Analysis of the Cullin Binding Sites of the E4orf6 Proteins of Human Adenovirus E3 Ubiquitin Ligases

Timra Gilson¹, Chi Ying Cheng¹, Woosuk Steve Hur¹*, Paola Blanchette¹ and Philip E. Branton¹,²,³#*

Departments of Biochemistry¹ and Oncology² and the Rosalind and Morris Goodman Cancer Research Centre³
¹²McGill University, McIntyre Medical Building, 3655 Promenade Sir William Osler, Montreal, Quebec, Canada, H3G 1Y6
³McGill University, 1160 Pine Ave. West, Montreal, Quebec, Canada, H3A 1A3

*Current address: Department of Biochemistry, University of British Columbia, Michael Smith Laboratories, 2185 East Mall, Vancouver, British Columbia, Canada, V6T 1Z4

#Corresponding author:
philip.branton@mcgill.ca

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Abstract

E4orf6 proteins of human adenoviruses form Cullin-based E3 ubiquitin ligase complexes that degrade cellular proteins that impede efficient viral replication. These complexes also include the viral E1B55K product believed to recruit most substrates for ubiquitination. Heterogeneity in the composition of these ligases exists as serotypes representing some species form Cul5-based complexes (species B2, C, D and E) whereas others utilize Cul2 (species A and F). Ad16 (species B1) binds significant levels of both. In this report we show that the Cul2 binding sequence in E4orf6 of Ad12 (species A) and Ad40 (species F) resembles the cellular consensus Cul2 box. Mutation of this Cul2 box prevents binding not only of Cul2 but also in some cases Elongin C, and reduces the ability to degrade target proteins such as Mre11 and p53. A comparable Cul2 box is not present in E4orf6 of Ad5 and other serotypes that bind Cul5; however, creation of this Cul2 box sequence in Ad5 E4orf6 promoted binding to Cul2 and Cul2-dependent degradation of Mre11. E4orf6 of Ad16 also bind Cul2 however, unlike Ad40, it does not contain an Ad12-like Cul2 box, suggesting that Ad16 binds Cul2 in a unique but perhaps non-functional manner as only Cul5 binding complexes appeared able to degrade Mre11. Our extensive analyses have thus far failed to identify a consensus Cul5 binding sequence, suggesting that association occurs via a novel and perhaps complex pattern of protein-protein interactions. Nevertheless the identification of the Cul2 box may allow prediction of Cullin specificity for all E4orf6-containing Adenoviridae.

Importance
The work described in this paper is a continuation of our in-depth studies on the Cullin-based E3 ligase complexes formed by the viral E4orf6 and E1B55K proteins of all human adenoviruses. This complex induces the degradation of a growing series of cellular proteins that impede efficient viral replication. Some human adenovirus species utilize Cul5 whereas others bind Cul2. In this paper we are the first to identify the E4orf6 Cul2 binding site, which conforms in sequence to a classic cellular Cul2 box. Ours is the first detailed biochemical and genetic analysis of a Cul2-based adenovirus ligase and provides both insights into the cooperative interactions in forming Cullin-based ligases as well as the universality of formation of all adenovirus ligase complexes. Our work now permits future analysis of the evolutionary significance of the ligase complex, work that is currently in progress in our lab.

Introduction

Viruses commonly encode proteins that extensively modify the cellular environment to promote viral replication. A frequent strategy is to produce polypeptides that disrupt cellular pathways through the formation of E3 ubiquitin ligases that target cellular proteins for degradation (1, 2). Several viruses, including human adenoviruses, utilize Cullin 2 (Cul2) and Cullin 5 (Cul5) dependent E3 ubiquitin ligase complexes (see Table 1) to target proteins for degradation that otherwise would interfere with viral propagation. Each of these viruses encodes a substrate acquisition protein which acts as a mediator to bring together the substrate protein to be degraded with Cul2/5 and Elongins B and C to form a complete E3 ligase complex. The target is subsequently polyubiquitinated (3–5), which is a signal for proteasome-mediated degradation (6).
Cellular substrate acquisition proteins bind to Cul2-based E3 complexes through a VHL box, and to Cul5 complexes through a SOCS box (7). Both of these boxes are composed of an Elongin B and C binding domain (BC box) and a Cul2 or Cul5 binding domain (Cul2/5 box). Viral proteins often form complexes with cellular proteins using canonical or closely related cellular binding domains. While the BC box domains have been identified in the early region 4 open reading frame 6 (E4 34K or E4orf6) protein of human adenovirus type 5 (Ad5), the location of the Cullin domains has remained elusive (3, 8). The Cullin binding domain within other viral proteins has been located in most of the examples listed in Table 1, only about half of which follow the cellular consensus sequence (LPxP for Cul5, and LxxxLxxxL for Cul2) (7, 9). Thus it seems equally likely that human adenoviruses might utilize either a cellular consensus domain or a novel sequence to bind Cullins 2 or 5.

The E3 complex formed with adenoviral proteins is unique in that two viral proteins are involved: the E4orf6 product and early region 1B 55kDa protein (E1B55K) (10, 11). The E4orf6 protein binds to the E3 ubiquitin ligase complex through its three BC box domains while the cellular targets are largely selected and brought to the E3 complex by E1B55K (3, 10). Together, E4orf6 and E1B55K of Ad5 target a growing list of cellular proteins for degradation, including DNA Ligase IV (12, 13), Mre11 (14), p53 (11), BLM (15), integrin α3 (16) and ATRX (17). We have found previously that among the seven human adenovirus species, thus far DNA ligase IV is the only common substrate (18).

Previous work by our group and others has shown that a heterogeneity exists in Cullin binding among human adenovirus species (19, 20). Whereas Ad5 representing species C, Ad34 (species B2), Ad9 (D) and Ad4 (E) bind essentially only Cul5, Ad12 (A) and Ad40 (F) bind essentially only Cul2. Ad16 (species B1) was found to bind significant amounts of both Cul5 and
Cul2. In the present studies we have taken a genetic approach to map the Cul2 and Cul5 binding sites on the E4orf6 products of Ad12, Ad40 and Ad5. We found that Cul2 binds both Ad12 and Ad40 E4orf6 by means of a sequence resembling the cellular consensus Cul2 box. No such functional sequence is present with Ad5 or other serotypes that utilize Cul5, which appears to associate with the E4orf6 protein via another as yet unidentified interaction.

**Materials and methods**

*Cells:*

Human small cell carcinoma H1299 (21) (ATCC CRL-5803) carrying a homologous deletion of the p53 gene as well as its knock-down derivatives were grown in Dulbecco Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS).

