Genome-wide RNAi screen identifies SEC61A and VCP as conserved regulators of Sindbis virus entry

Debasis Panda¹, Patrick P. Rose¹, Sheri L. Hanna¹, Beth Gold¹, Kaycie C. Hopkins¹, Randolph B. Lyde³, Michael S. Marks³, and Sara Cherry¹,²,*

¹Department of Microbiology, University of Pennsylvania, Philadelphia, PA 19104
²Penn Genome Frontiers Institute, University of Pennsylvania, Philadelphia, PA 19104
³Department of Pathology and Laboratory Medicine and Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104

SUMMARY

Alphaviruses are a large class of insect-borne human pathogens and little is known about the host factor requirements for infection. To identify such factors we performed a genome-wide RNAi screen using model Drosophila cells and validated 94 genes that impacted infection of Sindbis virus (SINV), the prototypical alphavirus. We identified a conserved role for SEC61A and VCP in facilitating SINV entry in insects and mammals. SEC61A and VCP selectively regulate trafficking of the entry receptor NRAMP2, and loss of these proteins, or pharmacological inhibition, leads to altered NRAMP2 trafficking to lysosomal compartments, and proteolytic digestion within lysosomes. NRAMP2 is the major iron transporter in cells, and loss of NRAMP2 attenuates intracellular iron transport. Thus, this study reveals new genes and pathways involved in both infection and iron homeostasis that may serve as targets for antiviral therapeutics or for iron imbalance disorders.

INTRODUCTION

Alphaviruses are a large group of emerging and re-emerging viruses, infecting more than a million people annually, in part due to lack of vaccines or antiviral agents (Gould et al., 2010; Strauss and Strauss, 1994; Weaver and Reisen, 2010). Many alphaviruses cause a self limiting disease characterized by chronic illness, arthralgia and myalgia, while others such as Venezuelan, Western and Eastern Equine Encephalitis viruses (VEEV, WEEV, EEEV), cause fatal disease in humans (Zacks and Paessler, 2010). Furthermore, VEEV, WEEV, EEEV, and Chikungunya virus are classified as biodefense agents (Weaver and Reisen, 2010). As arthropod-borne or arboviruses, the life-cycle of most alphaviruses is dependent on a mosquito vector and a vertebrate reservoir (Strauss and Strauss, 1994; Weaver and Barrett, 2004).

Alphaviruses have a nonsegmented single-stranded, positive sense RNA genome with a 5′-cap and 3′ poly (A) tail (Strauss and Strauss, 1994). The virus enters the cell by binding to a
plasma-membrane associated receptor and trafficking via clathrin-mediated endocytosis where fusion in a low pH compartment allows release of the viral genome into the cytoplasm and the launch of cytoplasmic replication (Strauss and Strauss, 1994). We have a limited knowledge of cellular factors involved in viral replication and pathogenesis of alphaviruses. Advances in genomic technologies and RNA interference (RNAi) methodologies has allowed for the development of high-throughput genome-wide RNAi screens to identify cellular factors that impact viral infection (Panda and Cherry, 2012). Robust in vitro and in vivo RNAi technology coupled with complete genome sequencing has made Drosophila an attractive model organism to conduct genome-scale RNAi screens (Cherry, 2008; Mohr and Perrimon, 2011). Importantly, a wide variety of viruses can infect Drosophila cells, including a number of mammalian arboviruses of medical and agricultural importance (Hughes et al., 2012). RNAi screening in Drosophila systems has advanced the discovery of host factors involved in virus infections including influenza, dengue and Drosophila C viruses (Cherry et al., 2005; Hao et al., 2008; Sessions et al., 2009).

To identify host genes that impact alphavirus infection, we took advantage of the prototypical alphavirus, Sindbis virus (SINV) (Strauss and Strauss, 1994). We performed a genome-wide high-throughput RNAi screen in Drosophila cells using SINV and identified a large number of genes as factors involved in SINV infection. Among the identified genes was dNRAMP, the major cellular iron transporter; we previously demonstrated that dNRAMP in insects and its ortholog NRAMP2 in vertebrates are entry receptors for SINV (Rose et al., 2011). Here we report the discovery of dSEC61A (Sec61α), dVCP (TER94), and dPMSD11 (Rpn6), as required factors for SINV infection at the level of entry. Moreover, we found that dSEC61A, dVCP and dPMSD11 positively regulate the expression level of the entry receptor protein dNRAMP. We extended these studies and found that human SEC61A1 and VCP are also required for SINV infection of mammalian cells. Depletion of SEC61A and VCP led to altered intracellular trafficking of NRAMP2 to lysosomes leading to its degradation. Since NRAMP2 is an essential iron transporter (Courville et al., 2006; Hentze et al., 2004), depletion of SEC61A or VCP led to decreased iron transport in human cells. Altogether, these results contribute towards a deeper understanding of alphavirus biology, regulatory mechanisms that impact selective trafficking, and iron homeostasis.

RESULTS
RNAi screen in Drosophila cells

Using a recombinant SINV (HRsp) that expresses GFP from a sub-genomic promoter (Burnham et al., 2007), we found that SINV productively infects Drosophila cells, consistent with previous work (Fig. S1A and (Hughes et al., 2012; Rose et al., 2011). Thus, we optimized a high-content assay in a 384-well format using dsRNAs against luciferase as a negative control and dsRNA against virus-encoded GFP and the vATPase component vha26 (dATP6V1E required for endosomal acidification) as positive controls (Fig. S1B and Fig. 1A). We performed the genome-wide screen in duplicate and identified 317 genes (2% of the Drosophila genome) whose silencing impacted SINV infection with a robust Z score of >2 or <2 in duplicate (p<0.001). The dataset was enriched for genes that have orthologs in both humans and mosquitoes (p<0.001) whereas Drosophila-specific genes were greatly under-represented (p<0.001) (Fig. 1B). Of the 317 genes identified in the primary screen, 70 were overtly cytotoxic (Robust Z score <2 in duplicate; ~40% decrease in cell number). Because cell death may influence data reliability, these genes were not further pursued. For validation, we generated independent dsRNAs against 227 genes. For large multiprotein complexes such as the proteasome, we chose to validate only a subset of those genes. Of the 227 genes directly tested, we validated 94 genes (42%) that impact SINV infection; 57 genes...
(60%) promoted infection and 37 genes (40%) restricted infection (Fig. 1C and Table S1). If we include the additional members of multi-protein complexes not directly tested, then we validated 51 additional genes (65% of the total gene set; Table S1). The SINV strain we used for our screen is a laboratory-adapted strain derived from Sindbis AR339 (Sherman and Griffin, 1990). Thus, we used a more virulent SINV strain, dsTE12H, carrying a similar eGFP reporter (Rose et al., 2011) and found that 75% of the genes that affected Sindbis HRsp infection also impacted dsTE12H infection (Fig. 1D).

