An Intrinsically Disordered Region of the DNA repair protein Nbs1 Is a Species-Specific Barrier to Herpes Simplex Virus 1 in Primates

Dianne I. Lou1,‡, Eui Tae Kim2,+, Nicholas R. Meyerson3, Neha J. Pancholi2,4, Kareem N. Mohni5, David Enard6, Dmitri A. Petrov6, Sandra K. Weller5, Matthew D. Weitzman2,*, and Sara L. Sawyer1,3,*

1Molecular Biosciences, University of Texas at Austin, Austin, TX 78712, USA
2Division of Cancer Pathobiology, Department of Pathology and Laboratory Medicine, The Perelman School of Medicine at the University of Pennsylvania and the Children’s Hospital of Philadelphia, Philadelphia, PA 19104, USA
3BioFrontiers Institute, Department of Molecular, Cellular, and Developmental Biology, University of Colorado Boulder, Boulder, CO 80303, USA
4Cell and Molecular Biology Graduate Program, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA
5Department of Molecular Biology and Biophysics, University of Connecticut Health Center, Farmington, Connecticut 06030, USA
6Department of Biology, Stanford University, Stanford, CA 94305, USA

Summary

Humans occasionally transmit herpes simplex virus-1 (HSV-1) to captive primates, who reciprocally harbor alphaherpesviruses poised for zoonotic transmission to humans. To understand the basis for the species-specific restriction of HSV-1 in primates, we simulated what might happen during the cross-species transmission of HSV-1 and found that the DNA repair protein Nbs1 from only some primate species are able to promote HSV-1 infection. The Nbs1 homologs that promote HSV-1 infection also interact with the HSV-1 ICP0 protein. ICP0 interaction mapped to a region of structural disorder in the Nbs1 protein. Chimeras reversing patterns of disorder in Nbs1 reversed titers of HSV-1 produced in the cell. By extending this analysis to 1,237 virus-interacting mammalian proteins, we show that proteins that interact with viruses are highly...
enriched in disorder, suggesting that viruses commonly interact with host proteins through intrinsically disordered domains.

**eTOC Blurb**

Herpes viruses occasionally transmit between humans and nonhuman primates. Lou et. al. report that the DNA repair protein Nbs1 interacts with the HSV-1 protein ICP0 and is required for optimal virus replication. Nbs1 of some primate species has escaped virus highjack through evolving increased structural disorder in the ICP0-interaction domain.

![Diagram](Image)

**Introduction**

Humans are endemically infected by three alphaherpesviruses: herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (the chickenpox virus). Of these, HSV-1 alone chronically infects more than 90% of adults over the age of 50 (Chayavichitsilp et al., 2009). In nature, alphaherpesviruses are found broadly in mammals, fish, amphibians, and invertebrates (Mocarski, 2007; Sharp, 2002). These viruses generally cause mild symptoms in their natural hosts, but are often associated with severe disease when transmitted to a new species. For example, *Cercopithecine herpesvirus 1* (CeHV-1 or “B virus”), an alphaherpesvirus of Asian macaques, is typically fatal in cases where it is transmitted to humans (Tischer and Osterrieder, 2010). B virus is a major obstacle to biomedical research because macaques are the most common nonhuman primate model for studying human disease. Primate research centers go to great lengths to monitor for the presence of B virus in macaque colonies. Should the B virus genome acquire mutations that optimize infection and
spread in humans, stable transmission chains could be established resulting in the emergence of a new human virus. Indeed, it is hypothesized that humans acquired herpes simplex virus 2 (HSV-2) through exposure to a chimpanzee virus that evolved to become stably transmitted in humans (Wertheim et al., 2014).

Not only do nonhuman primates pass alphaherpesviruses to humans, humans also reciprocate. The passage of HSV-1 from humans to captive nonhuman primates has been documented many times (Barnes et al., 2015; Gilardi et al., 2014; Heldstab et al., 1981; Kik et al., 2005; Landolfi et al., 2005; Matz-Rensing et al., 2003; Moontnick et al., 1998; Schrenzel et al., 2003). The macaque B virus and HSV-1 are similar in that they cause a neuro-virulent disease in unnatural hosts which is often fatal. In the reports describing HSV-1 outbreaks in captive primates, it is often speculated that the virus was passed to the affected animals by a human with herpes labialis (cold sores; in particular see (Matz-Rensing et al., 2003)). Given the very high prevalence of HSV-1 in humans, countless other transmissions to captive and wild nonhuman primates have almost certainly occurred. Despite this evidence for a healthy flow of HSV-1 from humans to nonhuman primates, no sustained transmission chains are known to have resulted.

