Adeno-associated viruses (AAVs) are members of the genus Dependovirus within the family Parvoviridae (Berns & Giraud, 1996; Bowles et al., 2006). AAV can only achieve efficient productive replication in the presence of a larger helper virus (Bowles et al., 2006).

We have previously shown that the adenovirus (Ad)-helper functions required for AAV replication provide a surprising combination of positively and negatively acting effects, and the Ad E4orf6/E1b 55 kDa E3 ubiquitin ligase (Ad E3 Ub-ligase) can target both AAV Rep52/40 and AAV capsid proteins for ubiquitination and subsequent degradation (Nayak et al., 2008; Nayak & Pintel, 2007). The role during infection for this activity is not yet fully understood. Steady-state levels of AAV protein are not seen to be reduced during co-infection with Ad, or when supported by the full panoply of Ad-helper functions, because enhancement of AAV mRNA translation by Ad virus-associated (VA) RNA restores AAV protein to levels required for efficient infection (Nayak & Pintel, 2007). Because the AAV large Rep protein is the major viral protein required for genome replication, and because it shares approximately 2/3 of its amino acid sequence with the targeted smaller Rep proteins, we sought to determine if the large Rep protein was also a target of the Ad E4orf6/E1b 55 kDa E3 Ub-ligase. It has previously been shown that when expressed alone in cells, large Rep is a stable protein exhibiting a long half-life (Weger et al., 2004), similar to AAV/Ad co-infection. Therefore, as seen previously during our analysis of Ad effects on AAV Rep 52/40 and Cap (Nayak et al., 2008), we expected that Ad E3 Ub-ligase-targeting of Rep for degradation would be only clearly apparent in the absence of VA RNA.

As can be seen in Fig. 1(a), accumulated levels of the large Rep protein expressed in Ad E1a- and E1b-expressing 293 cells are significantly reduced in the presence of Ad E4orf6 (compare lanes 1 and 2). Additionally, large Rep levels can be recovered in the presence of the proteasome inhibitor MG132 (Fig. 1a, lane 3), suggesting that the E4orf6-dependent degradation of large Rep is proteasome mediated. In these experiments, 293 cells were transfected with a human immunodeficiency virus (HIV) LTR-driven large Rep-expressing plasmid, either with or without HA-tagged E4orf6. Thirty-six hours later samples were prepared for immunoblotting as described previously (Farris et al., 2010), using anti-Rep78/68 monoclonal 7B73.2 (Hunter & Samulski, 1992) to detect large Rep, and anti-HA antibody (cat# H9658; Sigma) to detect E4orf6. MG132 (10 μM, cat# 474791; Calbiochem) was added to the samples during the last 6 h as indicated. Ad E4orf6 mutants described previously (Nayak & Pintel, 2007), which are deficient in Ad E3 Ub-ligase formation and hence incapable of causing targeted ubiquitination were unable to cause a reduction in accumulated Rep levels (data not shown). Taken together, these results suggest that in addition to a select group of previously reported cellular and viral proteins (Blanchette & Branton, 2009), the Ad E4orf6/E1b 55 kDa E3 Ub-ligase also targets AAV large Rep for proteasomal degradation.

If Rep is a target of the Ad E3 Ub-ligase, we should be able to demonstrate ubiquitination of Rep during AAV/Ad co-infection. As can be seen in Fig. 1(b), during AAV2/Ad5 co-infection, immunoblots of large Rep immunoprecipitated with the anti-Rep78/68 monoclonal 7B73.2 (Hunter & Samulski, 1992) and probed with an anti-ubiquitin antibody (cat # SC-8017; Santa Cruz Biotechnology) revealed high molecular mass ubiquitin conjugates of Rep both in the presence and absence of MG132 (Fig. 1b, lanes 4 and 5). Extracts from mock-infected cells, or cells infected with either AAV or Ad alone showed no specific ubiquitination reactivity (Fig. 1b, lanes 1–3). These experiments were done under stringent washing conditions that we have previously shown fully dissociate cellular proteins, including any potentially Rep-associated, ubiquitinated proteins from our immunoprecipitates (Farris et al., 2010).
To further characterize the ubiquitination of large Rep, we developed a ubiquitination assay utilizing transiently expressed HIV LTR-driven Rep and Flag-tagged ubiquitin. Ubiquitination of both AAV5 and AAV2 large Rep was assayed in 293T cells both in the presence and absence of added E4orf6. In these assays, large Rep was immunoprecipitated using the 7B73.2 antibody, and equivalent amounts of sample were immunoblotted with anti-Flag antibody to detect specific ubiquitination, as described previously (Farris et al., 2010). As expected, both AAV2 (left panel) and AAV5 (right panel) large Reps were ubiquitinated in the presence of E4orf6, which in 293 cells would reconstitute the Ad E3-Ub ligase (Fig. 1c, left panel lane 4 and right panel lane 3), but surprisingly, significant ubiquitination of Rep was also detected in the absence of E4orf6 (Fig. 1c, left panel lane 3 and right panel lane 2). The addition of MG132 had little effect in these experiments (Fig. 1c, left and right panel, lane 5), probably because the immunoprecipitations were subquantitative (data not shown).