We used a variety of functional annotation metrics to place these validated genes into cellular pathways and sub-cellular compartments, most likely relevant to SINV infection (Fig. 1E). Genes involved in clathrin-mediated endocytosis and vacuolar acidification were heavily represented as required for infection, and Gene ontology (GO) enrichment analysis further reinforced that SINV entry factors were significantly over-represented (Fig. S1C). In contrast, biological processes with a focus on transcription were highly enriched among the restriction factors (Fig. S1D). We further used Ingenuity Pathway Analysis to determine if the validated factors that influence SINV infection relate to specific cellular gene networks. Several gene networks were enriched in our dataset suggesting a functional role for these pathways in SINV infection (Fig. S1E).

Previously, genome-wide RNAi screens have been conducted to identify cellular factors that are required in the life-cycles of viruses including Drosophila C virus (DCV), influenza virus, and dengue virus (DENV) (Cherry et al., 2005; Hao et al., 2008; Sessions et al., 2009). When we analyzed the data sets from these published screens and compared them with the required factor dataset with our screen, we observed minimal overlap at the gene level. However, when we analyzed the over-represented gene ontology biological processes, we found that a number of biological processes were common to SINV and DENV as well as SINV and DCV (Fig. S1F). Furthermore, we directly tested whether the validated genes required for SINV infection were also required for the flavivirus West Nile virus (WNV) and DCV. Out of the 57 genes required for SINV, 35 genes and 14 genes are also required for WNV and DCV infection, respectively (Fig. S1G). Interestingly, the COP I complex and vATPase subunits are required for all three viruses examined suggesting that these complexes are co-opted by disparate viruses.

dSEC61A and the dVCP complex promote SINV infection

Our screen identified several cellular genes involved in the highly conserved ERAD pathway including dVCP, dUFD1L, dNPLOC4 and dSEC61A, and 22 components of the proteasome – which also functions in ERAD – as required factors for SINV infection (Fig. 1E). ERAD is central to maintaining protein quality control by preventing the toxic accumulation of misfolded proteins inside the endoplasmic reticulum (ER) (Buchberger et al., 2010; Vembar and Brodsky, 2008). So far the role of these genes in SINV or any alphavirus infection remained unknown, thus we explored the role of these factors in the SINV life cycle. We found that SINV infection was attenuated when we depleted cells for each of these genes using independent dsRNAs in two independent cell lines (Fig. 2A–B and Fig. S2A). Treatment of DL1 cells with these dsRNAs reduced the transcript or protein level of each gene (Fig. S2B–C) without any reduction in cell viability (data not shown). A reduction in SINV protein expression was also observed (Fig. 2C). The requirement for these proteins was specific for SINV since their depletion did not impact vesicular stomatitis virus (VSV) infection (Fig. 2D). In a complementary approach, we assessed the impact of a pharmacological inhibitor of VCP and SEC61A, Eyarestatin1 (Eer1) (Cross et al., 2009; Wang et al., 2008) on SINV infection in Drosophila cells. Eer1 was shown to inhibit VCP function in Drosophila (Griciuc et al., 2010) and treatment of DL1 cells with Eer1 reduced SINV infection, but not VSV infection, in a dose-dependent manner (Fig. 2E and Fig. S2D).
Protein quality control in the ER is monitored by the unfolded protein response (UPR), which acts in concert with the ERAD pathway to maintain ER homeostasis (Travers et al., 2000; Walter and Ron, 2011). Although we did not identify any genes involved in the UPR response in the genome-wide RNAi screen, we independently tested 5 key UPR genes (IRE1, PERK, ATF6, XBP1 and HSC70) in SINV replication and found that depletion of these factors did not impact SINV infection (Fig. S2E). Furthermore, upon SINV infection in Drosophila cells, we did not observe increased splicing of XBP1, a hallmark of UPR (data not shown). Altogether, these data suggest that SEC61A and the VCP-UFD1L-NPLOC4 complex play a specific role in SINV virus infection, and that this is not likely due to a global disturbance of ER quality control.

dSEC61A and the dVCP complex are required for SINV infection in adult flies

We next exploited the powerful genetic tools available in Drosophila to determine if these ERAD-associated proteins promote infection at the organismal level. dVCP, dUFD1L, dNPLOC4, and dSEC61A are essential genes (Leon and McKearin, 1999; Wang and Ward, 2010). Therefore, we used heat shock-inducible in vivo RNAi to deplete these proteins in adult flies and found that depletion of each component of the VCP-UFD1L-NPLOC4 complex as well as SEC61A significantly attenuated SINV infection (Fig. 2F).

SEC61A and VCP promote SINV infection of mosquito cells

Since the natural vector for SINV is a mosquito (Weaver and Barrett, 2004), we investigated the role of SEC61A and VCP during SINV infection of mosquito cells. We generated dsRNA against the annotated Aedes aegypti SEC61A ortholog (AAEL010716) and found that depletion of AESEC61A resulted in a modest reduction in SINV virus production (Fig. 2G). This may be due to redundancies since many organisms have multiple SEC61A orthologs and the Aedes aegypti genome is an incompletely annotated draft sequence. We were unable to identify clear orthologs of VCP, UFD1L or NPLOC4 in Aedes aegypti. Therefore, to examine the role of VCP we used two small molecule inhibitors Eer1 and DBeQ (Chou et al., 2011). We found that treatment with either Eer1 or DBeQ substantially reduced SINV infection in mosquito cells (Fig. 2H and S2F).

SINV entry is regulated by SEC61A and VCP

We next characterized the mechanism by which these proteins promote SINV infection. Since we observed a reduction in SINV protein expression upon the depletion of these components (Fig. 2C), we reasoned that they likely affect SINV at an early stage of infection. We first determined whether SEC61A, VCP, UFD1L or NPLOC4 regulate a pre- or post-entry step of SINV infection. To this end, we performed an ‘acid bypass’ assay which exploits the fusion of bound viruses with the plasma membrane when the extracellular pH is decreased to that of an endosomal compartment, thereby bypassing endosomal entry requirements (Liao and Kielian, 2005). First, we validated this assay by depleting the vacuolar ATPase (vATPase) component dATP6V1E, which is required for endosomal entry but should be dispensable for entry from the plasma membrane. Indeed, low pH treatment of plasma membrane bound SINV bypassed vATPase dependence and enhanced SINV infection as expected, while dsRNA treatment against the viral reporter GFP could not be bypassed (Fig. 3A). Under these conditions, we observed that low pH treatment bypassed the requirement for dSEC61A similar to that of dATP6V1E (Fig. 3A). Furthermore, we were able to bypass the requirements for VCP, UFD1L and NPLOC4, although the rescue was more modest (Fig. 3A).

To further explore the mechanistic requirement for SEC61A and VCP during SINV infection we took advantage of a time-of-addition assay. First, we used ammonium chloride (NH₄Cl), which blocks endosomal acidification and SINV entry, to identify the time after
which virus has entered cells and is thus insensitive to treatment. Pre-treatment of cells with \( \text{NH}_4\text{Cl} \) attenuated SINV infection, but SINV infection became resistant to \( \text{NH}_4\text{Cl} \) at 2 hr post infection (Fig. S3 A–B) suggesting that SINV has completed entry by 2 hrs post infection. Pretreatment of cells with the VCP inhibitor Eer1 reduced SINV infection while Eer1 treatment at 2 hrs post infection did not restrict SINV infection (Fig. 3B). In contrast, VSV infection was unaffected by Eer1 treatment at all time points (Fig. 3B). Altogether, these data demonstrate that dSEC61A and dVCP play a role in the early stage (likely during or immediately after virus entry) of SINV infection.

