), affects E4orf4-induced cell death. HEK293 cells were transfected with an E4orf4-expressing plasmid or with an empty vector together with plasmids expressing GFP-tagged WT Acf1, the Acf1-DN mutant or an empty vector. Data shown in Figure 4A demonstrate that Acf1-DN-GFP by itself did not affect HEK293 cell growth and E4orf4 altered cell morphology and reduced total cell number. When Acf1-DN-GFP was co-expressed with E4orf4, normal cell growth was partially restored. When E4orf4-induced cell death was measured by determination of the fraction of E4orf4-expressing cells with nuclear condensation or fragmentation, WT Acf1 was found not to affect E4orf4-induced cell death whereas the Acf1-DN mutant reduced cell death by 1.9-fold (Figure 4B). Western blot analyses revealed that the decrease in E4orf4 toxicity did not result from changes in E4orf4 levels or unequal expression of WT and mutant Acf1 proteins (Figure 4C), and both WT and Acf1 mutant proteins appeared to bind E4orf4 similarly (Figure 4D). In sum, these results suggest that E4orf4

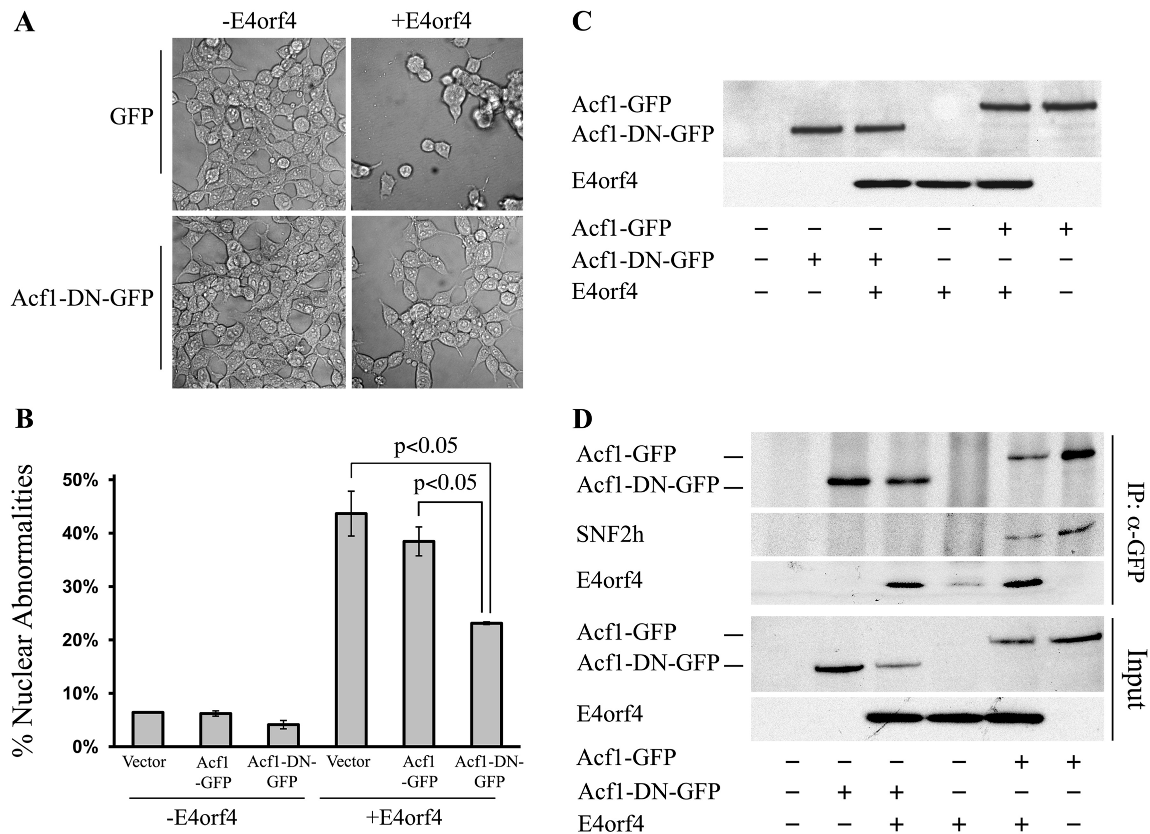

Figure 4. Expression of an Acf1 mutant which cannot interact with SNF2h interferes with E4orf4-induced cell death. HEK293 cells were transfected with plasmids expressing Acf1-GFP, Acf1-DN-GFP or GFP alone together with a plasmid expressing E4orf4 or its corresponding empty vector. (**A**) A representative picture of cells containing the indicated plasmids was taken 24 h after transfection, using a Zeiss Axioskop at a 400-fold magnification. (**B**) Cells in duplicate plates were fixed 24 h after transfection and stained with E4orf4-specific antibodies and with DAPI. The number of nuclei with abnormal morphology (condensed or fragmented) was counted in cells expressing both E4orf4 and GFP, and the percentage of cells with abnormal nuclei was calculated. The average of two independent experiments with two replicates each is shown. Error bars represent pooled standard deviation and statistical significance was determined using a paired Student's *t*-test. (**C**) Proteins were extracted from parallel plates as in (B) and a western blot was stained sequentially with antibodies to E4orf4 and to GFP. (**D**) Nuclear extracts were prepared from parallel plates as in (B) and were subjected to immunoprecipitation with antibodies to GFP. A western blot was stained sequentially with antibodies to GFP, SNF2h and E4orf4. Input lysates represent 10% of the extracts used for the immunoprecipitation.