There are multiple types of polyubiquitination. Polyubiquitination that extends through lysine 48 of ubiquitin is most often associated with proteasomal degradation (Ciechanover, 1998; Pickart, 1997). The other major form of ubiquitination, extending ubiquitin lysine 63, is most often associated with other modifications of protein function (Komander, 2009). We have previously reported that AAV5 Rep52 is ubiquitinated using both K48 and K63 polyubiquitination (Farris et al., 2010). One possibility that might explain both E4orf6-dependent and E4orf6-independent ubiquitination of large Rep would be that different forms of ubiquitin were added under these different conditions. To further characterize the ubiquitination of large Rep, we used transient expression assays, similar to those described above, and as described previously (Farris et al., 2010), in which mutant forms of HA-tagged ubiquitin that allowed extension only via K48, or alternatively only via K63 (Conze et al., 2008; Lim et al., 2005, 2006; Olzmann et al., 2007), were supplied in trans.

Fig. 1(d) demonstrates that large Rep undergoes both the K48-extension (left panel) and K63-extension (right panel) form of polyubiquitination. However, surprisingly, both types of ubiquitination were found both in the presence of Ad E4orf6 (Fig. 1d, left and right panel, lanes 4 and 5) and in its absence (Fig. 1d, left and right panels, lane 3), suggesting that the targeting of large Rep for both K48 and K63 ubiquitination can be accomplished by factors other than the Ad E3 Ub-ligase. Ubiquitination patterns were not detected in cell extracts from transfections lacking either Rep or HA-ubiquitin (Fig. 1d, left and right panels, lanes 1 and 2), or in which an HA-tagged ubiquitin that allowed extension only via K48, or alternatively only via K63 (Conze et al., 2008; Lim et al., 2005, 2006; Olzmann et al., 2007), were supplied in trans.

Although we have demonstrated that large Rep can be ubiquitinated via K48 and K63 extension both in the presence and absence of Ad E4orf6, ubiquitination by the Ad E3 Ub-ligase must be an important feature of Ad/AAV.
interaction because, as shown in Fig. 1(a), ubiquitin-tar
degradation of large Rep is dependent on the E4orf6/E1b 55 kDa Ad E3 Ub-ligase. To demonstrate this
involvement more directly, we examined the E4orf6-
dependent ubiquitination and targeted degradation of Rep in the presence of the dominant-negative ubiquitin, UBR7 (Sheafl et al., 2000), as described previously (Nayak et al., 2008). As can be seen in Fig. 2, both the E4orf6-
dependent ubiquitination (Fig. 2a, compare lanes 3 and 4
to lane 5), and the targeting of large Rep for degradation
(Fig. 2b, compare lane 2 to lane 3), was suppressed by the
addition of a dominant-negative ubiquitin, implicating the Ad E3-Ub-ligase in this process. For experiments shown in
Fig. 2(a), 48 h post-transfection of 293T cells HIV LTR-
driven Rep were immunoprecipitated with antibody
7B73.2, and immunoblotted with either anti-Flag (Fig.
2a, top panel) or anti-7B73.2 Rep (Fig. 2a, bottom panel)
to detect Flag-tagged ubiquitin or large Rep, respectively.
For experiments shown in Fig. 2(b), 293 cells were
transfected with HIV LTR-driven Rep either alone, or
together with E4orf6 or E4orf6 plus UBR7, as indicated.
Expression of HA-tagged E4orf6 was confirmed with an
anti-HA antibody (Fig. 2b, bottom panel). In Fig. 2(a),
substantial levels of Rep remain even in the presence of
E4orf6 (lane 3 bottom panel) because these immunopre-
cipitations are subquantitative (data not shown). We have
previously shown that the Ad E3Ub-ligase targeted loss of
AAV Rep52 evident by Western blotting was not apparent
following subquantitative immunoprecipitation of Rep
(Farris et al., 2010).