**NRAMP expression is altered by loss of SEC61A and VCP**

Because both SINV and VSV entry are dependent on clathrin-mediated endocytosis and acidification, and dSEC61A and dVCP specifically promote SINV entry, these data suggested that these genes promote a distinct aspect of SINV infection. Since SINV but not VSV uses NRAMP as a receptor (Rose et al., 2011), we examined whether the SINV-specific requirement for these proteins reflected changes in the level of NRAMP. Because antibodies to endogenous dNRAMP are unavailable, we generated a cell line stably expressing epitope-tagged dNRAMP (Fig. S3C). We validated that dNRAMP mRNA and protein levels are significantly reduced by dsRNA treatment against dNRAMP (Fig. 3C–D). RNAi against dSEC61A and dVCP reduced dNRAMP levels, while neither impacted mRNA levels (Fig. 3C–D). This effect is not likely due to a general requirement for these proteins in plasma membrane receptor expression, as the levels of plasma membrane receptor dToll7 or DE-Cadherin were not decreased by RNAi against this panel of genes (Fig. S3 D–E). Consistent with the observed decrease in NRAMP levels, SINV binding to the plasma membrane was significantly decreased when we knocked down dSEC61A and modestly decreased with VCP knockdown (Fig. S3F). Taken together, these data suggest that dSEC61A and dVCP play a role in SINV infection likely by positively regulating expression of dNRAMP.

**The proteasome promotes SINV entry**

Protein substrates of ERAD are targeted to the proteasome for subsequent degradation (Vembar and Brodsky, 2008). Our screen identified 22 proteasomal components as required for SINV infection (Fig. S1C). We focused on dPSMD11 which is a non-ATPase subunit of the 19S regulator complex of the 26S proteasome and whose depletion does not significantly impact cell viability (data not shown). Knockdown of dPSMD11 (Fig. S2B) significantly reduced SINV infection (Fig. 4A), whereas VSV infection was not affected (Fig. 4B), further suggesting that the proteasome plays a crucial and specific role in SINV infection. Like dSEC61A and dVCP, dPSMD11 functions during SINV entry since low pH treatment substantially bypassed the requirement of dPSMD11 in SINV infection (Fig. 4C) and dPSMD11-depletion leads to a substantial decrease in dNRAMP protein levels (Fig. 4D). Altogether, these data suggest that the proteasome regulates SINV entry likely by promoting dNRAMP expression. However, the incomplete rescue of SINV infection by low pH treatment suggests that the proteasome might function in additional downstream steps in the SINV life cycle.

**SEC61A1 and VCP promote SINV infection of human cells**

Since the SINV entry pathway is highly conserved and vertebrates are a natural host, we next tested a role for these components in SINV infection of mammalian cells and found that siRNAs to VCP and SEC61A1 induced a 2–3 fold reduction in SINV infection in human osteosarcoma (U2OS) cells (Fig. 5A–C) while VSV infection was not reduced (Fig. 5D). We verified the decrease in SINV infection using two independent siRNAs for each gene individually or together, which depleted SEC61A1 or VCP protein levels by more than 70% as measured by immunoblot (Fig. S4A–B). Moreover, pretreatment of U2OS cells with the
VCP inhibitors Ee1 (Fig. 5E) or DBeQ (Fig. 5F, S4C) or the proteasome inhibitors MG132 or lactacystin (Fig. 5G) attenuated SINV infection in a dose-dependent manner in the absence of cytotoxicity. Because sentinel cells such as macrophages are among the first cells infected during infection of the mammalian host and are required for efficient spread (Klimstra et al., 2003), we used a human monocytic cell line, and found that treatment of THP1 cells with the VCP inhibitors Ee1 or DBeQ significantly decreased infection (Fig. S4D).

To define the step in the viral lifecycle restricted by these drugs in human cells, we performed time-of-addition studies. SINV entry in U2OS cells occurs within 2 hr of infection since viral protein expression is no longer sensitive to ammonium chloride by 2 hr post infection (Fig. S4E). Whereas pretreatment of U2OS cells with either Ee1 or MG132 attenuated infection, infection was largely insensitive to treatment at 2 hr post infection (Fig. S4F–G). This indicates that VCP and the proteasome are required during or soon after virus entry in mammalian cells. Also, depletion of SEC61A or VCP led to reduction in the level of NRAMP2 protein (Fig. 5H). These results suggest that SEC61A and VCP impact SINV entry by likely regulating NRAMP2 expression across hosts.

**SEC61A and VCP regulate NRAMP2 localization**

Previous studies have shown that NRAMP2 rapidly shuttles between endosomal compartments and the plasma membrane (Lam-Yuk-Tseung and Gros, 2006; Tabuchi et al., 2002). Furthermore, VCP regulates endosomal dynamics and endosomal transport of transferrin receptor (TFR) and caveolin 1 (Ramanathan and Ye, 2012; Ritz et al., 2011). Since TFR traffics via a similar route as NRAMP2, we tested whether VCP or SEC61A impact the dynamics of NRAMP2. In control cells, NRAMP2 is largely present in early endosomes, as measured by colocalization with early endosomal antigen 1 (EEA1) (Fig. 6A), and overlaps poorly with the late endosome and lysosome marker LAMP1 (Fig. 6B; quantified in Fig S5A). Depletion of SEC61A and VCP resulted in enlargement and perinuclear clustering of EEA1-positive endosomes (Fig. 6A), as previously reported for VCP depletion (Ramanathan and Ye, 2012; Ritz et al., 2011), although EEA1 levels were not affected (Fig. 6E). Importantly, NRAMP2 overlap with EEA1 decreased and overlap with LAMP1 increased in both SEC61A- and VCP-depleted cells (Figs. 6A, B and S5A). Similarly, treatment of U2OS cells with Eer1 or DBeQ resulted in decreased localization of NRAMP2 to EEA1-positive early endosomes (Fig. S5C) and increased co-localization of NRAMP2 with LAMP1-positive vesicles (Fig. S5D). These data suggest that NRAMP2 is mis-targeted to late endosomes and lysosomes upon depletion of SEC61A and VCP. We next examined whether lysosomal proteases are responsible for the loss of NRAMP2 protein and found that treatment of cells with lysosomal protease inhibitors (Reusch et al., 1999) significantly restored NRAMP2 expression as measured by immunoblotting (Fig. 6C–D) and increased the degree of colocalization of NRAMP2 with LAMP1 (Fig. S5A–B).

To determine whether SEC61A and VCP impact endosomal trafficking globally, we assessed the effect of SEC61A and VCP depletion on infection by other viruses that, like SINV, require clathrin-mediated endocytosis for entry. In addition to VSV (Fig. 5D), infection of U2OS cells by two alphaviruses, Chikungunya virus (CHIKV) and Ross river virus (RRV), whose entry is clathrin-dependent but NRAMP2 independent (Rose et al., 2011; Sharkey et al., 2001; Sourisseau et al., 2007) and our unpublished results), were not impaired by depletion of SEC61A or VCP (Fig. S5E–F). These data suggest that SEC61A and VCP may not globally impact endocytosis or virus infection.