*Generation of Cul2/Cul5 knock down cell lines:*

Cul2 was knocked down in H1299 (19) cells by transfection with a plasmid DNA encoding short hairpin RNA (shRNA) against Cul2. The sequence for the shRNA was inserted between EcoR1 and Bg/II sites of pcDNA3 plasmid: 5’ AGCACCTGAACTGCTTGCT TTCAAGAGA 3’, with the hairpin listed in bold. Cloned upstream of the primer was a Sp6 site for sequencing followed by zeocin selection marker, U6 promoter and U6 leader sequences. H1299 cells were plated in 6 well dishes and transfected with the shRNA plasmid DNA and a plasmid DNA encoding the puromycin resistance gene using Lipofectamine 2000 as described by the manufacturer. Cells were selected with 2µg/mL puromycin and established colonies were then grown in the presence of 1µg/mL puromycin. Levels of Cul2 were examined by western blot analysis. The colony with the greatest decrease in Cul2 levels...
was used. H1299 cells with Cul5 knocked down (8) were re-made using a similar procedure as for Cul2, with the exception that a second round of RNAi was performed with hygromycin selection using the same plasmid DNA as used previously.

**Antibodies:**

Antibodies used were as follows: HA epitopes were detected using mouse monoclonal anti-HA antibody HA.11 (BabCO) for western blotting as well as for immunoprecipitation; FLAG epitopes with anti-FLAG M2 mouse monoclonal hydrogen peroxidase conjugated antibody FLAG-HRP (Sigma A8592) for western blotting or with rabbit polyclonal anti-FLAG antibody M2 (Sigma F7425) for immunoprecipitations; p53 epitopes with anti-p53 pAb1801 hybridoma supernatants as described previously for western blotting (22); HSV epitopes (QPELAPEDPEDC) with rabbit polyclonal anti-HSV antibody (Kamiya PC267) for western blotting or mouse monoclonal anti-HSV antibody (Novagen 69171) for immunoprecipitations; actin epitopes with mouse anti-Actin antibody (Fisher pan Ab-5), Mre11 epitope with rabbit polyclonal anti-Mre11 antibody (Novus 100-142) for western blotting; Cul2 epitopes with anti-Cul2 rabbit antibody (Novus NBP1-02780); Cul5 epitopes with anti-Cul5 rabbit antibody (Bethyl A302-173A); and TopBP1 epitopes with rabbit polyclonal anti-TopBP1 antibody (Bethyl A300-111A).

**Plasmids and E4orf6 chimeric proteins:** The pcDNA3 plasmids expressing FLAG-tagged E4orf6 and HA-tagged E1B55K from each serotype (19), HSV-tagged Elongin C (generous gift from Joan Conaway) and human p53 (3), were described previously. Chimeras between Ad5 and Ad12 E4orf6 were generated using a two-round PCR approach with a homologous sequence as
the hinge region. For the first set of chimeras, the homologous sequence ATGGCGCC (Ad5 449-456bp and Ad12 419-426bp) was used to generate fusion with approximately half of each protein. Primers used are indicated in Table 2. Following a first round of PCR for each half, the purified fragments were used as template for the second round of PCR which generated the full length chimera. These were then cloned into pcDNA3-FLAG with BamH1 and HindIII. For the second set of chimeras, the homologous sequence TGGGATATG (Ad5 241-249bp and Ad12 211-219bp) was used to generate chimeras in which the first half of the proteins was again divided into approximate halves. Specific mutations in FLAG tagged Ad5, Ad12, Ad16, and Ad40 E4orf6 were generated by PCR-based mutagenesis using the primers as indicated in Table 2. Incorporated within each primer were additional silent mutations which either added or removed a restriction site close to the mutation. Colonies were screened by restriction enzyme digestion then sequenced to confirm the incorporation of the mutation.

Cell Transfection and lysis:

H1299 cells plated in 10 cm dishes for immunoprecipitations or 6-well plates for degradation assays were transfected for 24h with plasmid DNAs using Lipofectamine 2000, as described by the manufacturer at a ratio of 3:1 (Lipo: DNA). DNA amounts were normalized within each experiment with pcDNA3 empty vector.

For degradation assays the amounts of plasmid DNA used were: 0.35µg p53, 1.5µg Ad5 or Ad12 HA-E1B55K or 2µg Ad16 or Ad40 HA-E1B55K, and the following amounts of FLAG-E4orf6: 1.5µg for wild-type Ad5 and Ad12, Ad12Y78E, and Ad12Y86C; 2µg for wild-type Ad16 and Ad40, all Ad40 mutants, Ad12 Y78E/Y86C, Ad5E88Y/C96Y and all Ad12 BC box mutants; and 2.25µg for Ad12 I74K and Ad12I82K. For immunoprecipitation experiments the following
amounts of plasmid DNAs were transfected: 3-4µg for HA-Cul2, 3-5µg for HA-Cul5, 2µg for
HSV-Elongin C, 2µg for Ad12 HA-E1B55K, and these amounts for the various forms of FLAG-E4orf6: 8µg for all forms of Ad40, 3µg for wild-type Ad16 and Ad16V92K, 4µg for Ad16E88R
and C96R, 1µg for wild-type Ad5, 2µg for Ad5E88Y/C96Y, 3µg for the Ad5/12 chimera, 6µg
for the Ad12/5/5, 5/12/5, 5/12/12 chimeras, 7µg for the Ad12/5/12 chimera, 9µg for the Ad12/5
chimera, 6µg for all Ad12 BC box mutants. In the immunoprecipitations with Cullins, all DNAs
echoicing Ad12 mutants were transfected at 9µg, but with the immunoprecipitations with either
HSV-Elongin C or E1B55K, DNAs were transfected as follows: 3µg for wild-type, Y78E, and
Y86C, 4µg for Y78E/Y86C, and 4.5 µg for I74K and I82K. Cells were lysed for 20 min on ice
in lysis buffer (20mM Tris pH 7.4, 150mM NaCl, 2mM EDTA, 5% Glycerol, 1% Triton-X,
2mM NaPP, 400mM NaF, 100mM Na3VO4, 0.2mg/mL PMSF, 1mM DTT, 2µg/mL aprotinin,
5µg/mL leupeptin, and 0.7µg/mL pepstatin A. For TopBP1 degradation assays, cells were lysed
for 20 min on ice in an NP40 based lysis buffer (50mM Tris-HCl, pH 8, 150mM NaCl,
5mMEDTA, 0.15% NP40, 2mM DTT, 4mM NaF, 2mM NaPP, 500µM Na3VO4, 200µg/ml
PMSF, 2µg/ml aprotinin, 5µg/ml leupeptin and 0.7µg/ml pepstatin A) followed by three rounds
of freeze/thaw cycles, and two rounds of sonication at 100% power output for 30 sec with a
VCX130 sonicator (SONICS & Materials, CT).

Immuno precipitations and Western blotting:

300-800µg lysate (consistent within each experiment) was used for immunoprecipications with 1
µL of appropriate antibody followed by incubation with a 50% slurry of ProteinA/ProteinG
beads. Antibody bound beads were extensively washed in lysis buffer, and immunoprecipitates
were eluted in 2x protein sample buffer + 5% β-Mercaptoethanol. Proteins were examined by
western blotting, essentially as described previously (3). Briefly, proteins separated by SDS-PAGE were transferred to PVDF membranes and blocked using 5% non-fat dry milk in TBST. Membranes were incubated with the indicated primary antibodies in TBS-1% Tween, followed by appropriate peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Polypeptides on membranes were then visualized by enhanced chemiluminescence with ECL plus western blotting reagents (Amersham-Pharmacia Biotech).