Evidence suggests that each mammalian species has long co-evolved with its specific endemic alphaherpesvirus(es), and that stable transmissions of these viruses from one species to another are rare (Sharp, 2002). Therefore, one could conclude that species-specific differences in mammalian genomes create unique environments for these viruses. A comprehensive understanding of viral disease emergence requires identification of host proteins that interact with viruses in a species-specific manner, fortifying barriers that keep viruses in their current hosts and make it difficult for them to move into new host species. Species-specific host proteins define how viruses must adapt in order to establish themselves in a new species (Meyerson and Sawyer, 2011; Sawyer and Elde, 2012). For instance, the infection of new host species by arenaviruses and parvoviruses involves adaptation of these viruses for improved binding to the TfR1 cell-surface receptor encoded by the new species (reviewed in (Choe et al., 2011; Kailasan et al., 2015)). This adaptation is required because TfR1 exhibits substantial genetic divergence between species (Demogines et al., 2013; Kaelber et al., 2012; Kerr et al., 2015).

The human DNA damage response has been found to enhance infection by several DNA viruses that replicate in the cell nucleus, including HSV-1 (Li et al., 2008; Lilley et al., 2005; Mohni et al., 2011; Wilkinson and Weller, 2004). The Mre11-Rad50-Nbs1 (MRN) complex is a key player in this phenomenon. MRN is a housekeeping complex normally involved in the detection of DNA double-strand breaks and in the signaling that leads to DNA repair (Ciccia and Elledge, 2010). HSV-1 infection introduces into the nucleus a double-stranded DNA molecule that may present exposed double-stranded ends and also contains lesions (nicks and gaps) acquired in the previous round of DNA replication (Garber et al., 1993; Poffenberger and Roizman, 1985; Smith et al., 2014; Strang and Stow, 2005). Presumably because they are attracted to these features, MRN proteins localize to HSV-1 replication centers (Lilley et al., 2005; Shirata, 2005; Taylor and Knipe, 2004; Wilkinson and Weller, 2004) and interact with HSV-1 proteins (Balasubramanian et al., 2010; Chaurushiya et al., 2012; Taylor and Knipe, 2004). Furthermore, exogenous expression of one MRN
component, Mre11, in an Mre11-deficient cell line increases HSV-1 DNA replication and virus production (Lilley et al., 2005). It remains a long-standing mystery in this field how the cellular DNA damage response benefits the virus lifecycle.

We find that Nbs1 is the MRN component that is most diverged in sequence between primate species. We simulated what might happen in a cross-species transmission of HSV-1, pitting this virus against the Nbs1 proteins of various nonhuman primates. We uncovered a high degree of species-specificity, with Nbs1 from different primate species having variable abilities to promote the HSV-1 lifecycle. We also find that binding to the viral ICP0 protein correlates with the ability of a particular Nbs1 to promote HSV-1 infection. In the cases where this interaction is observed, the DNA damage response is enhanced upon infection and virus replication is increased. In the Discussion, we propose that ICP0 may recruit Nbs1 to viral DNA genomes to trigger homologous recombination over non-homologous end joining, the latter of which is known to have antiviral effects. ICP0 interaction with Nbs1 maps to a region of structural disorder in the Nbs1 protein. We show that the evolution of increased disorder in the Nbs1 protein effectively achieves resistance to ICP0 interaction and virus hijack. We also show that host proteins that bind to viruses are generally enriched in intrinsically disordered domains, suggesting that the findings here may extend to other host-virus interactions as well.

**Results**

**Species-specific effects of Nbs1 on HSV-1 replication**

Host proteins that are extreme in their genetic divergence between species have sometimes been shown to create genetic barriers to the passage of viruses between species (Demogines et al., 2013; Elde et al., 2009; Kirmaier et al., 2010; Sawyer et al., 2005). Since the DNA damage response and MRN complex have been implicated in viral infections, we therefore examined genetic divergence in the three proteins, Mre11, Rad50, and Nbs1, that constitute the MRN complex. We gathered sequences of each from human and three other primate species: Sumatran orangutan (Pongo abelii), white-cheeked gibbon (Nomascus leucogenys), and rhesus macaque (Macaca mulatta). For each of the three proteins, we calculated the percentage amino acid identity between the human version and its homolog in each of the other primate species. We found that Nbs1 exhibits greater sequence variability than do Mre11 or Rad50 (Figure 1A). Based on this observation, we tested the hypothesis that genetic differences in Nbs1 may result in a species-specific interaction with HSV-1. We obtained a human cell line, NBS-1LBI, derived from a patient with Nijmegen Breakage Syndrome (NBS) (Kraakman-Van Der Zwet et al., 1999). In these cells, a frame-shifting mutation in the NBS1 gene leads to the production of a truncated Nbs1 protein which prevents formation of a functional MRN complex (Desai-Mehta et al., 2001). Herein we refer to these as nbs1 cells. Using retroviral transduction, we stably complemented nbs1 cells with an empty vector or a vector encoding the human NBS1 gene. Both cell lines were infected with a wild-type HSV-1 strain (in1863 derivative of strain 17+) at a multiplicity of infection (MOI) of 0.01. At 24 hours post infection (hpi), viral titers in the supernatant were significantly increased in cells expressing human Nbs1 when compared to negative control (Figure 1B). This was also true over a time-course of infection (Figure 1C). In line with
previously published Mre11 data (Lilley et al., 2005), this result supports a model where the MRN complex aids the HSV-1 lifecycle.

