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Short Article

siRNA Screen of Early Poxvirus Genes Identifies the AAA+ ATPase D5 as the Virus Genome-Uncoating Factor

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SUMMARY

Poxvirus genome uncoating is a two-step process. First, cytoplasmic viral cores are activated and early viral genes are expressed. Next, cores are disassembled and the genomes released. This second step depends on an early viral factor(s) that has eluded identification for over 40 years. We used a large-scale, high-throughput RNAi screen directed against vaccinia virus (VACV) to identify the VACV AAA+ ATPase D5 as the poxvirus uncoating factor. We show that the ATPase activity of D5 is required for uncoating. Superresolution microscopy suggests that D5 acts directly at viral cores for genome release. Thus, the putative helicase D5 is a multifunctional protein required for genome uncoating and replication. Additionally, in vivo delivery of anti-D5 siRNAs reduced virus production in a mouse model of VACV infection. These results demonstrate the use of virus-targeting RNAi libraries to investigate viral gene function and suggest therapeutic avenues.

INTRODUCTION

In virus particles, the genome is often packaged inside a stable, highly structured, proteinaceous core or capsid. It allows the viral DNA or RNA to be highly condensed and provides protection during the extracellular phase of the replication cycle. The structural design of a viral core must also include a built-in capacity for disassembly, because after entry into a new host cell, the genome is rapidly liberated in a process called uncoating. This must occur at the right time and place inside the host cell. The mechanisms of uncoating remain poorly understood for most animal virus capsids, yet it is evident that all viruses must undergo critical changes during entry that render them uncoating competent. The changes are often triggered by alterations in the milieu around the virus or by interactions with receptors or other host factors. They involve proteolytic cleavages, reduction or rearrangement of disulfide bonds, and changes in protein conformation (Suomalainen and Greber, 2013; Greber et al., 1994).

In this study, we focused on the genome uncoating of vaccinia virus (VACV), a poxvirus closely related to the causative agent of smallpox. The poxvirus replication cycle is exclusively cytoplasmic and begins with the delivery of a stable core, containing the viral DNA genome, into the host cytosol. Upon delivery, cores undergo activation and early genes are transcribed within the intact viral core; mRNAs are exported and translated in the cytosol, giving rise to a defined set of early proteins involved in intermediate gene transcription, DNA replication, host interaction, and immune modulation. Subsequent intermediate and late gene expression occurs from virus genomes only after DNA release and replication (Moss, 2007).

In classical poxvirus studies, uncoating was defined as the step that rendered viral DNA sensitive to DNase digestion in cell lysates (Joklik, 1964a). It was observed over 40 years ago that expression of one or more early viral proteins was required for this step (Joklik, 1964b; Prescott et al., 1971). The early viral protein(s) involved in uncoating has, however, not been identified to date. We recently demonstrated that VACV genome uncoating is a multistep process in which host cell proteasome activity is necessary but not sufficient (Mercer et al., 2012). Yet, the mechanism of uncoating remains elusive, and a detailed understanding requires the identification of the poxvirus uncoating factor(s).

Here, we addressed the problem by silencing VACV early genes using a high-throughput RNAi screen. Genome-wide RNAi screens have been used in recent years to address the role of host genes during virus infection (Kilcher and Mercer, 2014). By applying the same approach to viral genes, we could unambiguously identify one early protein as the viral uncoating factor. Consistent with its critical role in the VACV life cycle, siRNA-mediated depletion of the uncoating factor in infected mice reduced virus replication.

RESULTS

RNAi Screen Identifies 15 Uncoating Factor Candidates

To identify the VACV uncoating factor(s), we performed an automated high-throughput infection screen in HeLa cells with siRNAs targeting early VACV mRNAs. The custom-made library targeted 80 conserved viral proteins using three individual