must act through Acf1 molecules that bind SNF2h to induce cell death.

Next we determined whether SNF2h ATPase activity was required for induction of cell death by E4orf4. A SNF2h mutant was generated containing a K211R mutation. The K211 residue corresponds to a lysine residue that is essential for ATP hydrolysis and transcriptional activation by Swi2 and Brg1 ATP-dependent chromatin-remodeling factors. Substitution of this residue by arginine was reported to produce a dominant negative Brg1/Swi2 transcriptional activator (46). A similar mutation was also reported to eliminate ATPase activity of ISWI proteins in *Drosophila* and *Xenopus* (45,57). In measuring the effects of WT SNF2h and the SNF2h-K211R mutant on E4orf4-induced cell death we found that whereas the WT protein did not have any significant effect, the mutant decreased cell death by 1.9-fold (Figure 5A). This reduction in cell death did not result from a decrease in E4orf4 levels, as determined by western blot analysis (Figure 5B). Thus, SNF2h ATPase activity appears to be required for induction of cell death by E4orf4.

To further examine the contribution of SNF2h to E4orf4-induced cell death we generated a HEK293-derived cell line in which a SNF2h shRNA could be induced by doxycycline. E4orf4-induced cell death was measured by the appearance of nuclear aberrations following induction of SNF2h knockdown (Figure 5C). The results show a 2.4-fold decrease in E4orf4-induced cell death in cells with reduced SNF2h levels relative to the effect of E4orf4 in uninduced cells that contain normal levels of SNF2h. Knockdown of SNF2h in control cells did not induce cell death within the time frame of the experiment (Figure 5C, left). These alterations in the induction of cell death did not result from changes in E4orf4 levels, as determined by western blot analysis (Figure 5D). To confirm that the decrease in cell death was due to SNF2h knockdown and not to off-target effects of the shRNA or to effects of doxycycline treatment, E4orf4 was transfected into doxycycline-induced or uninduced cells together with a SNF2h mutant containing silent mutations that rendered it resistant to knockdown by the SNF2h shRNA. Restoration of SNF2h to the doxycycline-induced cells led to an increase in E4orf4-