We next tested the role that Nbs1 might play in blocking transmission of HSV-1 to nonhuman primate species. We used retroviral transduction to generate nbs1 cells stably expressing the NBS1 genes encoded by gibbon, orangutan, and rhesus macaque. These primate Nbs1 proteins are highly similar to human Nbs1, differing at only 21–36 amino acid positions (Figure 1A). The orangutan and rhesus macaque Nbs1 homologs supported lower levels of HSV-1 production than did human Nbs1 (p=0.09 and p=0.02, respectively, by two-tailed t-test) (Figure 1B). The gibbon Nbs1 was also significantly different than human Nbs1 (p=0.007 by two-tailed t-test), and provision of gibbon Nbs1 led to no gain in virus production compared to the nbs1 cell line. This pattern was also observed when progeny viruses were collected at various timepoints after infection, using a readout of either virus yield (Figure 1C) or viral DNA (Figure 1D). Because equal viral DNA is observed at 48 hours, the species-specific effect of Nbs1 may be a kinetic one. The differential effect of each Nbs1 on HSV-1 production was not due to differential Nbs1 expression levels, since the gibbon Nbs1 was expressed at levels slightly higher than human Nbs1 (Figure 1B, inset). The difference in virus yield from cells complemented with gibbon or human Nbs1 homologs reaches 2 logs at some timepoints and with some virus preps (Figure 1C), which is especially surprising considering that these are both naturally occurring, wildtype Nbs1 proteins.

Humans and gibbons are both members of the Hominoidea superfamily of primates (i.e. hominoids), which contains the great and lesser apes. Reflecting this close evolutionary relationship, the Nbs1 proteins encoded by human and gibbon differ at only 21 amino acid positions. Both of these proteins are wildtype variants in their respective species and are expected to support full DNA repair capabilities. In line with this expectation, we found that gibbon Nbs1 can complement human nbs1 cells for repair related functions. Gibbon Nbs1 interacts with the other human MRN components Rad50 and Mre11 (Figure 2A) and acts in concert with other human DNA repair factors to recover from damage caused by three different genotoxins: camptothecin (Figure 2B), X-rays (Figure 2C), and hydroxyurea (Figure S1). We also find that the differential effects of human and gibbon Nbs1 on HSV-1 replication are specific to this virus. Influenza A virus, which replicates in the nucleus but not in a manner that is known to involve Nbs1, replicates to equal levels in cell lines complemented with either human or gibbon NBS1 (Figure S1). Adenovirus also replicates within the nucleus, where the MRN complex orchestrates a well-described antiviral response against it (Evans and Hearing, 2005; Lakdawala et al., 2008; Stracker et al., 2002). Both human and gibbon Nbs1 proteins inhibited adenovirus replication to the same extent (Figure S1). Therefore, when expressed in human cells, gibbon Nbs1 is fully functional for DNA repair and inhibition of adenovirus, but does not promote HSV-1 replication.

We next explored the gibbon Nbs1 phenotype further. Gibbons are lesser apes of the family Hylobatidae, and siamangs are a genus within this family (Figure 3A). The Nbs1 proteins of white-cheeked gibbon (Nomascus leucogenys; tested above; herein referred to as “gibbon”) and siamang (Symphalangus syndactylus; herein referred to as “siamang”) differ by only 12 amino acids (an alignment is shown in Figure S2). Despite the close evolutionary
relationship of these two species, we find that their Nbs1 proteins have distinctly different effects on HSV-1 infection. Unlike the gibbon Nbs1, siamang Nbs1 expression enhanced virus yield in a manner similar to the human Nbs1 protein (Figure 3B). Likewise, using a qPCR assay we found that human and siamang Nbs1 promoted viral genome replication to a greater extent than the gibbon Nbs1 (Figure 3C). These experiments demonstrate that Nbs1 of closely related primate species can impact viral replication quite differently. In fact, as few as 12 amino acid differences in naturally occurring Nbs1 homologs can significantly impact HSV-1 replication.