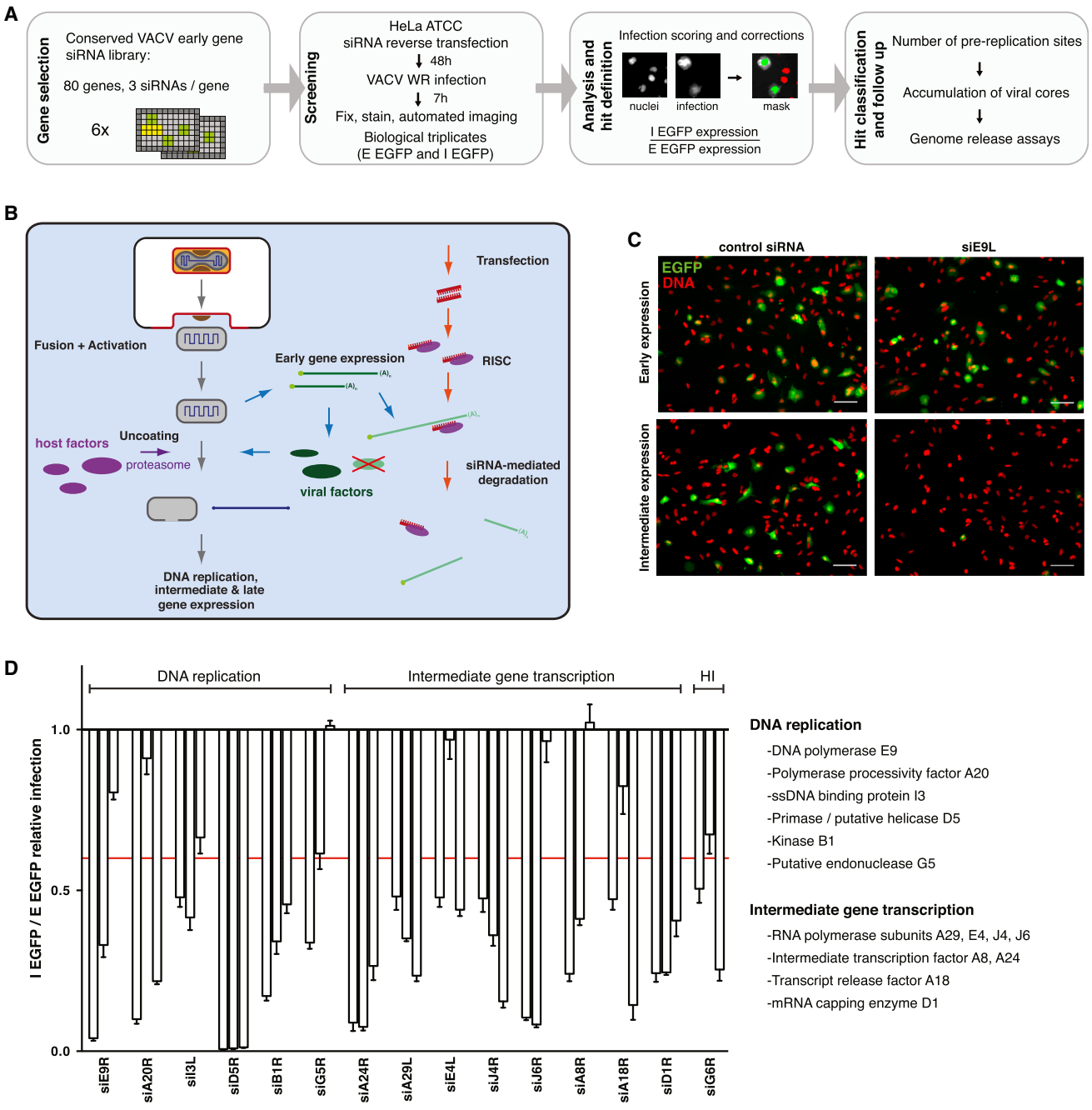


Figure 1. Antiviral siRNA Screen Reveals 15 Uncoating-Factor Candidates

(A–D) An siRNA screen targeting early mRNAs of VACV was used to identify viral proteins required for uncoating, DNA replication, or intermediate gene expression. Transfected HeLa cells were infected with VACV strains expressing EGFP from early or intermediate viral promoters (E EGFP and I EGFP, respectively), and the fraction of infected cells was quantified. Workflow and screening strategy are shown (A and B) along with exemplary images of the phenotypes after depletion of the viral DNA polymerase E9 (C). The I EGFP/E EGFP relative infection ratio of the final 15 candidate genes is shown in (D) with three siRNAs/gene (grouped bars). The threshold at a ratio of 0.6 is indicated (red line). Scale bars represent 100 μm. Data are mean ±SEM (n = 3). HI indicates host interaction; RISC indicates RNA-induced silencing complex. See also Figure S1 and Tables S1 and S2.

siRNAs per gene (see Table S1 available online). The screening strategy and workflow is outlined in Figures 1A and 1B (quality control is shown in Figure S1).

As VACV intermediate, but not early, gene expression depends on DNA release and replication, we reasoned that depletion of the poxvirus genome uncoating factor should impair intermediate, but not early, gene expression. The library was screened in parallel using recombinant reporter viruses expressing enhanced green fluorescent protein (EGFP) under the control of the early J2R promoter (VACV E EGFP; Stiefel et al., 2012) or

the intermediate G8R promoter (VACV I EGFP; all viruses used in this study are compiled in Table S3). The fraction of cells undergoing viral gene expression upon siRNA-mediated depletion of candidate proteins was quantified by automated fluorescence microscopy and compared to control siRNA (relative infection). Figure 1C exemplifies the expected phenotype in cells transfected with an siRNA against the viral DNA polymerase E9 (siE9L): EGFP expression from the intermediate, but not the early, gene promoter was abrogated.

As a readout, the ratios of relative I EGFP/E EGFP infection were determined. This allowed us to identify viral genes involved in the VACV life cycle between early and intermediate gene expression (i.e., for genome uncoating, DNA replication, or intermediate gene transcription). The I/E EGFP ratio excluded siRNAs that affected both E and I EGFP expression in an unspecific manner (off target). Fifteen candidates were identified with an I/E EGFP ratio below 0.6 for 2–3/3 siRNAs (Figure 1D). Most are required for DNA replication (six genes) or intermediate gene transcription (eight genes), including the DNA polymerase E9 and several RNA polymerase subunits. For full screening results, see Figure S1 and Table S2.

Four Candidate Genes Are Required for the Assembly of Prereplication Sites

We did not identify any factor lacking an assigned function and suspected that one of the 15 candidates may have an additional role in uncoating. Thus, we aimed at eliminating candidates that were only required after uncoating (i.e., for DNA replication and intermediate gene expression). We quantified "prereplication sites"—distinct cytoplasmic spots in which the DNA replication machinery assembles on released incoming viral genomes (Domi and Beaud, 2000; Rochester and Traktman, 1998; Tseng et al., 1999; Welsch et al., 2003). These sites form when new DNA synthesis is inhibited by cytosine arabinoside (AraC). As they can be visualized microscopically by staining for the viral ssDNA-binding protein I3, they have been used as an indirect measure for genome release (Mercer et al., 2012).