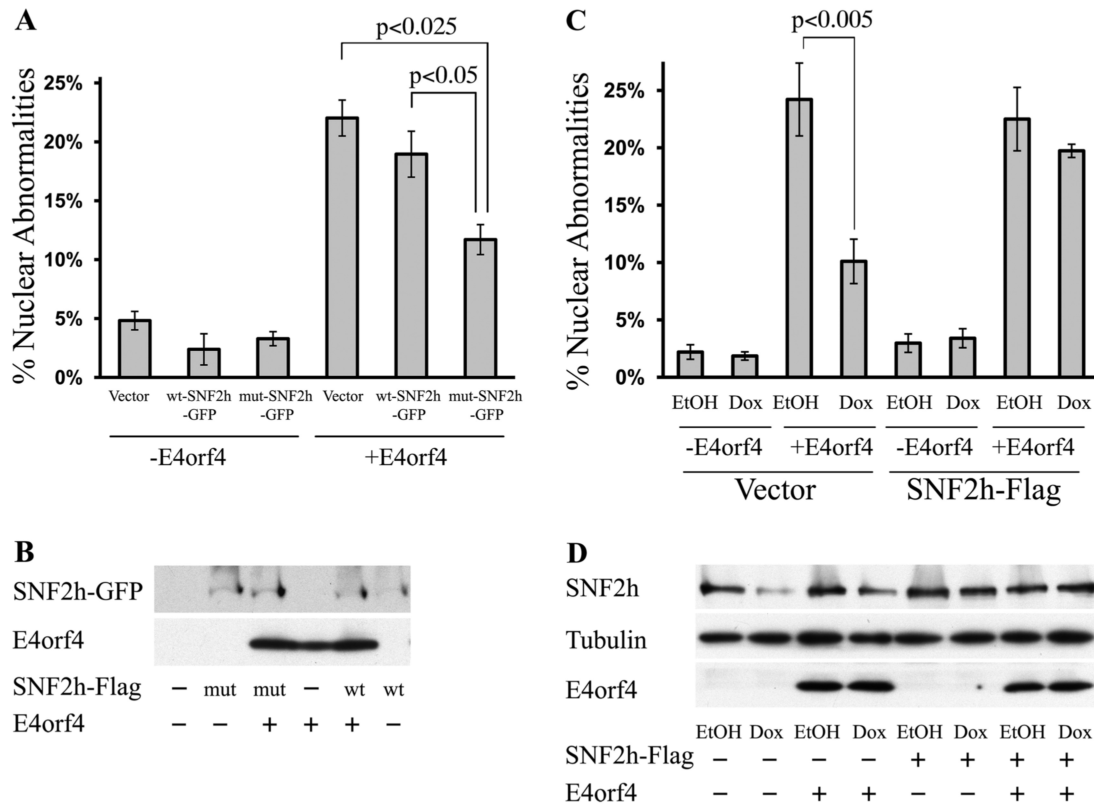

Figure 5. An active SNF2h ATPase is required for E4orf4-induced cell death. (**A**, **B**) HEK293 cells were transfected with plasmids expressing WT SNF2h (wt), a catalytically inactive SNF2h mutant (mut) or an empty vector (–) together with a plasmid expressing E4orf4 or its corresponding empty vector. The SNF2h proteins were GFP tagged. Twenty-four hours later, the cells were either stained with antibodies to E4orf4 and with DAPI or harvested for western blot analysis. Induction of cell death was measured by a DAPI assay (A), as described in the legend to Figure 4. The average of three experiments, each containing two replicates is shown. Error bars represent pooled standard deviation and statistical significance was determined using a paired Student's *t*-test. Proteins extracted from parallel plates were subjected to western blot analysis (B) using antibodies to GFP and E4orf4. (**C**, **D**) Cells from a HEK293-derived cell line expressing SNF2h–shRNA from a doxycycline-inducible promoter were induced with doxycycline (Dox) or with the solvent control (EtOH). After 3 days the cells were transfected with plasmids expressing E4orf4 or an empty vector together with a vector control or a plasmid expressing Flag-tagged SNF2h, which was rendered resistant to the shRNA by the introduction of silent mutations. Twenty-four hours after transfection the cells were either fixed and stained with antibodies to E4orf4 and the Flag tag and with DAPI or were extracted for western blot analysis. Induction of cell death (C) was measured by the DAPI assay described above. The average of two experiments with two replicates each is shown. Error bars represent pooled standard deviation and statistical significance was determined using a paired Student's *t*-test. A western blot of total lysates (D) was stained sequentially with antibodies to SNF2h, E4orf4 and α-Tubulin.

induced cell death in these cells, reaching levels of cell death as observed in uninduced cells (Figure 5C, right). These results are consistent with a requirement for SNF2h in E4orf4-induced cell death.

We next conducted similar knockdown experiments to investigate the contribution of Acf1 to E4orf4-induced cell death. Monitoring of E4orf4-induced cell death by measurement of the frequency of nuclear aberrations following induction of Acf1 knockdown revealed a 51% increase in cell death relative to the effect of E4orf4 in uninduced cells that contained normal Acf1 levels (Figure 6A, left). In contrast, knockdown of Acf1 in control cells did not induce cell death within the time frame of the experiment (Figure 6A, left). The results also indicate that the observed increase in cell death was not due to a change in the level of E4orf4 (Figure 6B). To confirm that the increase in cell death was due to Acf1 knockdown and not to off-target effects of the shRNA or to effects of doxycycline treatment, E4orf4 was transfected into doxycycline-induced or uninduced cells together with a shRNA knockdown-resistant Acf1 mutant. The uninhibited activity of Acf1 in the doxycycline-induced cells diminished E4orf4-induced cell death to levels observed in uninduced cells (Figure 6A, right).