Consistent with the involvement of the Ad E3-Ub-ligase in these processes, siRNA-mediated knockdown [performed exactly as described previously (Farris et al., 2010)] of cullin
5, the cullin present in the Ad E3-Ub-ligase, dramatically
reduced E4orf6-dependent (and to a much lesser extent
E4orf6-independent) K48 ubiquitination of large Rep (Fig.
3a, compare lanes 9 and 10 to 5 and 6). For these experiments 293T cells were first transfected with either siRNAs to cullin 5 (cat# GS8065; Qiagen) or scrambled siRNAs serving as negative controls. Twenty-four hours following siRNA delivery constructs expressing HIV LTR-
driven large Rep, HA-tagged K48 ubiquitin and E4orf6
were transiently transfected. MG132 or DMSO vehicle
controls were added in the last 6 h of the experiment.
Thirty-six hours later cell lysates were immunoprecipitated
with anti-7B73.2 large Rep antibody followed by immuno-
blotting with an anti-HA antibody to detect specific K48
ubiquitination of large Rep. Specific knockdown of cullin
5 was assessed by Western blotting of equivalent amounts
of pre-immunoprecipitation samples.

Additionally, in the presence of control siRNA, the
activated form of cullin 5 [apparent as the higher migrating
species of cullin 5 (Chew & Hagen, 2007)] was seen only in
the presence of E4orf6 (Fig. 3a, compare lanes 1, 2, 5 and 6
with lanes 3 and 4). This is consistent with cullin 5 participating in the Ad E3 Ub-ligase-targeted ubiquitina-
tion of Rep, but not in the ubiquitination of Rep in the
absence of this ligase described above. Similar to that observed in Fig. 2(a), significant levels of Rep remain in the
presence of E4orf6 (Fig. 3a, lanes 2, 5 and 6), probably
because these immunoprecipitations are performed under
sub-saturating antibody conditions and thus do not reveal
quantitative differences (Farris et al., 2010).

We next sought to begin to identify components that could
comprise the complex(es) that direct the ubiquitination of
large Rep. As can be seen in Fig. 3(b), immunoprecipita-
tion with an anti-E1b 55 kDa antibody (gift of A. J. Berk,
UCLA), followed by immunoblotting using the 7B73.2
anti-large Rep antibody demonstrated that in 293 cells
large Rep and E1b 55 kDa form a stable complex both in
the presence and absence of E4orf6. Extracts were pre-
pared under non-denaturing conditions and processed
as described previously (Farris et al., 2010) with one
modification: interfering IgG chains were stripped from
the immunoprecipitation elution using the ExactaCruz
reagent (cat# SC-45042; Santa Cruz Biotechnology) in
order to visualize the E1b 55 kDa protein. This interaction
has been reported previously by others as well as ourselves
(Nash et al., 2009; Nayak et al., 2008). These results suggest
a role for this interaction other than as part of the Ad E3
Ub-ligase and further suggest that within the Ad E3 Ub-
ligase complex the E1b 55 kDa protein may interact

Fig. 2. E4orf6-dependent ubiquitination and degradation of AAV
large Rep is suppressed by the addition of a dominant-negative ubiquitin. (a) Immunoblots of large Rep immunoprecipitated with
7B73.2 (top panel) or anti-Rep (7B73.2) (bottom panel) in the presence or absence of the dominant-negative UBR7 or MG132 as indicated, and as
described in the text. (b) Immunoblot of samples taken from 293
cells transfected with HIV LTR-driven large Rep alone (lane 1), or
with co-expression of E4orf6 (lane 2) or E4orf6 together with
UBR7 (lane 3). Large Rep was detected with 7B73.2 and E4orf6
expression was detected by HA (lower panel). Cellular tubulin was
used as a loading control.
AAV Rep is polyubiquitinated and proteasomal degradation of AAV large Rep proteins may represent important events in the AAV life cycle.

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**References**