The number of prereplication sites per infected cell was quantified after candidate gene knockdown using one potent nontoxic siRNA per gene (see Table S1 for selected siRNAs); nontargeting siRNA served as control (ASN). On average, ten I3-positive prereplication sites per cell were detected under the experimental conditions (Figures 2A and 2B). I3 foci were virtually absent when viral protein expression was inhibited (ASN + cycloheximide [CHX]) or when I3 was depleted by siRNA (Figures 2B and S2A). Of the 15 candidates, four showed more than 40% reduction in the number of prereplication sites per cell and were further pursued: the viral kinase B1, the primase/helicase D5, the putative nuclease G5, and I3 itself (positive control) (Figures 2A, 2B, and S2A).

The Viral Primase/Helicase D5 Is Required for Core Breakdown

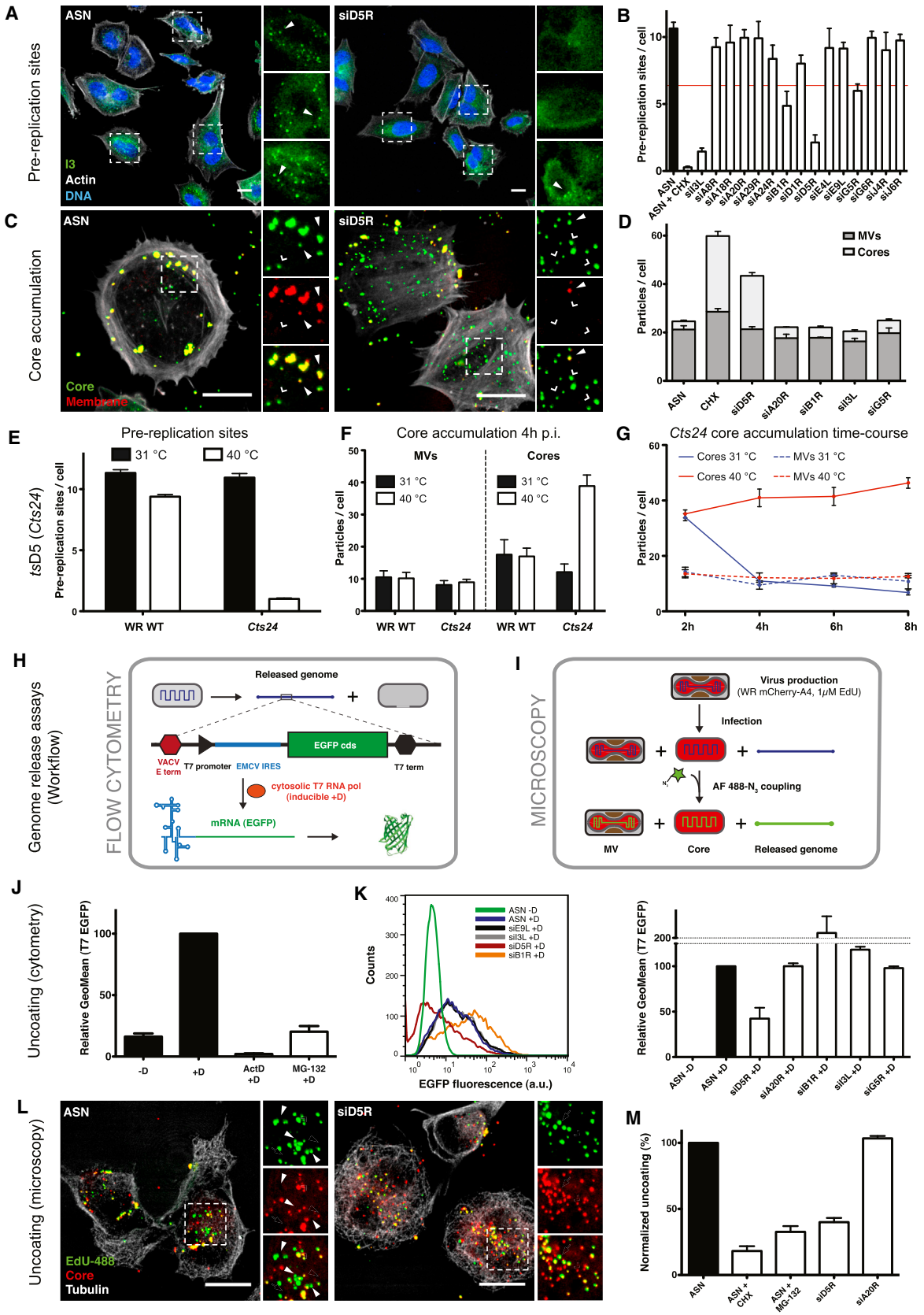
After delivery into the cytoplasm, VACV cores are rapidly disassembled. Using inhibitors of early gene expression (actinomycin D [ActD] or CHX) or proteasomal activity (MG-132) to prevent uncoating, we and others have shown that core disassembly and genome uncoating are concomitant events (Joklik, 1964b; Mercer et al., 2012; Sarov and Joklik, 1972). We assessed if knockdown of the remaining four candidates resulted in core accumulation (Figures 2C and 2D). HeLa cells were transfected with siRNAs against B1, D5, G5, I3, or A20 (control) and infected for 3.5 hr using a recombinant virus with a fluorescent fusion protein in the core (VACV WR EGFP-A4; Schmidt et al., 2011). Immunofluorescence (IF) staining of the viral membrane protein L1 was used to distinguish free cytoplasmic cores from mature virions (MVs). The number of bound or internalized MVs per cell was similar under all conditions (Figure 2D). As expected, few free cores were observed under control conditions (ASN), whereas the number of cores increased about 10-fold when uncoating was blocked (CHX). While depletion of B1, G5, I3, and A20 had no effect on the number of free cores, depletion of D5 resulted in core accumulation similar to CHX treatment (Figures 2C and 2D).