Species-specific effects of Nbs1 on HSV-1 viral transcription and translation

We next examined the mechanism and timing of the interaction between Nbs1 and HSV-1. We infected complemented nbs1 cells with HSV-1 at an MOI of 0.1 and used qRT-PCR to quantify abundance of ICP27 mRNA, one of the earliest (i.e. “immediate early”) HSV-1 transcripts to be expressed. Transcript levels were enhanced in the presence of human and siamang Nbs1, but not gibbon Nbs1 (Figure 4A). The differences were most apparent at 9 hours. This same trend was seen even in the presence of the viral polymerase inhibitor, phosphonoacetic acid (PAA), confirming that this effect is not entirely a result of variable levels of viral genome replication (Figure 4B). The complemented nbs1 cells were next infected with HSV-1 at an MOI of 3, where again virus yield was enhanced in the presence of human or siamang Nbs1 compared to gibbon Nbs1 (Figure 4C). Immunoblots of samples taken at different times after infection show the accumulation of three HSV-1 proteins known to be expressed at different stages during infection: ICP0 (immediate-early), ICP8 (early), and VP21 (late) (Figure 4D). All three viral proteins showed accelerated accumulation in the presence of siamang Nbs1 compared to gibbon Nbs1, consistent with a global effect on HSV-1 gene expression. We also find that Nbs1 co-localizes with the viral protein ICP4 (Figure 4E), a major transcriptional regulator of HSV-1 which marks replication compartments at early stages (Knipe et al., 1987; Livingston et al., 2008; Randall and Dinwoodie, 1986). Collectively, these data reveal a positive role for Nbs1 in the global HSV-1 lifecycle which is not specific to one viral gene or protein.

Species-specific effects of Nbs1 reflect differential susceptibility to viral hijack

Viral proteins often physically interact with host proteins as a means to manipulate cellular responses into creating an environment optimal for virus replication. Human Nbs1 was previously shown to interact physically with a peptide fragment of ICP0 (Chaurushiya et al., 2012). We detected an interaction between human endogenous Nbs1 and the full-length ICP0 protein in the context of HFF (human foreskin fibroblast) cells infected with HSV-1 at an MOI of 3 (Figure 5A). This interaction was most robust at 3 and 6 hours after infection. We next asked whether Nbs1 from different primate species would interact equally with ICP0. We first co-transfected HEK-293T cells with plasmids expressing Nbs1-FLAG from each species and ICP0-GFP. An immunoprecipitation was performed with anti-GFP, and again we observed human Nbs1 interacting with ICP0 (Figure 5B). Furthermore, we observed that ICP0 co-precipitates human and siamang Nbs1, but not gibbon Nbs1 (Figure 5B,C). We next infected nbs1 cells expressing each primate Nbs1 with HSV-1 at an MOI of 3 for 6 hours. Lysates were immunoprecipitated with an antibody against ICP0. Immunoblotting of the precipitate revealed that Nbs1 from human and siamang interacted
strongly with ICP0, while interaction with gibbon Nbs1 was barely detected (Figure 5D). Thus, HSV-1 ICP0 can interact with some but not all primate versions of Nbs1, and this successful interaction correlates perfectly with the ability of each Nbs1 to promote viral infection.

Nbs1 and MRN are critical to DNA damage responses, so we next examined the DNA damage response in nbs1 cells complemented with non-human primate Nbs1. Cells were infected with HSV-1 at an MOI of 3. Immunoblotting was used to monitor the accumulation of three HSV-1 proteins known to be expressed at different stages during infection: ICP0 (immediate-early), ICP8 (early), and VP21 (late) (Figure 6A). In this case, genome replication was inhibited by PAA so that we could examine responses to the early steps of infection and, as a result, the late protein VP21 does not accumulate. Protein extracts were probed with antibodies against phosphorylated proteins that serve as indicators of the DNA damage response: p-ATM and p-Chk2. Both p-ATM and p-Chk2 accumulate more rapidly in the presence of siamang Nbs1 than gibbon Nbs1 (Figure 6A). We also find that human but not gibbon Nbs1 drives accelerated accumulation of other markers of the DNA damage response, namely p-ATM, p-p53, and p-Nbs1, even in infections where PAA is not present (Figure 6B,C). Therefore, in the three cases studied (human, gibbon, and siamang Nbs1), the interaction with ICP0, enhanced cellular DNA damage response, and increased viral replication are all correlated. One possible model is that Nbs1 is hijacked to execute an activity beneficial to virus replication (enhancing a virus-specific DNA damage response, for example), but that Nbs1 proteins of some primate species are resistant to hijack.