Functional interaction between E4orf4 and Acf1 is conserved in yeast cells

Since the E4orf4-PP2A-mediated cell death pathway is conserved from yeast to mammals (14,16,18), and since Acf1 appears to participate in this pathway, we tested whether Itc1, the yeast Acf1 ortholog, plays a role in E4orf4-induced toxicity in yeast. WT and *itc1Δ* yeast cells were transformed with a plasmid expressing E4orf4 from a weak galactose-inducible promoter or with the empty vector and were plated in serial dilutions on glucose and galactose plates. As seen in Figure 6C, low E4orf4 levels driven by the weak galactose-inducible promoter caused minor inhibition of WT yeast growth, manifested by a small reduction in colony size on galactose relative to colonies containing only an empty

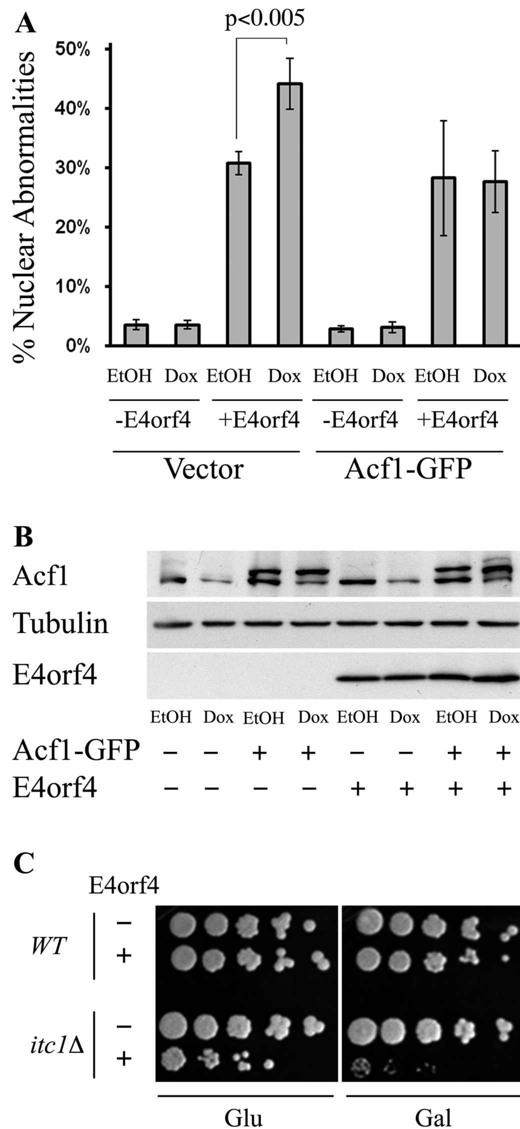

Figure 6. Acf1 knockdown enhances E4orf4-induced toxicity. (**A**, **B**) Cells from a HEK293-derived cell line expressing Acf1-shRNA from a doxycycline-inducible promoter were induced with doxycycline (Dox) or with the solvent control (EtOH). After 3 days the cells were transfected with plasmids expressing E4orf4 or an empty vector together with a plasmid expressing GFP-tagged Acf1, which was rendered resistant to the shRNA by the introduction of silent mutations or its corresponding empty vector. Twenty-four hours after transfection the cells were either fixed and stained with antibodies to E4orf4 and with DAPI, or were extracted for western blot analysis. Induction of cell death (A) was measured by the DAPI assay described above. The average of two experiments with two replicates each is shown. Error bars represent pooled standard deviation and statistical significance was determined using a paired Student's *t*-test. A western blot of total lysates (B) was stained sequentially with antibodies to Acf1, E4orf4 and α-Tubulin. (**C**) *WT300* and *itc1Δ* yeast cells were transformed with the plasmid pDAD2-E4orf4, expressing E4orf4 from a weak galactose-inducible promoter or with the empty vector. The yeast cells were serially diluted (1:5) and plated on glucose and galactose plates.

vector. However, inhibition of yeast growth was greatly increased when E4orf4 expression was induced from the same promoter in *itc1Δ* cells, as manifested by the much greater reduction in colony size. These results indicate that similarly to Acf1 deficiency in mammalian cells, deficit in Itc1 sensitizes yeast cells to E4orf4-induced toxicity, demonstrating high evolutionary conservation of the functional interactions between E4orf4 and Acf1.