D5 was the only candidate protein whose depletion prevented the formation of DNA prereplication sites and the loss of viral cores, strongly suggesting that it is involved in viral uncoating and/or core degradation. To test the role of D5 using an independent approach, we employed the temperature sensitive (ts) VACV strain *Cts24* (Condit and Motyczka, 1981), which encodes a ts mutant of D5 that is thermolabile at nonpermissive temperature (40°C) (Boyle et al., 2007; Evans and Traktman, 1992). We analyzed the formation of prereplication sites (Figure 2E) and constructed *Cts24* EGFP-A4 to address the core stability by IF, as described above (Figure 2F). At permissive temperature (31°C), the number of prereplication sites and cores in WR WT and *Cts24*-infected cells was comparable. At nonpermissive temperature, prereplication sites did not form, and cores accumulated in cells infected with *Cts24* viruses (Figures 2E, 2F, and S2B). As *Cts24* allows for efficient D5-depletion over an extended period of time, we used this virus in time course experiments to assess whether D5 depletion leads to a block or a delay of core breakdown. While cores were completely degraded within 4 hr at 31°C, they remained stable at nonpermissive temperature for at least 8 hr, proving that loss of functional D5 causes a profound block of core degradation (Figure 2G).

D5 Is Required for the Release of the Viral Genome from Incoming Cores

To probe the release of VACV genomes, we established a flow-cytometry- and a microscopy-based uncoating assay (workflows depicted in Figures 2H and 2I). The flow-cytometry-based uncoating assay quantifies the accessibility of viral genomes to cytosolic T7 RNA polymerase using a recombinant vaccinia virus encoding EGFP under the control of the T7 promoter (WR T7 EGFP). To test specificity, HeLa cells encoding T7 RNA polymerase under the control of a doxycycline-inducible promoter (HeLa T7i) were infected with WR T7 EGFP in the absence or presence of doxycycline. EGFP was expressed only when T7 RNA polymerase was present (Figure 2J; +D). The proteasome inhibitor MG-132—which blocks VACV genome uncoating—efficiently prevented EGFP expression (Figures 2J and S2C). Collectively, these results indicated that the viral genome within the core was not accessible to T7 RNA polymerase and that EGFP expression only occurred from released genomes.

Using this assay, the 15 initial candidate genes were reevaluated. Depletion of most factors showed only modest effects on EGFP expression and, thus, on uncoating. Interestingly, two



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candidates impacted genome release: depleting the viral kinase B1 increased EGFP expression two-fold, and D5 siRNA reduced genome release by more than 50% (Figures 2K and S2D). Thus, the role of D5 in genome uncoating was confirmed.

To directly visualize genome release, we developed a microscopy-based uncoating assay that relies on incorporation of 5-ethynyl-2′-deoxyuridine (EdU) into the viral DNA. VACV WR mCherry-A4 (red-fluorescent core) was produced in the presence of EdU (WR mCherry-A4 EdU-DNA) (Wang et al., 2013). After infection and fixation, EdU-containing viral genomes were labeled by coupling fluorescent azides to EdU. This allowed for simultaneous detection of viral DNA and cores and, thus, differentiation between released and nonreleased genomes. HeLa cells were infected with WR mCherry-A4 EdU-DNA for 4 hr in the presence of D5 siRNA. For these assays, CHX and MG-132 served as positive controls, and ASN and A20 siRNAs served as negative controls. Confocal microscopy images were acquired (Figure 2L), and genomes classified as uncoated or core associated. As expected, both CHX and MG-132 blocked genome release, whereas ASN and A20 siRNAs had no effect (Figure 2M). In confirmation of its role in genome uncoating, depletion of D5 prevented genome release as efficiently as MG-132 treatment (Figures 2L and 2M).

To compare the genome-release assays reported here to established DNase protection assays (Joklik, 1964a), we produced ^{3}H-thymidine-labeled *Cts24* MVs and quantified sensitivity of viral genomes to DNase I digest after infection of BSC-40 cells at permissive (31°C) and nonpermissive (40°C) temperatures. At 31°C, about 60% of the genomic DNA from cell lysates was sensitive to DNase I digestion. Consistent with our uncoating assays, VACV DNA remained largely protected from DNase I digestion in lysates from cells infected at 40°C or in the presence of CHX (Figure S2E).

The data obtained from these three independent uncoating assays demonstrate that D5 is required for genome release. Thus, in line with its requirement for prereplication site formation and core disassembly, D5 is the only viral factor from the 80 originally screened that fulfilled all of the requirements of a bona fide VACV genome uncoating factor.

Silent Mutations of D5R Render Uncoating Insensitive to siD5R

To monitor D5 expression, we generated recombinant viruses expressing N- or C-terminally HA-tagged D5 from its endogenous locus (WR HA-D5 and WR D5-HA). To test the specificity of siD5R, we generated virus strains encoding siRNA-insensitive HA-D5 or D5-HA (WR HA-D5 ins and WR D5-HA ins; Figure S2I). The I EGFP reporter cassette was introduced into the nonessential tk locus of the aforementioned virus strains to quantify infection. None of the viruses had plaque-formation defects, suggesting that the tags, silent mutations, and insertions did not compromise virus fitness (data not shown).