A recent paper demonstrated that transferrin receptor (TFR) recycling is delayed by VCP depletion but whether this delay had functional consequences is unknown (Ramanathan and Ye, 2012). Because both NRAMP2 and TFR expression are regulated by the level of iron...
and both the proteins traffic via similar routes, we tested whether TFR localization, degradation or function are also regulated by SEC61A and VCP. We found that the level of TFR protein was unaffected by depletion of SEC61A or VCP (Fig. 6E). We then examined whether SEC61A and VCP impacted entry of Junin virus, a virus that uses TFR as an entry receptor (Radoshitzky et al., 2007), using Junin virus glycoproteins pseudotyped on a VSV core and found that infection of the pseudotyped virus was not affected by depletion of SEC61A or VCP (Fig. S5G). These data suggest that the role for SEC61A and VCP in NRAMP2 trafficking is distinct from the previously described role for VCP in regulating the kinetics of TFR recycling. Rather, SEC61A and VCP might selectively regulate the lysosomal delivery of particular transmembrane cargo proteins. To test this, we screened a panel of membrane proteins in human cells depleted for SEC61A or VCP for changes in protein levels (Fig. 6E–H). In addition to TFR, we tested for effects on Polio Virus Receptor (PVR), Cadherin, Junctional Adhesion Molecule 1 (JAM1), and β1 Integrin which are all plasma membrane-associated proteins with single transmembrane domains. Because NRAMP2 is a multipass transmembrane protein (12 transmembrane domains), we also tested for effects on three ion transporters, ATP1A1, ATP7A and GLUT1, which have 8 or more transmembrane domains. We found that the expression of TFR, EEA1, Cadherin, JAM1, PVR, ATP7A, and ATP1A1 are not affected by the loss of SEC61A or VCP. In contrast, we found that in addition to NRAMP2, β1 Integrin and GLUT1 expression also require SEC61A and VCP.

SEC61A, VCP and the proteasome promote NRAMP-dependent iron transport

NRAMP is the major cellular iron transporter and its expression is tightly regulated to ensure proper iron uptake since either too much or too little iron can result in pathology (Hentze et al., 2004) and NRAMP levels largely dictate intracellular iron concentrations (Andrews and Schmidt, 2007). Therefore, we tested whether these factors also promote iron transport. First, we explored this in Drosophila cells where we adapted a well-established calcein AM-based fluorescence assay to monitor iron uptake (Cabantchik et al., 1996). Treatment of Drosophila cells with iron led to a reduction in fluorescence (Fig. 7A). This reduction was dependent on dNRAMP since depletion of dNRAMP significantly attenuated iron-induced changes in fluorescence (Fig. 7A). Similarly, depletion of dSEC61A or dPSMD11 also prevented quenching of fluorescence suggesting a reduction in iron transport (Fig. 7A–B). These data suggest that optimal dNRAMP-dependent iron import requires SEC61A and the proteasome. We extended these studies to human cells and examined whether SEC61A and VCP affected NRAMP2 activity by assaying for iron transport in human embryonic kidney (HEK-293T) cells that ectopically express Nramp2. (Wetli et al., 2006). Consistent with the results in Drosophila cells, we found that depletion of either SEC61A or VCP led to decreased iron uptake (Fig. 7C).

DISCUSSION

Over the past several decades, studies in Drosophila have been central to our understanding of various fundamental biological processes. In addition to being a public health threat, alphaviruses are gaining popularity as potential antitumor agents as well as a vaccine platform (Atkins et al., 2008; Quetglas et al., 2010; Riezebos-Brilman et al., 2006). To expand our knowledge of alphavirus-host interactions, we performed a genome-wide RNAi screen in Drosophila as a model insect. Importantly, we have identified several previously unrecognized factors and cellular pathways that either promote or restrict SINV infection. Additional plasma membrane associated genes such as dSLC22A13 (orct2) and dSCARB1 (peste) promote infection, raising the possibility that these factors act as co-receptors. A large number of genes that we validated have human orthologs suggesting that many of these genes may play similar roles in regulating SINV infection in mammalian systems;
thus, further exploration of these genes will undoubtedly reveal new insights into alphavirus-host interactions.

In this report we focused on a group of genes that are associated with ERAD, which included all three subunits of the VCP-UFD1L-NPLOC4 complex, SEC61A, and 22 subunits of the proteasome. Interestingly, while the proteasome impacts the replication of many viruses including positive strand RNA viruses such as flaviviruses (Gao and Luo, 2006; Gilfoy et al., 2009), to our knowledge, a role for the proteasome in alphavirus entry has not been previously described. We found that depletion of dSEC61A, dVCP and dPMSD11 reduce the levels of the entry receptor dNRAMP post-transcriptionally. The expression of Toll-7 and DE-Cadherin, two plasma membrane-associated proteins (Nakamoto et al., 2012; Tepass, 1999) was not altered by depletion of SEC61A and VCP suggesting that these genes may not regulate membrane proteins globally. Our findings with dNPLOC and dUFD1L demonstrate that they are also important in SINV entry, but more modestly. This may be due to technical reasons including differences in the efficiency of silencing or that the different assays employed have differential sensitivity. Alternatively, it is possible that these genes work through a different mode of action.

One possible mechanism for the control of NRAMP by these genes is that the ERAD-proteasome pathway targets a negative regulator of NRAMP stability for degradation. However, if the proteasome degraded NRAMP downstream of ERAD, loss of proteasomal components would lead to increased NRAMP levels. Furthermore, we could not rescue in NRAMP degradation upon simultaneous depletion of SEC61A or VCP with several known negative regulators of NRAMP in Drosophila cells such as NDFIP and WWP2 (data not shown) (Foot et al., 2008). This raised the possibility that SEC61A and VCP target a yet unknown negative regulator of NRAMP2 or more likely, act in a ERAD-independent manner. A recent study found that VCP regulates early endosomal morphology and the kinetics of transferrin trafficking (Ramanathan and Ye, 2012) and NRAMP2 can be mislocalized to lysosomes under some conditions (Lam-Yuk-Tseung and Gros, 2006). We found that SEC61A and VCP are required for the proper endosomal trafficking of NRAMP2. Thus, our data support and ERAD-independent role.