The Acf1 homolog, WSTF, is required for E4orf4-induced cell death

Our results indicated that knockdown of Acf1 increased E4orf4-induced cell death but knockdown of SNF2h or expression of a catalytically inactive SNF2h mutant inhibited E4orf4 toxicity. These observations suggest that E4orf4 inhibits Acf1-containing chromatin-remodeling complexes but that it requires at the same time other SNF2h-containing chromatin-remodeling factors to induce cell death. WSTF is a close homolog of Acf1, which associates with SNF2h to form WICH chromatin-remodeling complexes that may direct SNF2h to different chromatin targets than Acf1 (39). To examine whether WSTF contributes to E4orf4-induced cell death, its expression was reduced by doxycycline-induced shRNA expression in a similar manner to the above described knockdown of Acf1 and SNF2h. Data presented in Figure 7 demonstrate that WSTF knockdown inhibited E4orf4-induced toxicity. Conversely, recovery of normal WSTF expression levels by use of a shRNA-resistant mutant rescued the ability of E4orf4 to induce cell death. These results suggest that a WSTF-containing chromatin-remodeling complex is likely to contribute to E4orf4-induced non-classical apoptosis.

Acf1 overexpression inhibits E4orf4-induced downregulation of early viral gene expression during adenovirus infection

Very early in adenovirus infection, the viral E1A protein enhances cellular and early viral gene expression. E4orf4, which accumulates during the early phase of infection, downregulates genes that were upregulated by E1A, thus contributing to temporal control of the progression of virus infection (4). We next inquired whether the interaction of Acf1 with E4orf4 contributes to the downregulation of early viral gene expression. HEK293 cells were transfected with a plasmid expressing Acf1-GFP or with an empty vector and were subsequently infected with *dl366** or *dl366*+E4orf4* viruses. The cells were harvested 24 h post-infection and cell extracts were subjected to western blot analysis. Results shown in Figure 8 as well as a previous report (3), indicate that expression of the early adenovirus E2A-72 kDa protein was reduced when cells were infected with the *dl366*+E4orf4* virus as compared to its expression in cells infected with *dl366** that lacks E4orf4. However, in cells that overexpress Acf1 the E4orf4-induced reduction in E2A-72 kDa protein expression was inhibited. These results suggest that to achieve inhibition