**Virus hijack maps to an intrinsically disordered domain on Nbs1**

The close evolutionary relationship of gibbon and siamang Nbs1 allowed us to map the determinants of this species-specific interaction with HSV-1. The first three domains of Nbs1 form a compact structure, while everything downstream is disordered (Williams et al., 2009). Ten out of the twelve amino acid differences between gibbon and siamang Nbs1 fall in this disordered region (Figure 7A, top), focusing our attention here. To understand how these species-specific differences in Nbs1 might affect the degree of disorder in this region, we next analyzed the predicted disorder tendency in both gibbon and siamang Nbs1 (Figure 7A). Here, values above 0.5 indicate disorder and values below 0.5 indicate structured regions. We noticed that these two Nbs1 proteins diverge in their predicted disorder in a region spanning amino acids 590 to 710 (purple band in Figure 7A). This difference in predicted disorder is caused by only 4 amino acid differences between the two proteins, shown across the top of the schematic. In this region, gibbon Nbs1 is predicted to have higher disorder than siamang Nbs1 (Figure 7A). We considered whether this region could be identifying a point of contact with ICP0, because lower disorder in siamang Nbs1 could be consistent with a stronger binding site for ICP0 (Mohan et al., 2006).

To investigate whether or not the region of differential disorder identifies a potential ICP0 interaction motif, we generated constructs in which these four amino acid differences were exchanged between gibbon and siamang Nbs1 (Figure 7B, alignment in Figure S2). As shown in Figure 7C, four amino acid substitutions in gibbon Nbs1 (gibbon-4) restored the ability to interact with ICP0. Conversely, loss of the ICP0 interaction was observed with the
reciprocal substitution made in the siamang background (siamang-4) (Figure 7C,D). We next made nbs1 cell lines stably complemented with the chimeric Nbs1 constructs and infected them with HSV-1. In agreement with the ICP0 interaction data, gibbon-4 Nbs1 supported HSV-1 replication to the same extent as siamang Nbs1, while siamang-4 Nbs1 supported levels of virus production mirroring that of gibbon Nbs1 (Figure 7E). Thus, the 4 amino acid differences which alter the intrinsic disorder of this region change both ICP0 interaction and virus yield from the cell. It is interesting to speculate that certain mutations in this region of Nbs1, and the disorder changes that they cause, might be selected in primate genomes to reduce interaction with ICP0 and thus virus hijack.

**Intrinsically disordered domains are a common feature of proteins that interact with viruses**

We tested if disordered regions are common in proteins that mediate interactions with viruses. To do this, we contrasted the occurrence of intrinsic disorder within mammalian proteins known to interact with viruses (n = 1,237) versus those that do not interact with viruses (n= 8,356). These two sets of proteins were recently hand-curated as part of another study (Enard et al., 2016). Proteins were only included in the virus-interacting group if there was “low-throughput” evidence for their interaction with viral proteins, RNA, or DNA; proteins identified only in high-throughput screens were not included (Enard et al., 2016). For each of these two groups (mammalian proteins that do and do not interact with viruses), we plotted the proportion of proteins (Y-axis) against the fraction of each protein length predicted to be intrinsically disordered (X-axis; Figure 7F). In this large set, host proteins with little disorder are much less common among virus-interacting proteins (red curve) than among those that do not interact with viruses (blue curve) (left side of distribution, Figure 7F). In addition, the average fraction of the protein length that is disordered is markedly higher within virus-interacting proteins than within proteins that do not interact with viruses (25% versus 19%, observed p-value = 0 after 100,000 iterations of a standard permutation test, as described in the experimental procedures section). This strongly suggests that highly ordered host proteins are less likely to interact with viruses, and that proteins that contain disordered regions are much more likely to interact with viruses. Our datasets are certain to contain noise in that some proteins included in the virus-interacting group do not really interact with viruses (false positives), and vice versa. However, this noise would only serve to degrade the correlative signal, not strengthen it. Therefore, the correlation between interaction with viruses and the tendency to contain disordered regions is likely even stronger than we have measured here. The use of disordered regions by viruses is thus a common phenomenon not isolated to Nbs1.

**Discussion**

Here, we identify the essential DNA repair protein Nbs1 as a positive effector of the herpes simplex virus-1 (HSV-1) lifecycle. Surprisingly, the positive effects of Nbs1 on the HSV-1 lifecycle are species-specific in primates, since HSV-1 replication increases upon provision of human Nbs1 but not Nbs1 from some nonhuman primate species. We can begin to speculate about the specific mechanistic role of Nbs1 in the HSV-1 lifecycle. Once HSV-1 DNA enters the nucleus, an almost immediate host response is the recruitment of PML and...
other ND10 proteins to sites adjacent to incoming viral genomes (Everett, 2015; Everett and Murray, 2005; Everett et al., 2008; 2006; Glass and Everett, 2013; Lukashchuk and Everett, 2010). These proteins are then degraded by ICP0 to allow viral gene expression (Boutell and Everett, 2013; Everett et al., 2008; 2006; Lukashchuk and Everett, 2010). Although the state of the viral genome is unclear at this point, components of the DNA damage response machinery are also recruited to sites adjacent to incoming viral genomes (Lilley et al., 2011).