To explore the specificity of this SEC61A and VCP-dependent trafficking we first demonstrated that general endocytosis and expression of a number of plasma membrane and endosomal proteins are unaffected. Rather, specific cargoes (membrane receptors) are subject to regulation by SEC61A and VCP. In addition to NRAMP2, GLUT1 and β1 Integrin expression also require SEC61A and VCP activity. This specificity cannot be simply explained by structural similarities. First, NRAMP2 is a multipass transmembrane protein while β1 Integrin is not. Second, GLUT1 but not other multipass proteins tested were impacted by loss of SEC61A or VCP function. We considered whether SEC61A and VCP might regulate the activity of sorting nexins, which are known to retrieve selective cargo from lysosomal transport and degradation. However, whereas GLUT1 and ATP7A are cargoes for sorting nexin 27 (SNX27), β1 Integrin is a cargo for sorting nexin 17 (SNX17) (Steinberg et al., 2013; Steinberg et al., 2012), indicating that the cargo dependencies we identified are distinct from those identified for known sorting nexins. The cytoplasmic domain of NRAMP2 isoform1 has a ‘LL’ motif which is required for clathrin mediated endocytosis and targeting to endosomes and lysosomes (Lam-Yuk-Tseung et al., 2005). However such classic ‘LL’ motifs are absent from GLUT1 and β1 Integrin, both of which are internalized by clathrin-independent endocytosis (Maldonado-Baez et al., 2013), indicating that SEC61A1 and VCP regulate cargoes that are internalized by different mechanisms. This suggests that VCP and SEC61A1 might target these cargos not at the plasma membrane but rather at a downstream compartment such as the sorting endosome. Furthermore, VCP inhibition or depletion impacts different cargoes differently; while it
slowed TFR recycling, it did not target it for degradation ((Ramanathan and Ye, 2012) and our data) and Caveolin-1 was blocked from degradation while NRAMP2 is degraded ((Ritz et al., 2011) and our results). Altogether, our results found that only a subset of plasma membrane cargoes are specifically regulated by SEC61A and VCP pointing towards a yet uncharacterized role in endosomal sorting.

NRAMP is the major iron transporter in cells and it is known to be tightly regulated post-transcriptionally and post-translationally to tightly regulate intracellular iron concentrations (Brasse-Lagnel et al., 2011; Foot et al., 2008). We found that SEC61A and VCP promote iron import across hosts by regulating NRAMP expression by a mechanism that has not been previously described. This has important implications in pathologies associated with iron homeostasis dysfunction. The other two plasma membrane proteins we found that are dependent on SEC61A and VCP are GLUT1 and β1 Integrin. GLUT1 is a glucose transporter that plays essential roles in energy metabolism and β1 Integrin impacts diverse biological processes including cell adhesion suggesting a role for SEC61A and VCP in the regulation of cellular homeostasis more broadly. Further studies will clarify this new regulatory pathway and perhaps elucidate new therapeutic targets to treat alphaviruses or human disorders associated with an iron imbalance as well as provide insight into the regulation of energy metabolism.

MATERIALS AND METHODS

Cells, viruses and reagents

Insect cells were maintained as previously described (Rose et al., 2011). Drugs were purchased from Sigma. SINV (HRsp-GFP and dTE12H-GFP) stocks were propagated in C6/36 cells (Burnham et al., 2007). VSV-GFP was propagated in BHK-21 cells (Ramsburg et al., 2005). Viral titers for MOI calculations were determined in BHK-21 cells. The following antibodies were used in this study: Anti-SEC61A1(ab15575, Abcam), Anti-VCP (ab11433, Abcam), Anti-Actin (Sc 47778, Santa Cruz Biotechnology), Anti-GFP (Sc-9996, Santa Cruz Biotechnology), Anti-DE-cadherin (Developmental studies hybridoma bank), anti-LAMP1(H4A3, Developmental studies hybridoma bank), Anti-tubulin (T5168, Sigma), Anti-EEA1 (610457, BD Transduction laboratories), anti HA (Clone 3F10, Roche), Anti-ATP1A1 (Roche), Anti-Cadherin (Sc 1499, Santa Cruz Biotechnology), Anti-PVR (Sc 27754, Santa Cruz Biotechnology), Anti-JAM-1 (36-1700, Invitrogen), Anti-ATP7A (Setty et al., 2008), Anti-GLUT1 (a generous gift from M. Birnbaum) and anti-β1 Integrin (610467, BD transduction laboratories).

**Drosophila genome-wide RNAi screen**

DL1 cells (18,000/well) were seeded into 384-well plates pre-arrayed with 250ng/well dsRNA (Ambion) in 10μL of serum free media using automated liquid handling (Wellmate, Thermo Scientific). One hour later, 20μL of complete media was added, and the plates were incubated for three days and then infected with SINV (MOI of 10) for 36 hours. The plates were fixed in 4% formaldehyde in PBS for 10 minutes and washed twice in PBS. Cells were stained with 5μg/mL Hoechst 33432 and washed twice with PBS. GFP and DAPI images were captured, and 3 sites per well were imaged at 20X (ImageXpress Micro, Molecular Devices). Automated image analysis (MetaXpress, Molecular Devices) segmented the images and was used to determine the number of DAPI positive cells and the number of GFP positive cells. The percentage of infected cells was calculated, averaged for the three sites, and log transformed. The plate median and interquartile range was calculated. These metrics were used to calculate a robust Z score for each well using the following equation: 

\[
\left\{ \frac{\log_{10}(\%\text{infection}) - \log_{10}(\text{median})}{\text{IQR} * 0.74} \right\}
\]

(Zhang et al., 2006). Candidates were identified as positive if the robust Z score was < 2 or >2 in both independent replicates.

Cell Rep. Author manuscript; available in PMC 2014 December 26.
Cytotoxic candidates were identified based on nuclei counts and those with a Robust Z score < -2 in duplicate wells were considered toxic (~40% decrease in cell number).

**Secondary screen and statistical analysis**

Independent secondary amplicons were chosen from a different region within the target gene using SNAPdragon (Flybase.org) and used to generate dsRNA against candidate genes. Validation screening assay was performed as in the primary screen for SIN HRsp using either SIN HRsp or dsTE12H. Candidates were identified as positive if the robust Z score was < 1.5 or >1.5 in duplicate (p<0.009).

**Viral infection of insect cells**

DL1 cells in 96 or 384 well plates were infected with SINV at an MOI of 20 for 42 hrs. In 12 well plates, DL1 cells were infected with SINV at an MOI of 5 for 18–20 hrs. DL1 cells were infected with VSV at an MOI of 5. Aag2 cells were infected with SINV at an MOI of 3 for 16 hrs.

**Adult fly infections**

Flies obtained from the Bloomington Stock Center or VDRC (TER94: v24354, CG4673: v21917, Ufd1-like: v24700, Sec61alpha: v42763) were crossed to hs-GAL4 and the adult progeny (4–7 day old) were heat shocked at 37°C every other day to induce RNAi throughout the experiment. Infections were carried out as described previously using the indicated genotypes (Cherry and Perrimon, 2004). Virus production was assayed by crushing five flies and titering on BHK cells in three independent experiments (Rose et al., 2011).

**Human cells**

Human cells were transfected with a pool of two independent siRNAs for VCP (s14765, Ambion and J008727-11, Dharmacon) and SEC61A1 (s26721, s26722, Ambion) using Hiperfect (Qiagen, CA) at 20nM according to manufacturer’s instruction. At 72 hr post transfection, cells were replated in a 96 well format (30,000 cells per well) for infection or iron transport assays or replated in 12 well plate (300,000 cells per well) for virus infection. U2OS cells were infected with SINV at an MOI of 1 and processed for automated microscopy or immunoblot at 8 hpi. For all the experiments involving siRNA, a combination of pool of two siRNAs were used except for panels 6A–C where one siRNA targeting each gene was used.