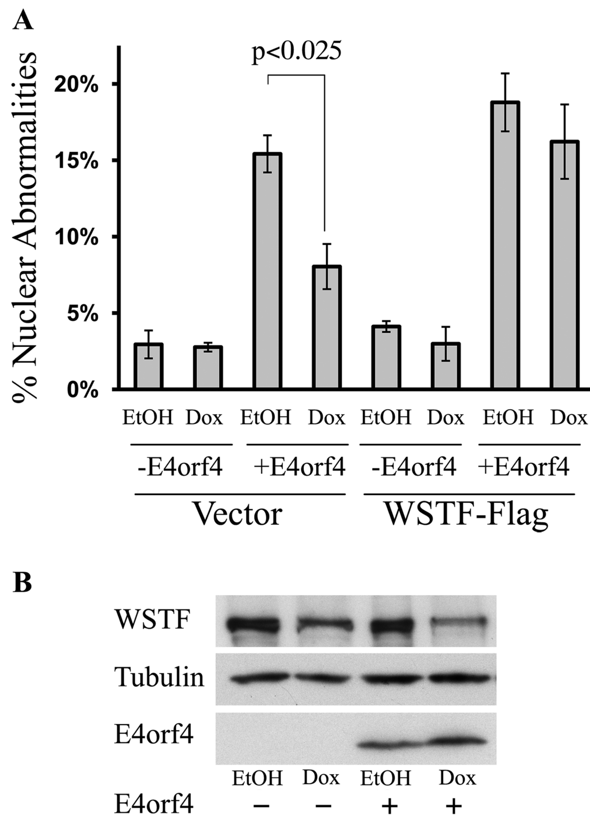

Figure 7. WSTF knockdown inhibits E4orf4-induced cell death. (**A**, **B**) Cells from a HEK293-derived cell line expressing WSTF-shRNA from a doxycycline-inducible promoter were induced with doxycycline (Dox) or with the solvent control (EtOH). After 3 days the cells were transfected with plasmids expressing E4orf4 or an empty vector together with a plasmid expressing Flag-tagged WSTF, which was rendered resistant to the shRNA by the introduction of silent mutations, or its corresponding empty vector. Twenty-four hours after transfection the cells were either fixed and stained with antibodies to the Flag tag and E4orf4 and with DAPI, or were extracted for western blot analysis. Induction of cell death (A) was measured by the DAPI assay described above. The average of two independent experiments with two replicates each is shown. Error bars represent pooled standard deviation and statistical significance was determined using a Student's *t*-test. A western blot of total lysates (B) was stained sequentially with antibodies to WSTF, E4orf4 and α-Tubulin.

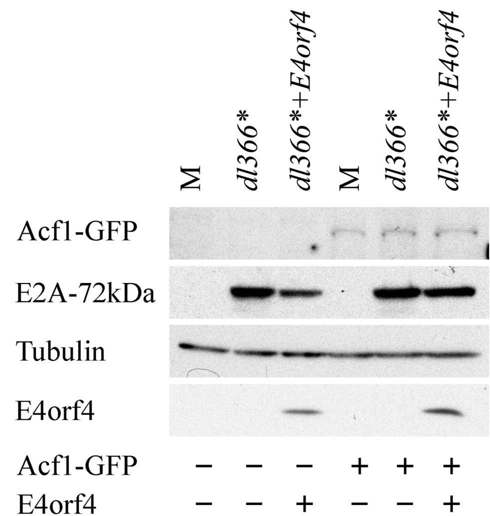

Figure 8. Acf1 overexpression inhibits downregulation of the early adenovirus E2A-72 kDa protein by E4orf4. HEK293 cells were transfected with plasmids expressing Acf1-GFP or GFP alone and were subsequently either mock-infected (M) or infected with the adenovirus mutants *dl366** or *dl366*+E4orf4*. The cells were harvested 24 h post-infection and protein extracts were prepared and chromatographed on SDS–PAGE. A western blot was stained sequentially with antibodies to GFP, E2A-72 kDa, α-Tubulin and E4orf4. This blot represents three independent experiments with similar results.

of early adenovirus gene expression, E4orf4 must inhibit Acf1.

DISCUSSION

E4orf4-induced cell death is a unique mode of non-classical programmed cell death and the mechanisms underlying this process are poorly understood. E4orf4 associates with two major partners, PP2A (2) and Src kinases (22), which contribute additively to its cell death-inducing activities (21). The association of E4orf4 with Src has been reported to lead to 'cytoplasmic cell death' (58) by perturbing cytoskeletal tension and endosomal traffic through a Src-Cdc42-Rab11a-dependent pathway (59). The present report shows, however, that the interaction of E4orf4 with PP2A is linked to its association with a chromatin-remodeling complex and with chromatin (Figures 1–3), enabling E4orf4 to exert its effect in the nucleus. The interaction between E4orf4 and Acf1 was observed as early as 2 h post-induction of E4orf4 expression (Figure 1D), suggesting that it is one of the earliest events induced by the viral protein. Previous reports showed that E4orf4 localizes to nuclei early after induction of its expression, and accumulates in the cytoplasm and membranes at a later stage, in correlation with its phosphorylation by Src kinases (24,60).

The E4orf4–PP2A–Acf1 interaction

Our results demonstrate that E4orf4 recruits PP2A to a complex with Acf1 and to chromatin (Figures 1–3). The low interaction levels between PP2A and Acf1 in the presence of E4orf4 might be due to their transient interaction or to the high salt concentration in the extraction buffer, which may have partially destabilized the complex. However, PP2A was detected above background levels in the Acf1 complexes, indicating a significant level of interaction between the two proteins. Although it has been previously proposed that E4orf4 induces cell death by inhibiting PP2A activity (61), results presented in that report were also consistent with inhibition of a PP2A-like phosphatase such as PP6. It has indeed been demonstrated that inhibition of PP6 contributes to E4orf4-induced cell death (62). Furthermore, it is not clear yet how E4orf4 affects PP2A activity towards the still unidentified physiological substrates of the E4orf4–PP2A complex. The novel finding presented here that E4orf4 targets PP2A to Acf1 and to chromatin suggests that, rather than to be inhibited, PP2A is recruited to dephosphorylate chromatin