Alignments and Phylogenetic tree:
Protein sequences were obtained from either Uniprot or NCBI protein database. The accession numbers of each adenovirus E4orf6 protein are: Ad2 P03239, Ad3 Q2KSJ0, Ad4 Q5VHA1, Ad5 Q2KRZ1, Ad6 E1U5Q7, Ad7 D0EF64, Ad8 B9A5K7, Ad9 P89083, Ad10 BAM66771.1, Ad11 D2DMB1, Ad12, Ad14 C3UZM1, Ad15 E1CIL8, Ad16 Q2KRV1, Ad17 F1DT87, HAd18 D3JU6, Ad19 B6C704, Ad21 Q2KS94, Ad22 C5HDT5, Ad26 33227-32349bp of EF153474, Ad28 C4P232, Ad29 E1C1Q4, Ad31 D0Z5U7, Ad34 Q3ZKX1, Ad35 Q7T924, Ad36 D4N3K8, Ad37 B9A6A9, Ad40 Q64865, Ad41 B5SNT8, Ad46 Q4KSI4, Ad48 33289-32411bp of EF153473, Ad49 33302-32424bp of DQ393829, Ad50 Q3ZKS5, Ad52 A0MK73, Ad53 E5RWD2, Ad54 B9A5P3, Ad55 C7SRR9, Ad56 E1A38, Ad57 E1ARS8, Ad58 E9P576, Ad60A AEL78894.1, Ad61 E4orf5 AEK79937.1, Ad62 AEL78858.1, Ad63 AEV92982.1, Ad64 AFA46707.1, Ad65 BAL41738.1, Ad67 BAL63204.1, Ad68 AET87249.1. Inclusion of nucleotide numbers beside the accession number indicates that the gene and protein were hand annotated. Protein sequences were aligned by ClustalW. The gap free region from 37-306 was selected for further analysis. The 49 sequences were first inserted into Seqboot for 100x bootstrapping. The distance matrix was generated by Protdist which was then analyzed by
Neighbor to generate phylogenies using the UPGMA method. Seqboot, Protdist, and Neighbor are part of the Phylip package 3.695 (23). The consensus phylogenetic tree was generated by BayesTrees 1.3 (24), and visualized with TreeView (25) using branch lengths.

**Results**

**Use of Ad5/Ad12 E4orf6 chimeras to localize Cul5 and Cul2 binding**

Cellular proteins bind to Cul2-based ligase complexes through a VHL box, and to Cul5-complexes through a SOCS box. Both are comprised of an Elongin B and C binding domain (BC box) followed by a Cul2 or Cul5 binding domain, with an average 10-25 amino acids separating the two (7, 9). In some viral proteins, however, these domains can also be quite distant, as is the case for the LANA protein where they are over 800 amino acids apart (26). Therefore the search for the Cullin binding domains must encompass the entire length of the protein.

The first step in localizing the Cullin domains was to narrow down the location to either the N- or C-terminus. Truncation mutants of E4orf6 have been utilized by some labs including our own (22), but this approach can often lead to improperly folded proteins, especially when few functional domains are known and the E4orf6 protein structure has yet to be determined. Instead, we chose to create chimeric E4orf6 proteins between Cul2 and Cul5 binding species (see Fig. 1A). The highest protein identity between the Cul2- and Cul5-binders is shared between species A (Ad12) and C (Ad5) at 52%, and therefore chimeric proteins created from these two would be the most likely to be functional. Chimeras were created in a two-step PCR cloning process as described in Materials and methods. Each chimera was N-terminally FLAG tagged and inserted into pcDNA3 vector. Plasmid DNAs were then transfected into H1299 cells along with DNAs encoding HA-tagged Cul2 or Cul5 and at 24h the soluble lysates were
immunoprecipitated using an anti-FLAG antibody and analyzed by western blotting using anti-HA antibody. Fig. 1B shows that the chimeric E4orf6 proteins were expressed at high levels and migrated at an appropriate full length size of about 34kDa. Some slight variation in gel mobility was noted in the chimeras relative to the wild-type species. We are uncertain of the basis of this effect; however, all constructs were sequenced and none contained unexpected gains or losses in the coding sequences. In addition, no study thus far has reported any form of post-translational modification of E4orf6. Western blotting indicated that in both cases Cullin binding appeared to be associated with the N-terminal regions as the Ad5-12 chimera bound exclusively Cul5 whereas the Ad12-5 chimera bound only Cul2 at high levels. Ad5 binds Cul5 and Ad12 binds Cul2 as expected (lane 1 of each blot). Very low levels of Cul2 binding were evident with both the Ad5 and Ad5/12 E4orf6 proteins, although in the case of Ad5 this association was not found to be of functional significance in terms of protein degradation (19). Therefore, the Cul2 and Cul5 binding domains both appeared to be located in the N-terminal half of these E4orf6 proteins.

To further localize the Cullin binding domains, we created Ad5/Ad12 chimeric proteins in which the N-terminal half was further divided (see Fig. 1C). Plasmid DNAs encoding all four chimeric proteins were transfected into H1299 cells along with DNAs expressing HA-Cul2 or HA-Cul5 and extracts immunoprecipitated with anti-FLAG antibody and analyzed by western blotting using anti-HA antibody. Fig. 1D shows that all chimeric proteins were stably expressed and of the expected size. With both Cul5 and Cul2, binding clearly occurred in the second portion of the N-terminal half of the E4orf6 proteins as chimeras 12-5-5 and 12-5-12 co-immunoprecipitated only Cul5, whereas chimeras 5-12-5 and 5-12-12 bound only Cul2. It is of interest to note that also located within the second quarter of the Ad5 E4orf6 proteins are BC
boxes 2 and 3 (3, 8). This pattern follows the cellular model of Cullin complex formation in that the Cullin box is located adjacent to the BC box.

**Identification of a functional Ad12 Cul2 box**

To identify the Cul2 binding domain, we performed site directed mutagenesis on Ad12 E4orf6 utilizing two strategies involving: differences in E4orf6 sequence relative to Cul2 and 5 specificity, and sequence homology to the cellular consensus Cul2 box. The amino acids involved in binding Cul2 by adenovirus species A and F should be unique, and absent or only partially present in other species. Fig. 2A shows an alignment of the sequences of the second quarter of E4orf6 protein of representatives of all seven adenovirus species. Remarkably, considering that the overall identity among each of the representative E4orf6 proteins averages only 57%, very few significant amino acid differences are present in this region, suggesting a high degree of localized conservation. Three amino acids are unique and present in all Cul5 binders: E88, L90, and D107 (Ad5 number based), and six unique to both Cul2 binders: Y78, Y86, N97, E99, R118, and Y140 (Ad12 number based). With so few differences between Cul2 and Cul5 binders, it appeared likely that at least a portion of the putative Cul2 box is probably present in Cul5 binders, and that alteration of just one or two of the essential amino acids may be responsible for failure of Cul2 binding. Thus five Ad12 E4orf6 mutants were made at these unique sites in which the amino acids were changed to the corresponding Ad5 residue: Y78E, Y86C, R118S, Y140D, and double mutant N97D/E99M.