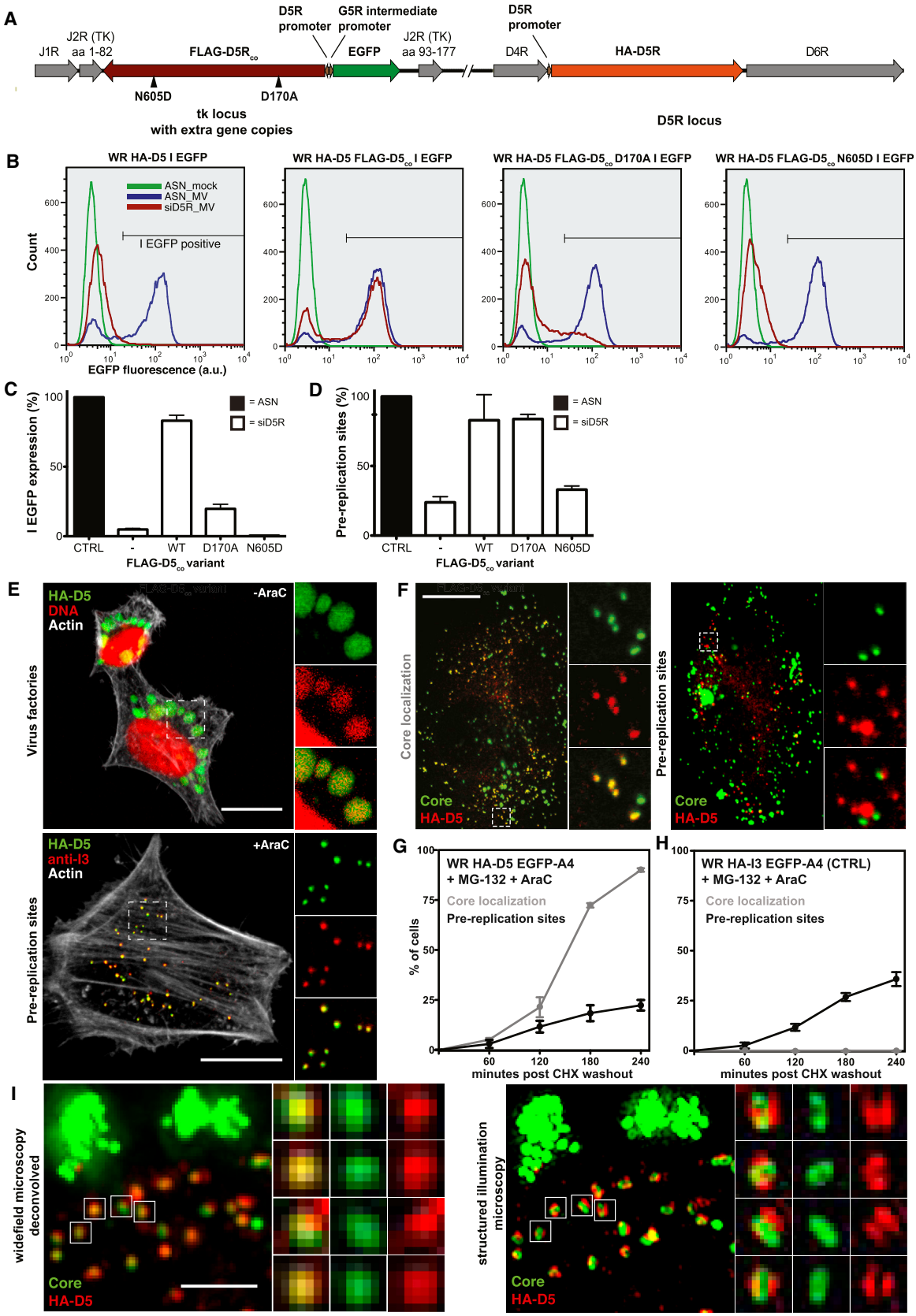
These viruses allowed us to assess intermediate gene expression and simultaneously monitor HA-tagged D5 by IF staining and flow cytometry. We confirmed that D5 knockdown almost completely blocked intermediate gene expression, which was rescued when the siRNA-insensitive variant of D5R was expressed (Figures S2F and S2G). Moreover, we could validate the knockdown of D5 by siD5R and verify that the introduced silent mutations rendered D5 expression insensitive to siD5R (Figures S2F and S2G). When the T7 EGFP cassette was inserted into WR D5-HA and WR D5-HA ins viruses to perform our T7 RNA-polymerase-based uncoating assay, uncoating of WR D5-HA T7 EGFP genomes was reduced by siD5R, while WR D5-HA ins T7 EGFP remained siRNA insensitive (Figures S2H and S2I). These results prove that depletion of D5 alone was responsible for the phenotypes observed in the presence of siD5R.