It is known that the MRN and the Ku70/80 heterodimer compete for binding at double-stranded DNA breaks in cellular DNA (Chapman et al., 2012; Ciccia and Elledge, 2010). Which proteins bind the ends will drive DNA repair pathway choice: MRN would promote a homologous recombination response, while Ku70/80 would promote non-homologous end joining (NHEJ) and recruit DNA-PKcs to double-strand breaks. Several lines of evidence suggest that NHEJ is antiviral and is inhibited in HSV-1 infected cells. HSV-1 infection has been shown to lead to an inactivation of NHEJ (Schumacher et al., 2012) and DNA-PKcs activity (Lees-Miller et al., 1996; Parkinson et al., 1999; Smith et al., 2014). HSV-1 replication is more efficient in cells lacking the catalytic subunit of DNA-PKcs (Parkinson et al., 1999), and in Ku-deficient murine embryonic fibroblasts viral yields are increased by almost 50-fold (Taylor and Knipe, 2004). Taken together with the results presented herein, we suggest that HSV-1 ICP0 may play a role in recruiting or retaining MRN complexes as “first responders” to viral genomes, thus affecting pathway choice by suppressing the antiviral NHEJ pathway. In line with this, Smith and Weller have recently reported that ICP0 is required to relieve suppression of HSV-1 DNA infectivity caused by DNA-PKcs (Smith et al., 2014).

The species-specific effects of Nbs1 on HSV-1 map to a disordered domain in Nbs1. Disordered domains in general evolve more rapidly than ordered domains and are therefore fertile ground for evolutionary innovation (Brown et al., 2010). New NBS1 alleles encoding increased disorder in the ICP0 binding site could experience positive selection in primate populations if they provide protection from infection. ICP0 may then evolve to re-establish this interaction, possibly through evolution to interact with a more ordered region of Nbs1. This would constitute a host-virus “arms race” scenario where Nbs1 experiences positive selection to increase the disorder of the ICP0 interaction site, and ICP0 evolves to interact with new and variant forms of Nbs1. Evaluation of this arms race model will require additional functional studies of ICP0. For instance, it would be interesting to determine whether ICP0 from different primate alphaherpesviruses can interact with Nbs1 proteins with which HSV-1 ICP0 cannot (such as gibbon Nbs1). By taking a genome-wide approach, we find that the use of disordered regions by viruses is likely to be a general phenomenon not isolated to Nbs1. What remains to be seen is whether structural divergence in these regions commonly manifests in species-specific interactions with viruses, as we have documented here for Nbs1. Given that these domains evolve more rapidly, they are very likely to be more highly differentiated between species. Identifying host genes that interact with viruses in a species-specific fashion will add to our understanding of the routes by which humans and nonhuman primates trade viruses back and forth.
Experimental Procedures

Cell lines

NBS-1LBI cells were grown in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C and 5% CO₂. Cell lines stably expressing each primate Nbs1 were then generated using a retroviral transduction system as described in the Supplemental Experimental Procedures (File S1). Vero and HEK-293Ts were purchased from ATCC and cultured in DMEM with 10% FBS and Pen/Strep.

Viruses

HSV-1 strain 17, and its variant in1863 containing the lacZ gene in the thymidine kinase region (a kind gift from Chris Preston), were used. Infections and plaque assays were conducted as described in the Supplemental Experimental Procedures (File S1).

Antibodies and Immunoblotting

Primary antibodies were purchased from Genetex (Nbs1 Y112, Mre11 12D7, GAPDH GTX41577), Santa Cruz (β-actin C4, Rad50 13B3/2C6, ICP0 11060, ICP8 11E2), Sigma (FLAG M2, FLAG rabbit F7425 for IF), Abcam (GFP ab290, p-ATM S1981), Novus Biologicals (p-Nbs1 S343), Cell Signaling (p-p53 S15, pChk2 T68), Syd Labs (FLAG C2). The polyclonal anti-HSV-1 serum (NR-4017) was obtained from the BEI Resources Catalog. ICP8 antibody (serum 4–83) was a gift from David M. Knipe. The VP21 antibody was a gift from Gary H. Cohen. The ICP4 antibody was generated from the 58S ATCC hybridoma cell line. All secondary antibodies were purchased from Thermo Scientific (goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies conjugated to HRP) or Jackson Laboratories (Peroxidase AffiniPure goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies). Immunoblotting was performed as described in the Supplemental Experimental Procedures (File S1).

Immunoprecipitation

Co-immunoprecipitations were conducted as described in the Supplemental Experimental Procedures (File S1).