Fig. 2A also shows that several possible cellular Cul2 box sequences may exist in this region of the Ad12 E4orf6 protein. The consensus cellular Cul2 box proposed by Mahour (9) is L(P/x)xxLxxL, but since multiple cellular Cul2 boxes contain hydrophobic residues other than...
leucine, for our purposes this motif was generalized to three hydrophobic residues (Ф):

ФxxxФxxxФ. Six potential motifs were found: 74I LTD YEMFI, 78Y EMF I LKKY, 83L KKY MSVC M,
96I NVE VTQL L, 130L LGR WFKMA, and one partial sequence which contains a proline residue
often found after the first hydrophobic residue: 69IP WD MILTD. Of these, two were immediately
disqualified, as residues 130-138 overlap with BC boxes 3, and the hydrophobic residues in
amino acids 83-91 are not conserved in Ad40 (assuming that Ad12 and Ad40 utilize the same
Cul2 box). The potential Cul2 boxes located at residues 74-82 and 78-86 appeared promising as
two of the hydrophobic residues (Y78, Y86) are part of the unique residues identified previously.
For these reasons, we began with mutations affecting residues 73, 74 and 82 in which these
hydrophobic residues were replaced by charged amino acids to yield mutants M73K, I74K, and
I82K.

Plasmid DNAs expressing each of the eight FLAG-tagged Ad12 E4orf6 mutants and HA-
Cul2 were transfected into H1299 cells, and after 24h soluble lysates were immunoprecipitated
and western blotted as above to examine Cul2 binding. Fig. 2B shows that each mutant was
stably expressed, and each of the I74K, Y78E, I82K, and Y86C mutations prevented binding to
Cul2. Although tagged versions of Cul2 and Cul5 were employed in these studies, previous
binding specificities have also been reported with endogenous Cul2 and Cul5 (11, 20). Thus
these results suggested that a cellular-like Cul2 box might exist involving hydrophobic amino
acids between residues 74 and 86.

We have noted that although adenovirus E4orf6 proteins exhibit strong Cullin specificity,
very low amounts of Cul2 binding can be detected with E4orf6 of Ad5 and conversely low
amounts of Cul5 binding is seen with Ad12. Thus we wondered if alteration of the Cul2 box of
Ad12 resulted in any change in Cul5 binding. Fig. 2C shows that indeed alteration of consensus
hydrophobic residues in the Ad12 E4orf6 mutants to residues found in Ad5 E4orf6 increased the ability of these proteins to bind Cul5. These results suggested that some overlap in the binding regions of Cul2 and Cul5 may exist (see more below).

One concern is always that the mutation may disrupt global protein structure and so we determined if our mutants could still associate with normal binding partners such as E1B55K and Elongin C. Thus H1299 cells were transfected with DNAs expressing the FLAG-tagged Ad12 E4orf6 mutants as well as HA-tagged Ad12 E1B55K, and 24h later the soluble lysate was immunoprecipitated with anti-HA antibody and western blotted using an anti-FLAG antibody. Fig. 2D shows that all but the I74K mutant were able to bind E1B55K, suggesting that the I74 mutation was either too severe, that the amino acid is key structurally, or that it is buried within the protein and disrupts the overall protein structure. To determine if these altered E4orf6 proteins could bind Elongin C, the Ad12 mutant cDNAs were co-transfected into H1299 cells along with a cDNA expressing HSV-Elongin C, and cell extracts were immunoprecipitated with an anti-HSV antibody. Fig. 2E shows that both wild-type and Y78E E4orf6 products were able to bind high levels of Elongin C. Interestingly mutants I74K, I82K and Y86C bound Elongin C poorly, suggesting that whereas binding of E1B55K was unaffected, interactions with both Cul2 and Elongin C were disturbed. As discussed below these findings may relate to the known cooperativity of Elongin B/C and Cullin interactions seen in the formation of some cellular Cullin-based ligase complexes (27). Nevertheless it seemed most likely that the Ad12 E4orf6 protein contains a Cul2 box similar to the canonical cellular sequence composed of three hydrophobic residues at 78YEMFILKKY86.

The spacing of the hydrophobic residues in the Cul2 box suggests that it lies within an alpha helix, with the hydrophobic residues aligned on one side. To form protein-protein
interactions, this alpha helix should be exposed at the surface of the E4orf6 protein. No crystal structure of any adenovirus E4orf6 protein has yet been obtained, but we generated a putative model structure using the Itasser protein structure prediction program (28, 29). Fig. 2F shows that the putative Cul2 box is predicted to be located in an alpha helix on the surface of the E4orf6 protein. Importantly, this structure predicts that residue 74 is buried within the protein and thus may explain why a mutation at this site might perturb overall protein conformation and thus binding to Cul2, Elongin C and E1B55K.

Ad12 E4orf6 protein and E1B55K together target Mre11 and several other cellular proteins for degradation (12–17). Mutants that prevent binding to Cul2 should therefore prevent degradation of Mre11. Plasmid DNAs expressing each of the Ad12 E4orf6 mutants that prevent binding to Cul2 along with DNAs encoding HA-tagged Ad12 E1B55K were transfected into cells and at 24h lysates were probed for endogenous Mre11 using anti-Mre11 antibodies. Fig. 2G shows that as expected, wild-type E4orf6 induced degradation of most of the Mre11, as found previously using this system (3, 8, 18, 19); however with I74K, which disrupts the overall structure of the protein, I82K and the double mutant Y78E/Y86C, degradation was significantly impaired. Mutants Y78E and Y86C still degraded Mre11 significantly (see further discussion below). Taken together, these results strongly supported the existence of a functional cellular-like Cul2 box in the Ad12 E4orf6 protein located between residues 78 and 86.