The AAA+ ATPase Domain of D5 Is Required for Uncoating

D5 is a well-established component of the VACV DNA replication machinery and contains a confirmed N-terminal primase and a predicted C-terminal SFIII helicase (AAA+ ATPase) domain, both of which are essential for DNA replication (Boyle et al., 2007; De Silva et al., 2007; Evans et al., 1995). As D5 is essential, it was impossible to disrupt the primase or ATPase (putative helicase) activity in the endogenous gene. Thus, to address the requirement of the two catalytic domains for intermediate gene expression and uncoating, we synthesized a codon-optimized, siRNA-insensitive version of D5 carrying an N-terminal FLAG tag (FLAG-D5$_{co}$) and introduced point mutations disrupting the primase (D170A) or ATPase (N605D) activity (Boyle et al., 2007; De Silva et al., 2007). In combination with the I EGFP cassette, these FLAG-D5 variants were introduced into the VACV genome as an additional copy using WR HA-D5 as the parental strain (Figure 3A). Upon infection with these recombinant virus strains, FLAG-D5 variants localized like HA-D5, were expressed to comparable amounts and were insensitive to D5 siRNA (Figures S3A and S3B). To assess the requirement of D5 primase and ATPase domains for intermediate gene expression, endogenous HA-D5 was depleted by siRNA, and I EGFP expression was quantified. While FLAG-D5 could readily substitute for loss of HA-D5, both primase and ATPase activities were required for intermediate gene expression (Figures 3B and 3C). The mutants were then assessed for their ability to support prereplication site formation. Interestingly, disruption of the D5 ATPase domain abrogated prereplication site formation to background levels,

Figure 2. D5 Is the VACV Genome-Uncoating Factor

(A–L) DNA prereplication sites (A, B, and E) and accumulated viral cores (C, D, F, and G) were visualized using confocal microscopy (A and C) and quantified (B and D–G) in VACV-infected cells either transfected with the indicated siRNA and/or infected with WR WT (A and B), WR EGFP-A4 (C and D), or with the indicated strain (E–G). T7 RNA-polymerase-driven EGFP expression from released genomes was measured by flow cytometry in drug- (J) and siRNA-treated (K) HeLa T7*i* cells, in which T7 RNA polymerase was expressed (+D) or not (−D). Released viral genomes in siRNA-transfected HeLa cells infected with WR mCherry-A4 EdU-DNA were visualized (L) and quantified (M) as EdU-DNA-positive spots negative for core fluorescence. Details of the genome release assays are presented in (H) and (I). Scale bars represent 15 μm. Data are mean ±SEM (n = 3). Arrowheads indicate prereplication sites (A), MVs (closed) or cores (open) (C), and released genomes (L), respectively. E term indicates early transcription terminator sequence; EMCV IRES indicates encephalomyocarditis virus internal ribosomal entry site; T7 term indicates T7 RNA polymerase transcription terminator sequence. See also Figure S2.



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while mutation of the primase domain had no impact (Figure 3D). These results indicate that the uncoating and DNA replication functions of D5 have different enzymatic requirements.

D5 Localizes to Distinct Subdomains of Incoming Viral Cores

The enzymatic activities of D5 have been studied in vitro, and a number of findings suggest that D5 is associated with the viral DNA replication complex during infection. These include the requirement of D5 for DNA replication, its function as a primase, and the predicted helicase activity, as well as a recent low-resolution structure of the DNA replication machinery (Boyle et al., 2007; De Silva et al., 2007; Evans et al., 1995; Iyer et al., 2001; Sèle et al., 2013). However, the localization of D5 in infected cells has never been investigated. HeLa cells were infected with WR HA-D5 for 4 hr. Consistent with an essential role during DNA replication, HA-D5 localized to virus factories in untreated cells and I3-positive prereplication sites in the presence of AraC (Figure 3E).

We hypothesized that D5 might act directly on released cytoplasmic viral cores to facilitate genome uncoating. To visualize VACV cores and D5 simultaneously, we generated a recombinant virus in which both HA-D5 and EGFP-A4 were expressed from their endogenous loci under the control of their natural promoters (WR HA-D5 EGFP-A4; Figure S3D). Using this virus, cytosolic cores were accumulated in the absence of early gene expression (CHX). After 100 min, when the majority of cores had reached the cytosol, cells were shifted into medium containing AraC and MG-132, allowing for the expression of early genes while preventing uncoating, core disassembly, and DNA replication. The localization of HA-D5 was then analyzed over time. We found that HA-D5 colocalized with free cytoplasmic cores in 90% of the cells 4 hr post-CHX washout (Figures 3F and 3G). At the 4 hr time point, we also found D5 spots close to, but not colocalizing with, cores in about 20% of the cells (Figure 3F, right image). These spots likely represent prereplication sites containing released DNA, due to an incomplete block of uncoating in the presence of MG-132 at high multiplicities of infection.

The association of D5 with incoming cores in the absence of proteasome activity further confirms a role for D5 prior to DNA replication. To exclude the possibility that viral DNA replication proteins generally associate with incoming cores, we generated WR HA-I3 EGFP-A4 (Figures S3C and S3D). When subjected to the same assay, we found that HA-I3 did not colocalize to incoming cores at any time point assayed (Figure 3H), while the levels of HA-I3 and HA-D5 in prereplication sites were comparable. We then employed 3D structured illumination microscopy (SIM) to address the localization of D5 on cores with higher resolution (Figure 3I). In most cytosolic particles, D5 localized to the long sides of the core, forming two distinct foci.

Taken together, our data suggested that D5 acts on distinct, possibly lateral, subdomains of viral cores before other components of the replication machinery have access to the DNA. It is tempting to speculate that these structures mark potential exit portals for the viral DNA.

In Vivo Delivery of Antiviral siRNAs Reduces Virus Production in a Mouse Model of VACV Infection

Our screen provides infection data on 240 individual siRNAs that can potentially be used to study specific functions of VACV early proteins in cell culture. From a more applied point of view, we have identified a number of siRNAs that efficiently block VACV infection at low concentrations with little associated cell toxicity (Figure S1 and Table S2). The most potent siRNAs targeted the uncoating and DNA replication factor D5 (D5R oligo number 2) and the viral DNA polymerase E9 (E9L oligo number 1). Both siD5R and siE9L completely abolished intermediate without strongly affecting early gene expression (Figure 4A).