**Immunofluorescence microscopy**

U2OS cells were transfected with the siRNAs and after 48h transfected with HA-NRAMP2. Twenty four hours later cells were replated on glass coverslips. Cells were fixed with 4% formaldehyde, permeabilized for 10 min with 0.1% Triton X-100 and stained with the indicated antibodies. Images were captured using 63X objective with a Leica DMI 4000 B fluorescent microscope and 3D deconvolution of the images were performed (AutoQuantX2).

**Bioinformatics and Statistical analysis**

Orthologs were determined using homologene (NCBI) and Chi squared test was used to calculate significance. Student’s T test was performed on each individual experiment. Experiments were performed at least three times and a p value < 0.05 was considered significant if the p value was < 0.05 in each independent experiment.
**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We would like to thank J. Rose for VSV-GFP, R. Hardy for SINV-GFP (HRsp), B. Levine for SINV (dsTE12H), A. Raikhel for Aag2 cells, P. Buckett and M. Wessling-Resnick for 293T-Nramp2 cells and Bloomington Stock Center for fly stocks. We thank C. Coyne for antibodies, and Ingenuity analysis; and M. Heise for Ross River virus and for critical comments on the manuscript. We thank M. Birnbaum for GLUT1 antibody, Susan Ross for Junin/VSV pseudotypes and M. Diamond for Chikungunya virus. We would like to thank members of the Cherry lab for helpful discussions and advice. This work was supported by National Institute of Health grants R01AI074951, U54AI057168, R21AI103441 and R01AI095500 to SC. SC is a recipient of the Burroughs Wellcome Investigators in the Pathogenesis of Infectious Disease Award.

**References**

Andrews NC, Schmidt PJ. Iron homeostasis. Annual review of physiology. 2007; 69:69–85.
Atkins GJ, Fleeton MN, Sheahan BJ. Therapeutic and prophylactic applications of alphavirus vectors. Expert reviews in molecular medicine. 2008; 10:e33. [PubMed: 1900329]
Brasse-Lagnel C, Karim Z, Letteron P, Bekri S, Bado A, Beaumont C. Intestinal DMT1 cotransporter is down-regulated by hepcidin via proteasome internalization and degradation. Gastroenterology. 2011; 140:1261–1271. e1261. [PubMed: 21199652]
Buchberger A, Bukau B, Sommer T. Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. Molecular cell. 2010; 40:238–252. [PubMed: 20965419]
Burnham AJ, Gong L, Hardy RW. Heterogeneous nuclear ribonucleoprotein protein K interacts with Sindbis virus nonstructural proteins and viral subgenomic mRNA. Virology. 2007; 367:212–221. [PubMed: 17561226]
Cabantchik ZI, Glickstein H, Milgram P, Breuer W. A fluorescence assay for assessing chelation of intracellular iron in a membrane model system and in mammalian cells. Analytical biochemistry. 1996; 233:221–227. [PubMed: 8789722]
Cherry S. Genomic RNAi screening in Drosophila S2 cells: what have we learned about host-pathogen interactions? Current opinion in microbiology. 2008; 11:262–270. [PubMed: 18539520]
Cherry S, Doukas T, Armknecht S, Whelan S, Wang H, Sarnow P, Perrimon N. Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition. Genes & development. 2005; 19:445–452. [PubMed: 15713840]
Cherry S, Perrimon N. Entry is a rate-limiting step for viral infection in a Drosophila melanogaster model of pathogenesis. Nat Immunol. 2004; 5:81–87. [PubMed: 14691479]
Chou TF, Brown SJ, Minond D, Nordin BE, Li K, Jones AC, Chase P, Porubsky PR, Stoltz BM, Schoonen FJ, et al. Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic protein clearance pathways. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:4834–4839. [PubMed: 21383145]
Courville P, Chaloupka R, Cellier MF. Recent progress in structure-function analyses of Nramp proton-dependent metal-ion transporters. Biochemistry and cell biology = Biochimie et biologie cellulaire. 2006; 84:960–978. [PubMed: 17215883]
Cross BC, McKibbin C, Callan AC, Robotti P, Piacenti M, Rabu C, Wilson CM, Whitehead R, Flitsch SL, Pool MR, et al. Eeyarestatin I inhibits Sec61-mediated protein translocation at the endoplasmic reticulum. Journal of cell science. 2009; 122:4393–4400. [PubMed: 19903691]
Foot NJ, Dalton HE, Shearwin-Whyatt LM, Dorstyn L, Tan SS, Yang B, Kumar S. Regulation of the divalent metal ion transporter DMT1 and iron homeostasis by a ubiquitin-dependent mechanism involving Ndfips and WWP2. Blood. 2008; 112:4268–4275. [PubMed: 18776082]
Gao G, Luo H. The ubiquitin-proteasome pathway in viral infections. Canadian journal of physiology and pharmacology. 2006; 84:5–14. [PubMed: 16845885]
Gilfoy F, Fayzulin R, Mason PW. West Nile virus genome amplification requires the functional activities of the proteasome. Virology. 2009; 385:74–84. [PubMed: 19101004]
Gould EA, Coutard B, Malet H, Morin B, Jamal S, Weaver S, Gorbunov G, Baronti C, Delogu I, et al. Understanding the alphaviruses: recent research on important emerging pathogens and progress towards their control. Antiviral research. 2010; 87:111–124. [PubMed: 19616028]

Griciuc A, Aron L, Piccoli G, Ueffing M. Clearance of Rhodopsin (P23H) aggregates requires the ERAD effector VCP. Biochimica et biophysica acta. 2010; 1803:424–434. [PubMed: 20097236]

Hao L, Sakurai A, Watanabe T, Sorensen E, Nidom CA, Newton MA, Ahlquist P, Kawaoka Y. Drosophila RNAi screen identifies host genes important for influenza virus replication. Nature. 2008; 454:890–893. [PubMed: 18615016]

Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. Cell. 2004; 117:285–297. [PubMed: 15109490]

Hughes TT, Allen AL, Bardin JE, Christian MN, Daimon K, Dozier KD, Hansen CL, Holcomb LM, Ahlander J. Drosophila as a genetic model for studying pathogenic human viruses. Virology. 2012; 423:1–5. [PubMed: 22177780]

Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. Journal of virology. 2003; 77:12022–12032. [PubMed: 14581539]

Lam-Yuk-Tseung S, Gros P. Distinct targeting and recycling properties of two isoforms of the iron transporter DMT1 (NRAMP2, Slc11A2). Biochemistry. 2006; 45:2294–2301. [PubMed: 16475818]

Lam-Yuk-Tseung S, Touret N, Grinstein S, Gros P. Carboxyl-terminus determinants of the iron transporter DMT1/SLC11A2 isoform II (-IRE/1B) mediate internalization from the plasma membrane into recycling endosomes. Biochemistry. 2005; 44:12149–12159. [PubMed: 16142913]

Leon A, McKeand D. Identification of TER94, an AAA ATPase protein, as a Bam-dependent component of the Drosophila fusome. Molecular biology of the cell. 1999; 10:3825–3834. [PubMed: 10564274]

Liao M, Kielian M. Domain III from class II fusion proteins functions as a dominant-negative inhibitor of virus membrane fusion. The Journal of cell biology. 2005; 171:111–120. [PubMed: 16216925]

Maldonado-Baez L, Williamson C, Donaldson JG. Clathrin-independent endocytosis: A cargo-centric view. Experimental cell research. 2013

Mohr, SE.; Perrimon, N. Wiley interdisciplinary reviews RNA. 2011. RNAi screening: new approaches, understandings, and organisms.