Ad40 but not Ad16 E4orf6 contains a functional Ad12-like Cul2 box

In addition to Ad12, and presumably other serotypes within the A species, both Ad40 (species F) and to a lesser degree Ad16 (species B1) have previously been shown to bind Cul2 (19). Fig. 3A shows the phylogenetic tree of E4orf6 of all species and indicates that species A, F and G more
recently branched out from the other species and are thus more closely related to each other than to the rest. It is therefore likely that Ad12 and Ad40 evolved similar mechanisms to bind Cul2. Fig. 3B presents the alignment of the E4orf6 protein sequences from the Ad12, Ad16, and Ad40 and shows that whereas Ad40 contains an Ad12-like Cul2 box with three hydrophobic residues from amino acids 79 to 87, Ad16 does not. To determine if this Ad40 sequence contains a functional Cul2 box, these three conserved hydrophobic residues were altered to lysine or arginine residues, and DNAs expressing mutant FLAG-tagged Ad40 E4orf6 proteins were transfected into H1299 cells along with plasmid DNA encoding HA-Cul2, and lysates were examined for Cul2 binding as in Fig. 2B. Fig. 3C shows that mutants V83R, Y87K and the double mutation W79K/V83R decreased binding to Cul2 relative to wild-type. The double mutant W79K/Y87K and triple mutant W79K/V83R/Y87K were both fully defective for binding to Cul2. To confirm the functionality of this putative Cul2 box studies similar to those in Fig. 2F were performed to assess the ability of the Ad40 mutant E4orf6 proteins to induce protein degradation, this time involving both exogenous p53 and the endogenous substrate Mre11. Each Ad40 mutant FLAG-E4orf6 protein was expressed along with Ad40 HA-E1B55K and human p53, and after 24h Mre11 and p53 levels were assessed by western blotting using appropriate antibodies. Fig. 3D shows that with both p53 and Mre11 an almost perfect correlation was observed between degradation and the ability to bind Cul2 presented in Fig. 3C, although degradation of p53 might have been slightly more sensitive to the overall conformation of the E3 ubiquitin ligase complex than Mre11 (e.g. see mutant Y87K). Nevertheless, taken together these results indicated that Ad40 E4orf6 protein possesses a Cul2 box similar to that of Ad12, although the binding dynamics to Cul2 may be slightly different.
Ad16 E4orf6 protein has only one of the three required hydrophobic residues in the putative Cul2 box region. Nevertheless, we mutated the three residues in Ad16 that align to the Ad12 Cul2 box (E88, V92, and C96) and plasmid DNAs expressing these mutant proteins were transfected into H1299 cells and Cul2 binding was assessed as in Fig. 3C. Fig. 3E shows that the E88R, V92K, and C96R mutants all were able to bind significant amounts of Cul2, suggesting that the interaction of Ad16 E4orf6 protein with Cul2 occurs via another type of interaction, perhaps even in another region of the protein. We made attempts to generate Ad5/Ad16 chimeric proteins as in Fig.1 to examine this issue further but several of the products were either expressed very poorly or gave inconclusive results on Cullin binding and we did not pursue this approach further (data not shown). That Ad16 contains a different Cul2 box from Ad12 and Ad40 was not entirely unexpected due not only to the lack of two of the conserved hydrophobic residues but also due to the reduced Cul2 binding levels relative to Ad12/Ad40 (19), and the completely separate branch of the B species on the phylogenetic tree (Fig. 3A) from species A and F.

To assess the functional activity of Ad16 Cul5- and Cul2-based ligase complexes studies on the degradation of Mre11 were carried out as in Fig. 3D except that in addition to using wild-type H1299 cells, those in which expression of either Cul5 (Cul5KD) or Cul2 (Cul2KD) had been knocked down through constitutive expression of appropriate RNAi sequences were also employed. Levels of Cul2 and Cul5 in each of the knock down cell lines as well as the parental H1299 cell line are shown in Fig 3F. Effects with Ad16 were compared with those obtained with Ad5 (Cul5) and Ad12 (Cul2) E4orf6 proteins, in all cases in combination with corresponding E1B55K species. Fig. 3G shows that the E4orf6/E1B55K ligase complexes of all three serotypes degraded Mre11 in wild-type H1299 cells although as shown previously (19), the degradation of...
Mre11 by Ad16 is only partial. Degradation with Ad5 was clearly unaffected in Cul2\textsuperscript{KD} cells; however, in Cul5\textsuperscript{KD} cells it was completely inhibited, thus confirming dependence on Cul5. The converse was true with Ad12, in accord with the known dependence of its E4orf6/E1B55K ligase on Cul2. With Ad16, degradation of Mre11 was inhibited to a similar degree as Ad5 in Cul5\textsuperscript{KD} cells whereas a significant amount of degradation still occurred in Cul2\textsuperscript{KD} cells. These results suggested that even though Ad16 E4orf6 protein binds to Cul2, this complex does not appear to degrade Mre11 efficiently. It remains possible however that targets other than Mre11 might depend upon Cul2 for degradation. It should be noted that some slight variation in the levels of E1B55K were apparent in Fig. 3G; however, in multiple repeat experiments these differences were not consistent.

Creation of an Ad12-like Cul2 box in Ad5 E4orf6 promotes functional Cul2 binding

To confirm the role of the putative Ad12 Cul2 box, studies were conducted in which we recreated this motif in the corresponding region of the Ad5 E4orf6 protein that binds Cul5 efficiently but only associates with Cul2 at extremely low levels. Fig. 4A shows that the Ad5 E4orf6 sequence lacks two of the three hallmark hydrophobic residues in the Cul2 box. Thus an Ad5 E4orf6 mutant was generated to recreate a Cul2 box (FLAG-Ad5 E4orf6 E88Y/C96Y) and expressed from plasmid DNA following transfection into H1299 cells along with DNAs encoding HA-Cul2 or HA-Cul5. Co-immunoprecipitation studies similar to those done previously were performed and Fig. 4B shows that in addition to having efficient binding to Cul5, like the parental Ad5 E4orf6, the mutant E88Y/C96Y E4orf6 protein bound Cul2 to the same degree as Ad12 E4orf6 bound Cul2. These results confirmed that this Cul2 box motif in
Ad12 and Ad40 is sufficient to promote binding to Cul2. Additionally, these findings suggested
that the residues important in binding Cul2 are not the same as those required for binding Cul5.

To test the functionality of these Cul2-based ligase complexes in the Ad5 E88Y/C96Y
mutant, H1299, Cul2KD and Cul5KD cells were transfected with plasmid DNAs expressing Ad12
E4orf6 and E1B55K, or Ad5 E4orf6 (wild-type or mutant E88Y/C96Y) and E1B55K, and the
ability to degrade transfected p53 was assessed as in Fig. 3G. Fig. 4C shows that all three ligase
complexes efficiently degraded p53 in H1299 cells and that in the H1299 Cul2KD and Cul5KD
cells degradation by wild-type Ad12 or Ad5 complexes was inhibited accordingly. Importantly,
the Ad5 E4orf6 E88Y/C96Y mutant was able to promote significant p53 degradation in Cul5KD
cells, even more so than Ad12, indicating a high level of activity of the novel Cul2-based Ad5
ligase complex. Degradation of p53 by mutant E88Y/C96Y also occurred in Cul2KD cells,
although at reduced levels, suggesting that the Cul5 based complex can still be formed and is
partially active. These results showed that the newly created Cul2-based complex with mutant
E88Y/E96Y is functional and that in all likelihood both Cul2- and Cul5-based complexes are
formed even with the presence of a newly created classic Cul2 box in this E4orf6 protein (see
more in the Discussion).