To test the antiviral potential of these siRNAs in an infected organism, we assessed virus production after siRNA administration in a mouse model of VACV infection. We found that delivery of siD5R and siE9L, but not control, siRNA significantly reduced virus production in the lung, as well as replication in liver and spleen (Figure 4B). These data indicate that the viral uncoating factor D5, and potentially other essential early genes, is a promising antiviral target and highlight the therapeutic potential of RNAi-based antiviral treatment.

DISCUSSION

The proteinaceous structure that encapsidates the poxvirus genome is not inherently unstable once it is released into the host cell cytosol (Pedersen et al., 2000). Instead, poxviruses undergo a stepwise uncoating process that involves core activation and genome release. While core activation occurs immediately after membrane fusion (Schmidt et al., 2013), genome release requires proteasome activity and the expression of early viral genes. Here, we demonstrate that the VACV AAA+ ATPase D5 is a multifunctional protein required for genome uncoating and DNA replication. Evolutionarily, D5 is conserved, encoded by all poxviruses, and identified as one of the proteins shared by all nucleo-cytoplasmic large DNA viruses (Iyer et al., 2006).

Figure 3. D5 Localizes to Incoming Cores and Requires ATPase Activity for Uncoating

(A–E) To address the requirement of the primase and AAA+ ATPase domains of D5 for DNA replication and uncoating, we constructed viruses encoding HA-D5 in the endogenous locus and an additional codon-optimized version of the D5R gene (FLAG-D5$_{co}$), in combination with an I EGFP reporter cassette in the nonessential tk locus of VACV. FLAG-D5$_{co}$ encodes either the WT protein or alternatively D5 bearing mutations inactivating the primase (D170A) or ATPase (N605D) domain (A). Intermediate gene expression (B and C) and viral prereplication sites (D) were quantified in cells transfected with ASN or siD5R and infected with the indicated viruses. To test the localization of HA-D5 with respect to virus factories (positive for viral DNA) and prereplication sites (positive for I3), HeLa cells were infected with WR HA-D5 for 4 hr in the absence or presence of AraC to accumulate virus factories and prereplication sites, respectively, followed by staining with anti-HA, phalloidin, and DRAQ5 (DNA) or anti-I3, where indicated (E).

(F–H) To visualize cores and HA-D5/HA-I3 simultaneously, HeLa cells were infected with WR HA-D5 EGFP-A4 (F and G) or WR HA-I3 EGFP-A4 (H) in the presence of CHX and chased into MG-132 + AraC for 4 hr. The percentage of cells in which HA-D5/HA-I3 colocalized with cores and/or prereplication sites was quantified over time (G and H). Representative images of both phenotypes observed 4 hr post-CHX washout are shown in (F). SIM images of HA-D5 on cytosolic cores are shown (Z projection) and compared to conventional fluorescence microscopy in (I). All data are mean ±SEM (n = 3). Scale bars represent 15 μm (E and F) or 2 μm (I). See also Figure S3.

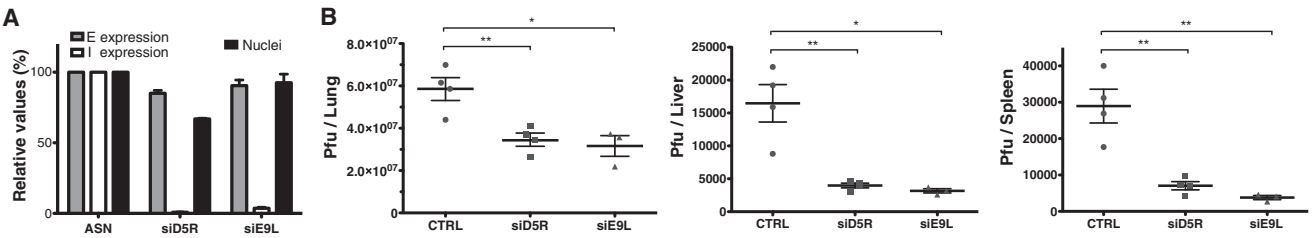

Figure 4. Antiviral siRNAs Can Curtail VACV Replication In Vivo

(A–D) Early and intermediate gene expression in VACV-infected HeLa cells treated with the indicated siRNAs was quantified along with the number of cells (A). C57BL/6 mice were treated with the indicated siRNAs and infected 8 hr postdelivery. Three days later, lungs (B), livers (C), and spleens (D) were collected, and virus titers determined. Data are mean ±SEM. p values are indicated (*$p < 0.05$; **$p < 0.01$, unpaired t test).

It forms hexamers (Boyle et al., 2007; Sèle et al., 2013) and contains an N-terminal primase domain, as well as a C-terminal AAA+ ATPase domain classified as a putative SFIII helicase (Iyer et al., 2006). Primase and NTPase activity of D5 have been verified in vitro, and experiments with temperature-sensitive mutants of D5 show that it plays a direct role in DNA replication (Boyle et al., 2007; De Silva et al., 2007; Evans et al., 1995). Our mutagenesis experiments reveal that the uncoating function of D5 is independent of its primase activity. Therefore, the two functions of D5 during the viral life cycle can be mechanistically separated. That D5 is recruited to incoming cores in the absence of proteasomal activity suggests that it acts directly on virus cores to release the genome.

