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# Mechanisms of Immune Evasion by Mousepox Virus in Mammalian Cells

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College Honors

Departmental Distinction Biology



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# Mechanisms of Immune Evasion by Mousepox Virus in Mammalian Cells

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# ABSTRACT

Ectromelia virus (ECTV; also referred to as “mousepox”) is a double-stranded DNA virus in the *Poxviridae* family. In nature, ECTV typically infects mice through abrasions in the skin. Following initial replication at the site of infection, the virus disseminates to multiple organs. Among the mice that survive the initial infection, characteristic pock lesions manifest on the skin in a similar fashion to the disease manifestations of humans infected with smallpox.

Viruses cannot replicate themselves unless they first enter a host cell. Once inside, viruses essentially take over host processes and hijack the cellular machinery to produce new virus particles. In order to increase their replication potential, many viruses produce proteins that actively inhibit various components of the host organism’s immune system. Interestingly, mammalian cells have numerous mechanisms to detect viral invaders and fight back.

In order to counteract host defenses, many viruses – including poxviruses – have evolved strategies to hide dsRNA. One such strategy is to express a protein that binds to viral dsRNA and prevent recognition by the host cell. Erin Hand ’15 created a mutant version of ECTV that lacks the E3L gene earlier this year. The study of this “knockout” virus (ECTV□E3L) was the focus of my research. This work is important to learn more about basic virus biology and host immune responses. Additionally, ECTV may prove to be a useful vaccine delivery vehicle in the future. Therefore, the more we know about its biology, the better we will be to manipulate its replication for therapeutic benefit.

Our results indicated that the formation of dsRNA was a late viral event. This dsRNA is more predominate in VACV infections vs. ECTV infections. The dsRNA may

be more predominate in the WT VACV for several reasons: unequal levels of E3L expression in WT ECTV vs. WT VACV, more precise termination of transcription in ECTV vs. VACV, or the fact that VACV infection is in general a much faster infection. Levels of secreted IFN are negligible across all cell lines and viruses, but cell lines do produce IFN mRNA during ECTVΔE3L infections. Our data showed that PKR activation is widespread for VACVΔE3LΔK3L infections, and some mutant ECTV infections. This points to the activation of PKR being cell line specific.

# INTRODUCTION

Viruses are obligate intracellular parasites, which means they rely on the infected host cell enzymes and proteins to replicate their viral genome. Viruses lack several basic elements required for growth and replication such as: mechanisms for the synthesis of nucleotides, amino acids, carbohydrates, and lipids; enzyme systems to produce energy in the form of ATP; ribosomes and associated proteins for protein synthesis. Viruses can infect virtually all life forms including plants and insects and are the most abundant life form on Earth. Scientists have concluded that there are approximately $10^{31}$ viruses and bacteriophages in the world (1). With such a diverse host range, the range in virus morphology is also as impressive. Virus size, both of their genome and diameter, can vary greatly. The smallest known viruses are approximately 20nm in diameter while the largest known viruses can be as large as 500nm in diameter. The respective genomes can be anywhere from 2000 nucleotides to 1.2 million nucleotides (1). Virus's genetic information is stored in the form of either DNA or RNA, but never both. An additional layer of complexity is that viruses can have either single stranded or double stranded genomes. These genomes can come in the form of linear or circular structure. The basic

structure of a virus consists of the genome, which can be RNA or DNA, enclosed in a protective protein coat referred to as a capsid. Some viruses have an additional layer known as a envelope that consists of a lipid bilayer, usually derived from the host cell bilayer, that can assist in immune evasion and protection. A complete infection virus particle is known as a virion.

Viral replication occurs in 6 main steps. The first of step is the virion must bind to a specific receptor on the host cell. These receptors generally consist of glycoproteins or glycolipids. A particular virus can bind only to a specific receptor on any given cell, and if the cell does not have this receptor the virus cannot infect it. This restriction on what cells the virus is able to infect is known as the tropism of the specific virus. After the virus binds to its receptor on the cell the virion or the genome must gain access to the cell. There are several mechanisms by which a virus can enter the cell. One common way is through the endosomal pathway.  Once the virus has entered the cell and reached its destination, which can be the nucleus in the case of some DNA viruses or just the cytoplasm for other usually RNA viruses, it can begin to replicate its genome using host cell machinery. Early viral protein synthesis mainly consists of proteins aimed to participate in the replication of the genome. Once they are synthesized they can begin to promote the replication of the genome. Late viral protein synthesis consists mainly of structural genes, such as the formation of capsid subunits for the packaging of viral genome. The final step in the virus life cycle is the release of progeny virions from the infected host cell. This process can either destroy the cell through lysis or the virus can be released thought the plasma membrane through a process known as budding (1).

Although viruses have evolved specifically to hijack host cell machinery in order to survive and replicate, the host cell is by no means helpless. Mammalian cells have evolved highly complex intrinsic cellular defense systems that allow for the detection of virus particles. The activation of these systems begins with the detection of the virus. Viruses have structures that are unique only to viruses and are not normally found inside of the host cell, one such example is the presence of dsRNA (1). Cells have evolved receptors that can recognized these molecules, which in turn induces a signal cascade initiating downstream transcription factors upregulating specific genes that can help to fight a virus infection. Some examples of these genes that can be turned on by the presence of viral components are interferon, protein kinase R (PKR), and RNase L (2'-5' oligoadenylate sythetase) (1).