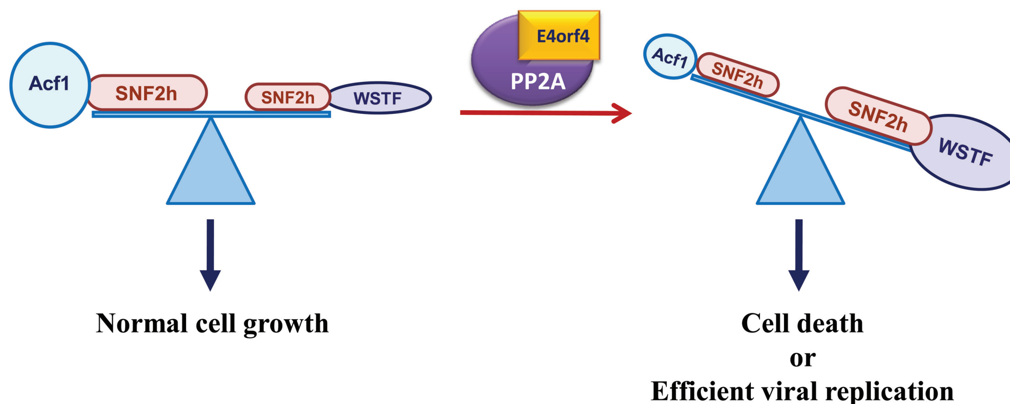

Figure 9. A working model: E4orf4 functions by shifting the balance between different SNF2h-containing chromatin-remodeling complexes. Various SNF2h-containing complexes participate in chromatin remodeling and affect transcription, DNA replication, DNA repair, etc. E4orf4 targets PP2A to Acf1-containing chromatin remodelers and inhibits them. This inhibition leads to a shift in the balance between various SNF2h remodeling complexes, allowing, for example, more activity of a WSTF–SNF2h complex. The variation in chromatin remodeler activity alters chromatin remodeling and produces changes in transcription, DNA replication, DNA repair or other processes that require remodeling. These events contribute to E4orf4 functions during virus infection and lead to cell death when E4orf4 is expressed individually.

substrates. Dephosphorylation of chromatin substrates by the E4orf4–PP2A complex may then lead to alterations in local chromatin structure, resulting in altered transcription, DNA replication, or other processes requiring chromatin-remodeling. Examples showing that the phosphorylation status of subunits of ATP-dependent chromatin-remodeling factors can affect their function have been previously described. WSTF, an Acf1 homolog, is phosphorylated in response to MAPK activation, and as a result is targeted to the Swi/Snf-containing WINAC chromatin-remodeling complex rather than to the SNF2h-containing WICH complex (63). The Swi/Snf ATPases Brm and Brg-1 are phosphorylated in mitosis, and thus are excluded from the chromosomes (64). However, at this point we cannot rule out an alternative possibility by which the recruitment of PP2A to chromatin provides a non-catalytic function, analogous to its function in the chromosomal association of condensin II (65). These possibilities are currently under investigation.

Functional interactions between E4orf4 and the ACF chromatin-remodeling factor

In addition to their physical association with E4orf4, we show here that both Acf1 and SNF2h subunits of the ACF chromatin-remodeling factor interact functionally with the viral protein. A catalytically inactive SNF2h protein and SNF2h knockdown were shown to inhibit E4orf4-induced cell death (Figure 5), whereas Acf1 knockdown enhanced it (Figure 6). The functional interaction between E4orf4 and Acf1 is highly conserved in evolution as demonstrated by the enhanced toxicity of E4orf4 in yeast following deletion of the yeast Acf1 ortholog *Itc1* (Figure 6C). The data indicate that SNF2h activity is required for E4orf4-induced cell death whereas Acf1 or Acf1-containing complexes must be inhibited. Based on these results we offer a model, schematically portrayed in Figure 9, of the mode of action of E4orf4. This model posits that E4orf4 inhibits an Acf1-containing complex by targeting PP2A to it, which consequently results in enhancement of the activity of other SNF2h-containing complexes. The shift in chromatin-remodeling activity induced by E4orf4 could lead to changes in transcription or DNA replication, which might result in cell death. This model is supported by the finding that knockdown of WSTF, an Acf1 homolog that is also found in SNF2h-containing complexes, leads to inhibition of E4orf4-induced cell death (Figure 7). According to the model, WSTF knockdown prevents replacement of the inhibited Acf1–SNF2h complexes by WSTF–SNF2h complexes, and therefore obstructs induction of cell death. It should be noted that we could not detect a physical interaction between E4orf4 and WSTF. However, such an interaction is not required by the suggested model. The Acf1 mutant, which is unable to bind SNF2h, was found to inhibit E4orf4-induced cell death (Figure 4). This finding is also consistent with the model proposed here since this mutant can be targeted to chromatin even without binding SNF2h (32) and could thus block the accessibility of other SNF2h-containing complexes to the relevant chromatin sites, preventing them from inducing cell death.