D5 could facilitate uncoating in several different ways: Genome release may involve the complete disruption of viral cores (e.g., by the global degradation of core structural proteins or the destruction/release of critical core stabilizing components). In support of this, inhibitors of uncoating lead to the accumulation of cores (Mercer et al., 2012; Pedersen et al., 2000), and broken VACV cores were observed in infected cells by electron microscopy (Cyrklaff et al., 2007; Pedersen et al., 2000). As a AAA+ ATPase that converts the energy released by NTP hydrolysis into conformational changes in the hexameric ring, D5 may be suitable to actively contribute to core disassembly.

Alternatively, it is conceivable that genome uncoating involves the opening of a channel or defined exit site in the core wall, as observed in the case of herpesviruses (Brown and Newcomb, 2011) and certain bacteriophages. In support of an exit portal, D5 localizes to distinct core domains, as revealed by our SIM data. Mechanistically, T7 and N4 phages use the enzymatic activity of their RNA polymerases to pull the genome into the cell (Zavriev and Shemyakin, 1982; Choi et al., 2008). It is possible that, in the case of VACV, genome release is intimately linked to the putative helicase activity of D5, which may lead to the release of single-stranded DNA (ssDNA). In fact, released genomes in prereplication sites can be stained with antibodies directed against the viral ssDNA binding protein I3 (Rochester and Traktman, 1998; Tseng et al., 1999), even in the absence of ongoing DNA replication (AraC). Releasing ssDNA would, in theory, allow the virus to avoid recognition by double-stranded DNA receptors that could trigger the intrinsic immune response.

Taken together, results in this and previous studies suggest that cores released into the cytosol by fusion first undergo activation. This involves morphological changes, as well as the reduction of viral disulfide bonds (Cyrklaff et al., 2007; Dales, 1963; Locker and Griffiths, 1999; Schmidt et al., 2012, 2013). Transcription within activated cores allows the expression of early proteins, such as D5. The activity of both host cell proteasomes and D5 subsequently permits the genuine uncoating step, the release of the viral genome. Interestingly, recruitment of D5 to cores was only observed in the presence of the proteasome inhibitor MG-132, indicating that the presence of D5 on the core is transient and perhaps reversed by proteasomes. It remains to be determined how these complexes cooperate to facilitate uncoating and whether additional cellular factors are involved.

Our study describes a comprehensive siRNA screen targeting viral genes. A number of advantages make antiviral siRNA screens an attractive strategy to identify viral genes required for specific steps in the life cycle of other complex DNA viruses, such as herpesviruses and NCLDVs: as no cellular mRNAs are targeted, the siRNAs exhibit relatively low cell toxicity and circumvent compensatory mechanisms often induced in cells upon depletion of critical cell factors. Since the siRNAs are administered before infection and production of early genes, depletion efficiency is high and independent of protein stability. As shown here in an in vivo mouse model of VACV infection, such screens are likely to reveal sensitive viral target genes and may lead to the identification of potent antiviral siRNAs. We envision that similar screens may be used to systematically probe the function of viral genes in other complex DNA viruses and are confident that analogous approaches will prove beneficial for both basic and applied virus research.

EXPERIMENTAL PROCEDURES

Antiviral siRNA Screen

Antiviral siRNA screens were performed in HeLa ATCC cells transfected with siRNAs against 80 conserved early genes of VACV WR (20 nM, three oligos/gene). Cells were infected with WR E EGFP or WR I EGFP 48 hr posttransfection, fixed 7 hr postinfection, and stained with Hoechst and, in case of WR E EGFP, with anti-EGFP antibodies. Images were acquired with a Molecular Devices screening microscope, and infection was quantified using a custom-written Matlab program. See also Supplemental Experimental Procedures.

Prereplication Site Formation, Core Accumulation, and Uncoating Assays

To quantify prereplication sites, HeLa cells were infected in the presence of AraC for 4–5.5 hr and stained with anti-I3 antibodies. To measure released

cores, cells were infected with WR EGFP-A4 in the presence of AraC for 4 hr and stained with anti-L1 (7D11) antibody to distinguish free cores from L1-postive MVs.

To detect released genomes, WR mCherry-A4 EdU DNA was used to infect cells in the presence of AraC for 4 hr, and EdU-containing genomes were labeled with AF 488 azide. Prereplication sites, fluorescent cores and MVs, and released viral genomes were quantified using the spot-detection function of Imaris. To measure the accessibility of viral genomes to cytosolic enzymes, HeLa cells inducibly expressing T7 RNA polymerase were infected with WR T7 EGFP in the presence of AraC. Cells were harvested 6 hr postinfection, and EGFP expression was analyzed by flow cytometry. See Supplemental Experimental Procedures for details.

In Vivo Delivery of Antiviral siRNAs

For delivery, 60 mg siRNA was complexed with in vivo jetPEI reagent and injected into the tail vein of C57BL/6 mice. Eight hours posttransfection, mice were infected intratracheally with 5×10^6 plaque-forming units WR WT. Virus titers in lungs, spleen, and liver were quantified 3 days postinfection.

Cell lines, viruses, used reagents, and detailed methods are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.12.008.

AUTHOR CONTRIBUTIONS

S.N.K., F.I.S., C.S., and J.M. performed experiments. S.N.K. and F.I.S. analyzed data. S.N.K., F.I.S., C.S., M.K., A.H., and J.M. conceived of and designed experiments. S.N.K., F.I.S., A.H., and J.M. wrote the manuscript.

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