DNA repair assays

Cells were plated at a density of 200 cells per well in 6 well plates. The following day, media containing variable concentrations of camptothecin (Sigma) was added to the cells. 24 hours later, the media was replaced with fresh media. For X-ray irradiation experiments, the cells were subjected to the doses indicated using the Faxitron X-ray system at 120 kV and 5mA. All cells were grown to allow for colony formation at 37°C for at least 7 days. Colonies were stained with a crystal violet staining solution, washed, and counted. Cell counts from treated wells were normalized to untreated controls and expressed as percent survival.
Viral DNA and mRNA detection

Viral DNA and mRNA levels were measured by quantitative PCR. More details and primer sequences are given in the Supplemental Experimental Procedures.

Immunofluorescence

Cells were seeded at a density of $8 \times 10^4$ per well in 24 well plates containing glass coverslips coated with poly-L lysine. The next day, cells were infected with HSV-1 at an MOI of 10. At 2 hour post-infection, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Coverslips were incubated for 1 hour with the ICP4 (1:200) or FLAG rabbit F7425 (1:2000) antibody in PBS at room temperature and then incubated with secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (1:200) or Alexa Fluor 568 goat anti-rabbit IgG (1:200) (Life Technologies). Nuclei were visualized by staining with 1 μg/ml DAPI (Thermo Fisher, D1306).

Analysis of Intrinsic Disorder

IUPred (http://iupred.enzim.hu) (Dosztanyi et al., 2005) was used to predict structural disorder in Nbs1 amino acid sequences. A long disorder prediction type was selected for all analysis. For the analysis of disorder in mammalian proteins in general, disordered proportions predicted by iupred-l were directly downloaded from http://d2p2.pro/ (Oates et al., 2012). To test if the degree of structural disorder is different between the two groups of proteins, we used simple random permutations as described in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


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Highlights

- The DNA repair protein Nbs1 promotes early steps of HSV-1 replication
- Nbs1 is species-specific in its interaction with HSV-1 immediate-early protein ICP0
- Nbs1’s ICP0-interacting domain is structurally disordered and determines infectivity
- Intrinsically disordered domains are a common feature of virus-interacting proteins
Figure 1. Species-specific effects of Nbs1 on HSV-1 replication

(A) The percentage amino acid identity between human MRN proteins and their homologs in three other species. For Nbs1, the actual number of amino acid differences (ΔAAs) is also shown. Although orangutan and gibbon Nbs1 each differ from human Nbs1 by 21 amino acids, they differ from each another by 26 amino acids. (B) nbs1 cells stably expressing the indicated human or primate Nbs1 protein were infected with HSV-1 at an MOI of 0.01. Virus in the supernatant was harvested at 24 hours post infection and titrated on Vero cells. The resulting plaques (examples shown below graph) were counted and yield calculated as plaque forming units (PFU) per milliliter (mL). Results are an average of three independent replicates with error bars representing standard error. Expression levels of the different Nbs1 proteins in these stably complemented cell lines are also shown: (–) empty vector, (h) human, (g) gibbon, (o) orangutan, (r) rhesus Nbs1. (C) nbs1 cells stably expressing the indicated Nbs1 protein were infected with HSV-1 at an MOI of 0.01. Supernatants were collected at 12, 24, 36, and 48 hours post infection and viral yield was determined as in panel B. Results are presented as an average of three replicates with error bars representing standard deviations. (D) Infected cells in panel C were collected at the indicated time points and DNA was extracted. Viral DNA was quantified using ICP27 primers and normalized to the endogenous control gene RPLP0. Data is expressed as fold over the 4 hour time point (input) and error bars represent standard error.
Figure 2. DNA repair-related functions of human and gibbon Nbs1 are the same

(A) Extracts from nbs1 cells stably expressing human or gibbon Nbs1 were immunoprecipitated with an Nbs1 antibody. Precipitates were immunoblotted with antibodies to Nbs1, Rad50, Mre11. (B, C) nbs1 cells stably expressing human or gibbon Nbs1 were exposed to increasing doses of (B) camptothecin or (C) X-rays. Percent survival was calculated by normalizing the number of colonies after 7–10 days to untreated controls. Experiments were performed in triplicate and error bars represent standard deviations from the mean. See also Figure S1.
Figure 3. Two closely related lesser apes encode Nbs1 with opposite effects on HSV-1

(A) The phylogeny of gibbons is adapted from (Müller et al., 2003). The two species from which Nbs1 is tested in this study are in bold type. (B) nbs1 cells stably expressing human or siamang Nbs1 were infected with HSV-1 at an MOI of 0.01. Viral titers were determined by plaque assay on Vero cells. The results are an average of three experimental replicates with standard deviations. (C) nbs1 cells stably expressing Nbs1 were infected with HSV-1 at an MOI of 0.1. DNA was extracted after 9 hours and viral genome copy number was determined by qPCR using primers specific for the ICP27 gene. Viral DNA is expressed as fold over the vector only control cells. Error bars represent standard deviations. See also Figure S2.
Figure 4. Nbs1 is involved in the early steps of HSV-1 replication