How does recruitment of PP2A to ACF by E4orf4 inhibit this complex? It is possible that PP2A activity causes the ACF complex to dissociate thus facilitating SNF2h association with other regulatory subunits. We could not detect a significant decrease in Acf1 binding to SNF2h in co-immunoprecipitation experiments, nor changes in co-localization of Acf1 and SNF2h in cells by confocal microscopy (results not shown). We also found both SNF2h and Acf1 in E4orf4-containing complexes (Figure 1). Although these observations exclude the possibility of global dissociation, they are not inconsistent with local dissociation events that are restricted to and affect specific chromosomal sites. Alternatively, the ACF complex could be released from chromatin, to be replaced by other SNF2h-containing complexes. Here too, we did not observe global changes in chromatin association of Acf1 or SNF2h (Figure 3). A third possibility is that

E4orf4-PP2A inhibits ACF remodeling activity. These possibilities will have to be addressed in future studies.

Chromatin remodeling and cell death

It is not currently known how the physical and functional interactions between E4orf4 and the ACF chromatin-remodeling complex as well as the functional interactions with WSTF contribute to E4orf4-induced cell death. These interactions may affect DNA-dependent processes, including transcription, replication or repair. WSTF has been shown to be involved in regulation of the DNA damage response by phosphorylating H2A.X on Tyr142 (41). This novel histone modification appears to determine the relative recruitment of either DNA repair or pro-apoptotic factors to sites of DNA damage, and thus, the balance between phosphorylation and dephosphorylation of H2A.X Tyr142 may constitute a switch mechanism that modulates survival/apoptotic decisions (66). Based on our results, a switch regulating survival versus cell death may also be achieved by an E4orf4–PP2A-induced shift in the balance between Acf1-SNF2h and WSTF-SNF2h in chromatin. ACF was shown to be required for the regulation of both transcription and DNA replication (32,37), and Acf1 and Snf2h have recently been shown to contribute to double-strand break repair as well (38). Modulation of any of these processes may entail a situation that is not compatible with cell survival. In any event, our current study establishes a novel function for the Acf1 chromatin-remodeling factor in life and death decisions.

Chromatin remodeling during adenovirus infection

We have shown here that E4orf4 binds Acf1 within the context of adenovirus infection (Figure 1E), suggesting that their interaction contributes to the viral life cycle. E4orf4 is a downregulator of both cellular and early adenoviral gene expression, and the interaction with PP2A is required for this activity (1–4,24). Acf1 was also shown to contribute to downregulation of the expression of specific genes, including wingless-regulated and vitamin D3 receptor-regulated genes (34,35). In this report, we showed that Acf1 overexpression inhibited E4orf4-induced downregulation of one of the early adenovirus proteins, the E2A-72 kDa protein, whereas Acf1 over-expression in the absence of E4orf4 did not affect E2A-72 kDa expression (Figure 8). These results suggest that activation and downregulation of E2A-72 kDa expression are carried out by distinct mechanisms. Whereas activation of E2A expression has been described and is achieved by E1A-induced displacement of Rb proteins from the E2F transcription factor and subsequent E2F dimerization facilitated by the adenovirus E4orf6/7 protein [reviewed in (67)], it remains to be seen whether downregulation of expression of this early viral protein results from a direct or an indirect effect of Acf1. However, the finding that Acf1 counteracts E4orf4 activity during virus infection is consistent with the finding that Acf1 knockdown enhances E4orf4-induced cell death, and both results indicate that the activities of E4orf4 and Acf1 counteract each other.

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