Nakamoto M, Moy RH, Xu J, Bambina S, Yasunaga A, Shelly SS, Gold B, Cherry S. Virus recognition by Toll-7 activates antiviral autophagy in Drosophila. Immunity. 2012; 36:658–667. [PubMed: 22464169]

Panda D, Cherry S. Cell-based genomic screening: elucidating virus-host interactions. Current opinion in virology. 2012; 2:784–792. [PubMed: 23122855]

Quetglas JI, Ruiz-Guillem M, Aranda A, Casales E, Beznarte J, Smerdou C. Alphavirus vectors for cancer therapy. Virus research. 2010; 153:179–196. [PubMed: 20692305]

Radoshitzky SR, Abraham J, Spiropoulou CF, Kuhn JH, Nguyen D, Li W, Nagel J, Schmidt PJ, Nunberg JH, Andrews NC, et al. Transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses. Nature. 2007; 446:92–96. [PubMed: 17287727]

Ramanathan HN, Ye Y. The p97 ATPase associates with EEA1 to regulate the size of early endosomes. Cell research. 2012; 22:346–359. [PubMed: 21556036]

Ramsburg E, Publicover J, Buonocore L, Poolek M, Robek M, Palin A, Rose JK. A vesicular stomatitis virus recombinant expressing granulocyte-macrophage colony-stimulating factor induces enhanced T-cell responses and is highly attenuated for replication in animals. Journal of virology. 2005; 79:15043–15053. [PubMed: 16306575]

Reusch U, Muranyi W, Lucin P, Burgert HG, Hengel H, Koszinowski UH. A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. The EMBO journal. 1999; 18:1081–1091. [PubMed: 10022849]

Riezebos-Brilman A, de Mare A, Bungener L, Huckriede A, Wilschut J, Daemen T. Recombinant alphaviruses as vectors for anti-tumour and antimicrobial immunotherapy. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology. 2006; 35:233–243. [PubMed: 16448844]
Ritz D, Vuk M, Kirchner P, Bug M, Schult S, Hayer A, Bremer S, Lusk C, Baloh RH, Lee H, et al. Endolysosomal sorting of ubiquitylated caveolin-1 is regulated by VCP and UBXD1 and impaired by VCP disease mutations. Nature cell biology. 2011; 13:1116–1123.

Rose PP, Hanna SL, Spiridigliozzi A, Wannisorn N, Beitig DF, Ross SR, Hardy RW, Bambina SA, Heise MT, Cherry S. Natural resistance-associated macrophage protein is a cellular receptor for sindbis virus in both insect and mammalian hosts. Cell host & microbe. 2011; 10:97–104. [PubMed: 21843867]

Sessions OM, Barrows NJ, Souza-Neto JA, Robinson TJ, Hershey CL, Rodgers MA, Ramirez JL, Dimopoulos G, Yang PL, Pearson JL, et al. Discovery of insect and human dengue virus host factors. Nature. 2009; 458:1047–1050. [PubMed: 19396146]

Setty SR, Tenza D, Sviderskaya EV, Bennett DC, Raposo G, Marks MS. Cell-specific ATP7A transport sustains copper-dependent tyrosinase activity in melanosomes. Nature. 2008; 454:1142–1146. [PubMed: 18650808]

Sharkey CM, North CL, Kuhn RJ, Sanders DA. Ross River virus glycoprotein-pseudotyped retroviruses and stable cell lines for their production. Journal of virology. 2001; 75:2653–2659. [PubMed: 11222688]

Sherman LA, Griffin DE. Pathogenesis of encephalitis induced in newborn mice by virulent and avirulent strains of Sindbis virus. Journal of virology. 1990; 64:2041–2046. [PubMed: 1691310]

Sourisseau M, Schilt C, Casartelli N, Trouillet C, Guivel-Benhassine F, Rudnicka D, Sol-Foulon N, Le Roux K, Prevost MC, Fsihi H, et al. Characterization of reemerging chikungunya virus. PLoS pathogens. 2007; 3:e89. [PubMed: 17604450]

Steinberg F, Gallon M, Winfield M, Thomas EC, Bell AJ, Heesom KJ, Tavare JM, Cullen PJ. A global analysis of SNX27-retromer assembly and cargo specificity reveals a function in glucose and metal ion transport. Nature cell biology. 2013; 15:461–471.

Steinberg F, Heesom KJ, Bass MD, Cullen PJ. SNX17 protects integrins from degradation by sorting between lysosomal and recycling pathways. The Journal of cell biology. 2012; 197:219–230. [PubMed: 22492727]

Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. Microbiological reviews. 1994; 58:491–562. [PubMed: 7968923]

Tabuchi M, Tanaka N, Nishida-Kitayama J, Ohno H, Kishi F. Alternative splicing regulates the subcellular localization of divalent metal transporter 1 isoforms. Molecular biology of the cell. 2002; 13:4371–4387. [PubMed: 12475959]

Tepass U. Genetic analysis of cadherin function in animal morphogenesis. Current opinion in cell biology. 1999; 11:540–548. [PubMed: 10508657]

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell. 2000; 101:249–258. [PubMed: 10847680]

Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. Nature reviews Molecular cell biology. 2008; 9:944–957.

Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science. 2011; 334:1081–1086. [PubMed: 22116877]

Wang Q, Li L, Ye Y. Inhibition of p97-dependent protein degradation by Eeyarestatin I. The Journal of biological chemistry. 2008; 283:7445–7454. [PubMed: 18199748]

Wang X, Ward RE. Sec61alpha is required for dorsal closure during Drosophila embryogenesis through its regulation of Dpp signaling. Developmental dynamics: an official publication of the American Association of Anatomists. 2010; 239:784–797. [PubMed: 20112345]

Weaver SC, Barrett AD. Transmission cycles, host range, evolution and emergence of arboviral disease. Nature reviews Microbiology. 2004; 2:789–801.