(A) nbs1 cells stably expressing human or primate Nbs1 were infected with HSV-1 at an MOI of 0.1 and cells were collected at 3, 6, and 9 hours post infection (hpi). RNA was extracted and reverse transcribed. The resulting cDNA was then used for qPCR using ICP27 specific primers. Results are presented as fold over the endogenous RPLP0 mRNA control. Error bars represent standard deviations from three independent samples. (B) As in panel A, except PAA was added during infection. (C) nbs1 cells stably expressing human or primate Nbs1 were infected with HSV-1 at an MOI of 3 for 12 hours and then virus was titrated. Error bars represent standard deviations from three independent samples. (D) As in panel C except lysates were collected at 3, 6, 9, and 12 hours post infection (hpi). Immunoblotting detected viral proteins ICP0, ICP8, VP21, and the host protein GAPDH. (E) nbs1 cells stably expressing human or primate Nbs1 were infected with HSV-1 at an MOI of 10. After 2 hours, cells were fixed and ICP4 (green) and Nbs1 (red) were visualized via immunofluorescence. DAPI stain highlights the nucleus.
Figure 5. Nbs1 is species-specific in its interaction with HSV-1 ICP0

(A) HFF cells were infected with HSV-1 at an MOI of 3. Lysates were harvested at 3, 6, and 9 hours and subject to immunoprecipitation with an antibody against ICP0. Immunoblotting was performed with antibodies against ICP0 or Nbs1. (B) HEK-293T cells were co-transfected with plasmids encoding Nbs1-Flag and ICP0-GFP. Immunoprecipitation was performed using an anti-GFP antibody to precipitate GFP-ICP0 and immunoblotting was performed to detect Nbs1 binding. (C) The intensity of the bands in the top two rows in panel B were quantified using ImageJ and a ratio of the Nbs1/ICP0 signal is shown. (D) nbs1 cells stably expressing each primate Nbs1 were infected with HSV-1 at an MOI of 3 for 6 hours. Lysates were immunoprecipitated with an antibody against ICP0, and immunoblotting was performed with antibodies against ICP0 and Nbs1. In all panels, WCL is whole-cell lysate.
Figure 6. The species-specific effects of Nbs1 correlate to the induction of a DNA damage response in infected cells

(A) nbs1 cells complemented with gibbon or siamang Nbs1 were infected with HSV-1 in the presence of the viral DNA replication inhibitor PAA. Immunoblotting was performed to detect the viral proteins ICP0, ICP8, and VP21, as well as phosphorylated ATM and Chk2, in the hours post infection (hpi). GAPDH was detected as a loading control. (B) As in panel A, except at a different MOI as indicated. Immunoblotting detected host MRN proteins (Mre11, Rad50, and Nbs1), viral proteins (ICP0, ICP8, and those recognized by anti-HSV-1 serum), and phosphorylated host DNA damage response proteins (p-ATM, p-p53, and p-Nbs1). (C) The signals on three sets of immunoblots in panel B (p-ATM, p-p53, and p-Nbs1) were quantified using ImageJ.
Figure 7. Nbs1 interaction with ICP0 maps to a region of intrinsic disorder

(A) The gibbon and siamang Nbs1 protein sequences were analyzed for disorder tendency using IUPred (Dosztanyi et al., 2005). The domain structure of Nbs1 is shown above, and as expected the N-terminal globular domains (Williams et al., 2009) show low predicted disorder tendency. Tick marks on the domain structure indicate the 12 residue differences between gibbon and siamang Nbs1. See an alignment in Figure S2. The purple band indicates a region where the intrinsic disorder of siamang and gibbon Nbs1 is predicted to diverge. (B) Chimeric gibbon and siamang Nbs1 constructs were generated. Tick marks represent the amino acid residues exchanged between the gibbon and siamang Nbs1 proteins in the region of interest (purple band). (C) HEK-293T cells were co-transfected with plasmids encoding Nbs1-Flag and ICP0-GFP. Immunoprecipitation was performed using an anti-GFP antibody. WCL is whole cell lysate. (D) The top two rows in panel C was quantified using ImageJ, and a ratio of the Nbs1/ICP0 signal was taken. (E) nbs1 cells stably complemented with the indicated Nbs1 homologs or chimeras (X-axis) were infected with HSV-1 at an MOI of 0.01. Viruses were harvested from the supernatant at 30 hours post infection and titrated on Vero cells. The results are an average of three independent replicates with error bars representing standard deviations. (F) The proportion of proteins in each group (those that do and do not interact with viruses) was plotted against the fraction of each protein predicted to be disordered. Distribution curves were calculated using a smoothing window of 0.05.