Identification of Ad12 BC boxes

Our group previously identified three BC boxes in the Ad5 E4orf6 protein (3, 8) but these motifs
have not been functionally identified in other adenovirus serotypes. Ad12 Eorf6 contains three
BC box domains analogous to the three identified in Ad5, and their alignment is shown in Fig.
5A. To confirm the identity of the Ad12 BC boxes and to determine which are active, mutants
were generated with alterations in each box individually (BC1, BC2, BC3), as well as all three
together (BC1-3). Binding of Elongin C to each of the BC box mutants was assessed in Fig. 5B, where H1299 cells were transfected with plasmid DNAs encoding HSV-Elongin C and FLAG-Ad12 E4orf6 wild type or BC box mutants, and lysates were immunoprecipitated with an antibody to HSV as in Fig 2D. While mutation of BC box 1 was insufficient to prevent binding to Elongin C, the BC2, BC3, and BC1-3 mutants successfully prevented binding to Elongin C, suggesting that BC boxes 2 and 3 are the major functional binding sites. The BC boxes of Ad12 therefore exhibit properties quite similar to those of Ad5 (8).

The order in which cellular substrate acquisition proteins bind Elongins B/C and Cullin has been established, but it is unknown if Ad12 E4orf6 follows this cellular model. For cellular substrate acquisition proteins, the Elongins must first bind before Cullin can stably bind and form the complete E3 complex (27). If Ad12 E4orf6 follows the cellular model, mutation of the BC boxes in Ad12 therefore should prevent binding to Cul2. To test this possibility cells were transfected with plasmid DNAs expressing each of the FLAG-Ad12 E4orf6 BC box mutants and HA-Cul2, and Cul2 binding was assessed as above. Fig. 5C shows that only wild-type and the BC1 mutant stably associated with Cul2, while BC2, BC3, and BC1-3 each showed very low levels of Cul2 binding. Fig. 5D shows results obtained in studies to measure binding of E1B55K by the Ad12 E4orf6 BC box mutants. Results mimicked those of Cul2 binding in that BC2 and BC3 showed reduced binding to E1B55K. BC1-3 however showed complete absence of E1B55K binding.

To this point the BC box mutants of Ad12 E4orf6 mimic the previously published results of Ad5 BC box mutants (3, 8). It should follow then that the decreased Elongin B/C (and subsequently decreased Cul2 and E1B55K), should result in an inability to degrade targets such as Mre11. Fig. 5E shows that alteration of individual BC boxes (BC2, BC3) had some effect on
Mre11 degradation; however, the triple mutant BC1-3 was defective in degradation of Mre11. The Ad12 ligase (but not Ad5) has been shown recently to degrade TopBP1, a checkpoint signaling protein for stalled replication forks and DNA damage (30, 31) and its degradation was also studied in a similar fashion. TopBP1 degradation was more sensitive to BC box mutations as BC2, BC3, and BC1-3 were all defective for degradation. While the basis for this substrate-related difference is unknown, it should be remembered that TopBP1 (20) can be degraded by the Ad12 E4orf6 protein alone whereas Mre11 (14) requires both E4orf6 and E1B55K. One explanation could be that the placement of the target lysine to be ubiquitinated on the target protein is more restrictive when only E4orf6 is involved whereas with substrates relying on E1B55K for binding to the ligase complex more flexibility in orientation of the target may be permissible. Nevertheless the results shown in Fig. 5 confirmed the general important role of BC boxes 2 and 3 in adenovirus E4orf6 proteins and the inability of E1B55K to form stable complexes with E4orf6 protein in the absence of ligase complex formation involving Elongins B and C.

Most targets of the adenoviral E4orf6/E1B55K complex are only degraded by a subset of the species. All targets identified so far are degraded by at least two adenovirus species, and only one target, DNA Ligase IV, is degraded by all serotypes examined thus far (19). TopBP1, on the other hand, was found only to be degraded upon infection with Ad12 (species A), in a study conducted with species A-E (32). Lacking in this analysis however was species F (represented by Ad40 and also a Cul2 binder). According to the phylogenetic sequence analysis, species F evolved closely with species A (Fig. 3A), and therefore it was of interest to determine if Ad40 also degrades TopBP1. To determine the pattern of TopBP1 degradation H1299 cells were transfected with plasmid DNAs encoding E4orf6 and E1B55K representative of all species...
and the levels of endogenous TopBP1 were assessed by western blotting as before but using antibodies against TopBP1. Fig. 5F shows that species B-E as well as F (Ad40) were unable to degrade TopBP1, and that only expression of Ad12 E4orf6 (species A) was sufficient to promote degradation. Thus only species A appears able to degrade TopBP1, which thus far represents the only known single species-specific substrate.

Discussion

Adenoviral E4orf6 proteins associate with either Cul2 or Cul5 to form E3 ubiquitin ligase complexes which target cellular proteins for proteasome mediated degradation. Considerable heterogeneity exists in the pattern of substrates degraded by different human adenovirus species, although this diversity seems due not to Cullin specificity but rather largely to differences in the E1B55K protein, the major substrate acquisition component of the ligase complex (3, 10). Nevertheless details of the biochemistry of complex formation will be informative in many ways, including providing insights into the evolution of human and other adenoviruses.

Our studies indicated that Ad12 and Ad40 E4orf6 products bind Cul2 via a cellular-like consensus Cul2 box domain: Ad12 78YEMFILKKY86; Ad40 79WEKHVCKIY87. We had previously identified three functional BC boxes in Ad5 E4orf6 (3, 8) and in the present study a similar arrangement was demonstrated in the Ad12 E4orf6 protein. In both cases BC boxes 2 and 3 appeared to predominate functionally, and as was the case with Ad5, complex formation with Elongins B/C was necessary for stable interactions with E1B55K. These results suggested that a general feature of human adenoviruses is that binding of E1B55K to E4orf6 protein is highly dependent on an association with Elongins B and C (see more below). Together, this makes the
E4orf6 product the first viral protein for which the complete VHL box (BC box + Cul2 box) has been identified. Only two other viral proteins thus far have been found to form active E3 ligase complexes with Cul2: HPV16 E7 (5) and EBV BZLF1 (4); however, the Cul2 box has only been identified in BZLF1 (Table 1).

Phylogenetic analysis of the E4orf6 sequences suggests that species A, F, and G were more closely related to each other than the rest. Thus, perhaps not surprisingly, both Ad12 (species A) and Ad40 (species F) bind Cul2, utilize similar Cul2 boxes, degrade similar targets, and infect similar tissues. It was thus surprising that these two serotypes diverged in their ability to degrade TOPBP1, so far the only ligase-related difference observed between them. Very few studies have been done on Ad52, the only human species G serotype; however, we noted that it does contain a putative Cul2 box similar to those of Ad12 and Ad40, 76WEEKVVMKY84, strongly suggesting that it also forms a Cul2 based complex. The hallmark of these Cul2 boxes is three hydrophobic residues spaced four amino acids apart, whereas all Cul5 binding adenovirus species contain only the central hydrophobic residue (see alignment in Fig. 2A), thus explaining why they are unable to bind Cul2.