Weaver SC, Reisen WK. Present and future arboviral threats. Antiviral research. 2010; 85:328–345. [PubMed: 19857523]

Wetli HA, Buckett PD, Wessling-Resnick M. Small-molecule screening identifies the selanazal drug ebselen as a potent inhibitor of DMT1-mediated iron uptake. Chemistry & biology. 2006; 13:965–972. [PubMed: 16984886]
Zacks MA, Paessler S. Encephalitic alphaviruses. Veterinary microbiology. 2010; 140:281–286. [PubMed: 19775836]

Zhang XD, Yang XC, Chung N, Gates A, Stec E, Kunapuli P, Holder DJ, Ferrer M, Espeseth AS. Robust statistical methods for hit selection in RNA interference high-throughput screening experiments. Pharmacogenomics. 2006; 7:299–309. [PubMed: 16610941]
Highlights

- Genome-wide RNAi screen reveals novel regulators of SINV infection
- SEC61A and VCP promote SINV entry by regulating NRAMP expression
- SEC61A and VCP regulate intracellular trafficking of NRAMP2, GLUT1 and β1 Integrin
- SEC61A and VCP regulate NRAMP-dependent iron transport
Figure 1. Genome-wide RNAi Screen
(A) Schematic of the RNAi screen with Robust Z scores plotted for each replicate (B) Fraction of candidates that have homologs in the indicated genera. Significant (p<0.001) enrichment of conserved genes and under-enrichment of Drosophila-specific genes (p<0.001). (C) Pie chart showing number of genes identified as restriction factor or required factors. (D) Overlap of factors regulating SINV infection using HRsp and dsTE12S strains of SINV. Number of genes in each category is in parenthesis. (E) Cellular map of SINV-host interactions. Validated genes were classified according to sub-cellular compartments and cellular processes using information from Gene Ontology, PANTHER, InterPro, and literature curation. Factors that facilitate infection (red) and factors that restrict infection (green) are shown. Genes conserved in humans have an asterisk and the ones in black are the additional components of a complex identified in the primary screen but not retested.
Figure 2. SEC61A and VCP complex promote SINV infection

(A–D) *Drosophila* cells were pretreated with the indicated dsRNA and infected by the indicated virus. (A) Representative images showing SINV infection after the indicated dsRNA treatment. (B) Normalized percent SINV infection from (A) Mean±SD is shown from four independent experiments; *p<0.05. (C) Virally-expressed GFP was examined by immunoblot and compared to the control tubulin. Representative blot shown. (D) Normalized percent VSV infection after the indicated dsRNA treatment. Mean±SD is shown from four independent experiments; *p<0.05. (E) *Drosophila* cells were treated with the indicated concentrations of Eer1 1h before infection and then infected with SINV for 18h. GFP and tubulin expression was examined by immunoblot. Representative blot shown. (F) Adult flies expressing dsRNA against the indicated ERAD-associated components or control flies were infected with SINV and virus production was monitored at day 3 post infection by plaque assay on BHK21 cells. Mean±SD from three independent experiments is shown; *p<0.05. (G) Aag2 cells were treated with dsRNA against Aedes aegypti SEC61A or control and infected with SINV. Mean±SD from three independent experiment is shown; *p<0.05. (H) Aag2 cells were treated with the indicated concentrations of Eer1 1h before infection and then infected with SINV for 16h. GFP and tubulin expression was examined by immunoblot. Representative blot shown.
Figure 3. SEC61 and VCP are required for SINV entry

(A) Drosophila cells pretreated with the indicated dsRNAs were infected with SINV-GFP and subsequently treated with PBS pH 7.2 or pH 5.5. Relative percent infection for each treatment is shown normalized to control dsRNA. Data represent mean±SE from five independent experiments; * p< 0.05. (B) Drosophila cells were treated with Eer1 at the indicated time pre or post infection by SINV-GFP (upper panel) or VSV-GFP (lower panel) and infection was monitored by immunoblot. Representative blot shown. (C) Drosophila cells stably expressing HA-dNRAMP were treated with the indicated dsRNA and NRAMP levels were examined by immunoblot against HA compared to actin control. Representative blot shown. (D) Endogenous dNRAMP mRNA was examined by RT-PCR after the indicated dsRNA treatment. Relative level of dNRAMP was normalized to control RP49. Mean±SD from three independent experiments is shown; * p< 0.05.
Figure 4. The proteasomal component dPSMD11 regulates SINV entry

*Drosophila* cells depleted of dPSMD11 were infected with (A) SINV or (B) VSV. Normalized fold change in infection from three independent experiments is presented as mean±SD; * p< 0.05. (C) *Drosophila* cells pretreated with dPSMD11 or control dsRNA were infected with SINV-GFP and subsequently treated with PBS pH 7.2 or pH 5.5. Relative percent infection is shown. Data represents mean±SE from three independent experiments; *p<0.05. (D) *Drosophila* cells expressing HA-dNRAMP were treated with the indicated dsRNA and the level of dNRAMP was examined by immunoblot against HA and compared to the actin control. Representative blot shown.
Figure 5. SEC61A and VCP regulate SINV infection in human cells

(A–B) U2OS cells were transfected with the indicated siRNAs. At 96 h post transfection, cells were infected with SINV (MOI of 1). Cells were fixed and processed at 8 hpi. Representative image is shown in A and quantification from three independent experiments is shown in B *p<0.05. (C) Experiment was performed as in (A) but was processed for immunoblot analysis for GFP and actin. Representative blot shown. (D) Experiment was performed as in (A) but was infected with VSV (MOI of 2) for 8 h. Quantification from three independent experiments is shown. (E–G) U2OS cells were pretreated with the indicated drugs and then infected with SINV (MOI of 1) for 7 h. GFP and actin expression was examined by immunoblot. Representative blot shown. (H) U2OS cells were transfected with the indicated siRNAs. After 48 h, the cells were transfected with HA-NRAMP2 and processed for immunoblot at 30 h for NRAMP2 and actin. Representative blot shown.
Figure 6. SEC61A and VCP regulate NRAMP2 trafficking

(A–B) U2OS cells were transfected with the indicated siRNAs and 48h later transfected with HA-NRAMP2. Cells were replated on glass coverslips and processed for microscopy 30h later and probed with the indicated antibodies. Representative images shown and magnification of the dotted area is shown below. (C–D) U2OS cells were transfected with the indicated siRNAs and 48h later transfected with HA-NRAMP2 and supplemented with media without or with protease inhibitors (2 μM Pepstatin, 10 μM Leupeptin and 10 μM E64) 5h later. Cells were collected for immunoblot 26h later and the levels of NRAMP2 and actin are shown for a representative experiment and quantification of NRAMP2 levels (mean±SE) from four independent experiments is shown; *p<0.05. (E–H) U2OS cells were treated with the indicated siRNAs for 72h and the levels of indicated proteins were analyzed by immunoblot. Representative blots shown.

Cell Rep. Author manuscript; available in PMC 2014 December 26.
Figure 7. SEC61A and VCP regulate iron transport

*Drosophila* cells expressing dNRAMP were treated with the indicated dsRNA. After 72h (A) or 48h (B), cells were treated with calcein AM for 1hr and subsequently treated with vehicle (no iron) or 20 μM iron (iron). Relative fluorescence intensity is shown. Data represent mean±SE from three independent experiments; *p<0.05. (C) HEK 293T cells expressing mouse Nramp2 were transfected with the indicated siRNAs. After 72h, iron transport was examined by Calcein AM assay. Mean ±SE of the relative fluorescence intensity for five independent experiments is shown; *p<0.05.