While the BC and Cul2 boxes used by human adenoviruses resemble the cellular consensus sequences, the order of domains is different. In human proteins, all BC box domains are on average within 10-25 amino acids upstream of the Cul2 box, but in Ad12 and Ad40 E4orf6, the active BC boxes 2 and 3 are downstream of the Cul2 box. Another unique aspect of E4orf6 proteins is the presence of multiple BC boxes, as all known cellular proteins contain only one BC box. It is possible that binding of E4orf6 protein to Elongins B/C via multiple BC boxes might allow for different conformations, and therefore potentially different substrate degradation profiles. Our group previously examined this possibility with respect to p53 and Mre11.
degradation, and found that both Ad5 BC boxes 2 and 3 could direct degradation of both of these
two targets (3, 8), although other substrates might be BC box specific. It is also possible that the
multiple adjacent BC boxes help to enhance reformation of the complex after its transient
dissociation from the Elongins.

Ad16, and presumably other B1 species, encode the only E4orf6 products that bind high
levels of both Cul5 and Cul2; however, only the Cul5 Ad16 complex was able to degrade Mre11.
These viral E4orf6 proteins do not contain a consensus Cul2 box and thus the interaction with
Cul2 appears both exceptional and non-functional.

It is of interest to note that the nuclear export signal (NES) from residues 76-84 in Ad12
E4orf6 overlaps with the Cul2 box. Binding of the large 80kDa Cul2 protein at this site likely
prevents NES-dependent nuclear export of E4orf6. Other binding partners associated with
E4orf6 which contain an NES however could still alter the localization of the complex. An NES
within E1B55K had previously been identified in Ad5 (species C) from amino acids 83-93 (33).
Interestingly, examination of E1B55K coding sequences from the other species reveals that this
NES is only present in Cul5 complex forming species (B, C, D, and E), but not in Ad12 (A), or
Ad40 (F). This finding suggests that this ligase complex may function largely within the nucleus.

Based upon the binding data of the Ad12 E4orf6 mutants we suggest that a cooperatively
of components exists in assembling the E3 ubiquitin ligase complex. As mutation in the BC box
disrupted binding of both Elongin B/C and E1B55K, it is likely that binding of E4orf6 to Elongin
B/C is required to promote stable association to E1B55K. The Cul2 box mutants were defective
in Cul2 binding but also exhibited some decrease in Elongin B/C binding, though presumably
enough occurred to permit E1B55K binding. It is possible that binding of Elongin B/C in this
Cul2-based ligase complex induces a conformational change in E4orf6 to permit the interaction with E1B55K, as we have also proposed with the Cul5-based complex formed by Ad5 (8).

We generated an extensive collection of Ad5 E4orf6 mutants to map the Cul5 binding site; however, in no case did we identify any meaningful single mutation or combination of mutations that eliminated Cul5 binding (data not shown). Mapping studies using Ad5/Ad12 chimeric E4orf6 proteins identified the second N-terminal quarter of the protein as being involved, the same region implicated in Cul2 binding with the Ad12 E4orf6 product. Thus it is likely that overlapping regions of these proteins play a role in the binding of these two Cullins.

The fact that it was possible to generate a functional Ad12-like Cul2 box in this region of Ad5 E4orf6 confirmed the function of this motif in binding Cul2; however, this mutant nevertheless was still able to bind Cul5. We think it unlikely that both Cullins bind simultaneously and favor the model wherein two populations exist, one associated with Cul2 and the other Cul5. We prefer this possibility because expression of Ad5 E4orf6 E88Y/C96Y in CulKD cells induced both Cul2- and Cul5-dependent protein degradation. Given the difficulties in identifying the Cul5 box it seems likely that the interaction of Cul5 with E4orf6 proteins must be complex and involve multiple contacts that are difficult to identify by a limited site-directed mutagenesis approach. Perhaps it will only be possible to determine the nature of this interaction by obtaining high resolution crystal structures of these complexes.

Finally, identification of the Cul2 box sequence and other features related to formation of ligase complexes could provide additional new biochemical E4orf6 properties with which to analyze the evolution of all E4orf6-containing Adenoviridae. Most insights into adenovirus evolution have been based on partial or full genomic sequence variations and, in human
adenoviruses, biological properties such as serum neutralization or cell transformation (34–36).

The identification of the Cul2 box sequence should provide a means to compare a highly specific biochemical function, Cul2 binding by E4orf6, with broader patterns of viral evolution. For example, the human adenoviruses that bind Cul2 (species A and F) largely target the gastrointestinal tract whereas the other species utilize Cul5 and infect the upper respiratory tract and other tissues. It has been found in rabbit and rat that the levels of Cul5-specific mRNAs are higher in lung tissue than in the gut (37, 38), suggesting possibly that Cullin specificity could represent a selective pressure in promoting tissue specificity of the virus. Thus an extensive analysis of all known E4orf6-like sequences might lead to important new insights into adenovirus evolution.

Acknowledgements

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### Table 1 Viral Cul2 and Cul5 boxes*

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### Cellular Cullin 2 box: ΦxxΦxxxΦ

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*The canonical cellular consensus Cul2 and Cul5 box domains are listed where x can be any amino acid, and Φ represents a hydrophobic residue. All viral target acquisition proteins shown to bind Cul2 or Cul5 are listed along with the degradation target and the Cullin box (within the viral protein) if known. Virus abbreviations used: Epstein Barr Virus (EBV), chicken embryo lethal orphan avian adenovirus (CELO, or fowl adenovirus 1, FAdV1), human papilloma virus 16 (HPV16), human immunodeficiency virus (HIV), Kaposi sarcoma-associated herpes virus (KSHV), murine herpes virus 68 (MuHV68).
Figure Legends

Fig. 1 Localization of E4orf6 Cul2 and Cul5 boxes using Ad5/Ad12 chimeric E4orf6 proteins.

Chimeric Ad5 and Ad12 E4orf6 proteins were constructed and tested for Cul2 and Cul5 binding as described in Materials and Methods. A. Chimeras consisting of the N- and C-terminal half of E4orf6. N-terminally FLAG-tagged chimeric proteins were made between E4orf6 from Ad5 and Ad12. Amino acids numbers of Ad5 or Ad12 E4orf6 products are listed to the right using position numbers relative to wild-type E4orf6.

B. Binding of chimeric proteins to Cul5 and Cul2. H1299 cells were transfected with plasmid DNAs expressing wild-type or chimeric FLAG-E4orf6 proteins and HA-Cul2 or HA-Cul5, and immunoprecipitates obtained using anti-FLAG antibodies and whole cell extracts (Load) were immunoblotted using anti-HA or anti-FLAG antibodies. C. Illustration of chimeras consisting of the N-terminal quarters and C-terminal half of E4orf6. D. Binding of chimeric proteins to Cul5 and Cul2. Immunoprecipitations and western blotting analyses were performed as in B. * indicates non-specific background bands.
Fig. 2  Identification of the Ad12 E4orf6 Cul2 box.  A. Alignment of the second N-terminal quarter of representative E4orf6 protein sequences from each of the seven species, grouped according to Cul5 binders (top), and Cul2 binders (bottom). Areas of protein similarity are highlighted in light gray. In dark gray are areas unique to either Cul2 or Cul5 binders.  B and C. Cul2 or Cul5 binding to Ad12 E4orf6 Cul2 box mutant proteins. Cul2 binding to wild-type or mutant FLAG-E4orf6 was assessed as in Figs. 1B and 1D.  D. Binding of Ad12 E1B55K to E4orf6 Cul2 box mutant proteins. H1299 cells were transfected with plasmid DNAs expressing Ad12 HA-E1B55K and FLAG-E4orf6 and immunoprecipitates obtained using anti-HA antibodies or whole cell extracts (Load) were immunoblotted using the indicated antibodies.  E. Association of Elongin C with E4orf6 Cul2 box mutant proteins. H1299 cells were transfected with plasmid DNAs expressing HSV-Elongin C and Ad12 FLAG-E4orf6 and immunoprecipitates obtained using anti-HSV antibodies and whole cell extracts (Load) were immunoblotted using anti-FLAG or anti-HSV antibodies.  F. Ribbon diagram of predicted structure of Ad12 E4orf6 generated by the Itasser program (see Materials and methods). Residues 74, 78, 82, and 86 are drawn in stick format.  G. Degradation of Mre11 by Ad12 E1B55K and Cul2 box mutant E4orf6 proteins. H1299 cells were transfected with plasmid DNAs encoding Ad12 FLAG-E4orf6 and HA-E1B55K as indicated, and whole cell extracts were immunoblotted for Mre11, Actin, FLAG and HA using appropriate antibodies.  * indicates non-specific background bands.

Fig. 3  Identification of the Cul2 box in Ad40 E4orf6 and Activity of Cul2- and Cul5-based Ad16 E4orf6/E1B55k complexes. A. Phylogenetic tree (using Neighbor Joining method) of all human
sequenced E4orf6 proteins. Bootstrap confidence values from 100 replicats are listed at each main branch. Mouse adenovirus 1 was used as an outgroup. **B.** Alignment of Cul2 box from Ad12 to homologous regions of Ad16 and Ad40 E4orf6 proteins. **C.** Binding of Cul2 to Ad40 E4orf6 Cul2 box mutant proteins. H1299 cells were transfected with plasmid DNAs expressing wild-type or Cul2 box mutant Ad40 FLAG-E4orf6 and HA-Cul2 and immunoprecipitates obtained using anti-FLAG antibodies and whole cell extracts (Load) were immunoblotted using anti-HA or anti-FLAG antibodies. **D.** Degradation of p53 and Mre11 by Ad40 E1B55K and E4orf6 Cul2 box mutants. H1299 cells were transfected with plasmid DNAs encoding human p53 or Mre11, Ad40 FLAG-E4orf6 and HA-E1B55K and whole cell extracts were immunoblotted for p53, Mre11, Actin, FLAG and HA using appropriate antibodies. **E.** Binding of Cul2 to Ad16 E4orf6 putative Cul2 box mutant proteins. H1299 cells were transfected with plasmid DNAs expressing wild-type or Cul2 box mutant Ad16 FLAG-E4orf6 and HA-Cul2 and immunoprecipitates obtained using anti-FLAG antibodies and whole cell extracts (Load) were immunoblotted using anti-HA or anti-FLAG antibodies. **F.** Cullin levels in Cul2KD and Cul5KD cell lines. Lysates from the parental H1299 and Cul2KD and Cul5KD cell lines were examined by western blotting with anti-Cul5, anti-Cul2, and anti-actin antibodies. **G.** Activity of Cul2- and Cul5-based Ad16 E4orf6/E1B55K complexes. H1299, Cul5KD, and Cul2KD cells were transfected with plasmid DNAs encoding human Mre11, and FLAG-E4orf6 and HA-E1B55K from Ad5, Ad12, or Ad16 and whole cell extracts were immunoblotted for Mre11, Actin, FLAG and HA using appropriate antibodies.

**Fig. 4** Insertion of the Ad12 E4orf6 Cul2 box sequence into Ad5 E4orf6. **A.** Alignment of Cul2 box region of Ad12, Ad5, and Ad5 E88Y/C96Y E4orf6. **B.** Association of Cul2 and Cul5 with
E4orf6 proteins. H1299 cells were transfected with plasmid DNAs expressing Ad12, Ad5, or Ad5 E88Y/C96Y FLAG-E4orf6 proteins and HA-Cul2 or HA-Cul5 and immunoprecipitates obtained using anti-FLAG antibodies or whole cell extracts (Load) were immunoblotted using anti-HA or anti-FLAG antibodies. C. Activity of Cul2 and Cul5 based Ad12, Ad5 or Ad5 E88Y/C96Y E4orf6/E1B55K complexes. H1299, Cul5KD, and Cul2KD cells were transfected with plasmid DNAs encoding human p53, and FLAG-E4orf6/HA-E1B55K from Ad5 or Ad12, as indicated, and whole cell extracts were immunoblotted for p53, Actin, FLAG and HA using appropriate antibodies.

Fig. 5 Identification of BC boxes in Ad12 E4orf6. A. Alignment of BC boxes from Ad5 E4orf6 with homologous regions within Ad12 E4orf6. B. Association of Elongin C with Ad12 E4orf6 BC box mutant proteins. H1299 cells were transfected with plasmid DNAs expressing HSV-Elongin C and Ad12 FLAG-E4orf6 and immunoprecipitates obtained using anti-HSV antibodies and whole cell extracts (Load) were immunoblotted using anti-FLAG or anti-HSV antibodies. C. Association of Cul2 with Ad12 E4orf6 BC box mutant proteins. H1299 cells were transfected with plasmid DNAs expressing wild-type or mutant FLAG-E4orf6 and HA-Cul2 and immunoprecipitates obtained using anti-FLAG (E4orf6) antibodies and whole cell extracts (Load) were immunoblotted using anti-HA or anti-FLAG (Cul2) antibodies antibodies. D. Binding of Ad12 E1B55K to E4orf6 BC box mutant proteins. H1299 cells were transfected with plasmid DNAs expressing Ad12 HA-E1B55K and FLAG-E4orf6 and immunoprecipitates obtained using anti-HA antibodies and whole cell extracts (Load) were immunoblotted using anti-FLAG or anti-HA antibodies. E. Degradation of TopBP1 and Mre11 by Ad12 E1B55K and E4orf6 BC box mutants. H1299 cells were transfected with plasmid DNAs encoding Mre11,
Ad12 FLAG-E4orf6 and HA-E1B55K, as indicated, and whole cell extracts were immunoblotted for endogenous TopBP1, Mre11, Actin, FLAG and HA using appropriate antibodies. F. Serotype specificity of TopBP1 degradation. H1299 cells were transfected with plasmid DNAs encoding FLAG-E4orf6 and HA-E1B55K from representative adenoviruses of species and whole cell extracts were immunoblotted as in E.