Interferon, a subclass of cytokines, is a signaling protein that can be grouped into three major species (alpha, beta, and gamma) depending on their cellular origin and what mechanism they induce. Upon binding of interferon to the appropriate receptors on a cell interferon (IFN) induces the activation of certain genes that are involved in antiviral pathways. Infected cells releasing the interferon can sense their own interferon, which can protect from later infection, or more commonly the interferon can act on adjacent cells to protect them from infected and activate and antiviral state. Some of the most well known IFN induced genes are 2'-5' oligoadenylate sythetase, the RNA-dependent protein kinase (PKR) and the Mx proteins. Recently, the effects of IFN on cancerous tumors have been studied and have shown promise in the fight against cancer.



PKR is a IFN induced protein that is a component in signal transduction pathways mediating cell growth control and response to stress. The most well studied role of PKR

is the inhibition of translation through the phosphorylation of eukaryotic initiation factor 2 α-subunit (eIF2-α). While this is the best understood role of PKR it is thought there may be additional roles relating to antiviral activity. PKR can be activated by both dsRNA, which is not normally found in uninfected cells, as well as cytokine signals like IFN. The activation of PKR is necessary to upregulate expression of genes involved in the cellular inflammatory response (2). The main point of PKR is to halt the initiation of protein synthesis and to activate the transcription of genes involved in the inflammatory response in the hopes of limiting the spread of the virus to neighboring cells (2,13,14).

RNase L also known as 2'-5' oligoadenylate sythetase dependent ribonuclease is an IFN induced ribonuclease. When activated RNase L degrades all RNA in the cell, often resulting in cell death. The goal of this mechanism is to sacrifice the cell for the good of the whole organism. Degradation of all cellular RNAs includes any viral RNAs that are ready to be transcribed at the time thus ending the virus life cycle in that cell. RNase L is usually present at very low levels in the absence of infection. Upon binding of IFN the transcription of the RNase L and related genes gets upregulated. RNase L is produced in an inactive form and is activated after 2'-5' oligoadenylate sythetase, in combination with ATP, bind to the RNase L molecule causing it to dimerize (3,16). The dimerized form of RNase L then degrades cellular RNA leading to apoptosis. Interestingly, some of the fragments of RNA from the destroyed cell can induce the production of IFN in neighboring cells further spreading the signal to employ an antiviral state among cells (1,3,4,17).



Although mammalian cells have evolved multiple detection mechanisms for viral invaders, viruses are not helpless. They too have developed mechanism for host immune

evasion. Viruses can posses multiple genes that can interfere with the host cells immune system, either by producing proteins that mimic cytokines and cytokine receptors or proteins that mask viral components from being detected by the host immune system. Many times viruses can also evade host cell immune responses by their capability to change their genome through random mutations, which occurs at a higher rate in viruses than most mammalian cells (1).

The virus of interest while doing research during my senior year at Albright was Ectromelia virus (ECTV). ECTV is a virus that belongs to the family Poxviridae that has the genus Orthopoxvirus (1,5). Poxviruses have large double-stranded DNA genomes. Typically their genomes are approximately 200kb and encode roughly 200 proteins. Poxviruses are generally 300 by 250 by 200 nm in size and can have one or two lipid envelops. Unlike many DNA viruses poxviruses, including ECTV, replicate their genomes in the cytoplasm. Because host cell genome replication machinery is located in the nucleus, poxviruses must bring their own enzymes for DNA replication and RNA synthesis. Poxviruses are unique in that they form virions within DNA factories that are visible as large crescent shaped structures outside the nucleus using light microscopy. It is thought that this DNA is then packaged into the viral capsid proteins to make viral progeny (1). Poxviruses encode multiple proteins to evade host cell defenses. Because one of the first lines of defense against viruses is the production of IFN that induces an antiviral state among mammalian cells, poxviruses encode proteins that inhibit the activation of antiviral mechanisms (6). Examples of these proteins are K3L, which acts as a competitive inhibitor of PKR and E3L that sequesters dsRNA in the cytoplasm essentially hiding it from the PKR and RNase L pathway. Poxviruses are also known to

produce soluble forms of cytokine receptors that act to sequester free antiviral cytokines in hopes of reducing or eliminating an antiviral state among cells (7,8). Variola virus, the causative agent of smallpox, is also an orthopox virus that caused millions of deaths around the world. Through extensive research and development of a vaccine for this disease, global eradication was achieved in 1977. Within all poxviruses the central 100kb segment is approximately 90% homologous, which makes the study of these viruses, such as ECTV, a sought after area of research for the understanding of molecular aspects of host cell immune evasion (9).

ECTV is commonly referred to as Mousepox because it is the only poxvirus that infects mice. ECTV infection presents itself as skin lesions, hunched posture and conjunctivitis, which can be fatal in susceptible mice. Studies have shown that ECTV encodes for one of these E3L proteins mentioned earlier (10,15). Past work, done by a student by the name of Erin Hand, was focused on making a virus that lacked this specific protein. She produced this mutant virus (ECTV**Δ**E3L) using a technique referred to as homologous recombination. During this process the segment of DNA encoding for the E3L protein is essentially "knocked out" of the genome so the virus is no longer able to produce it during replication. The objective of this is to study the role of the E3L protein in the ability to effectively replicate in mammalian cell lines.



The E3L gene encodes a 190 amino acid protein with a highly conserved carboxy-terminal dsRNA-binding domain and N-terminal DNA binding domain. The main function of the E3L gene is to specifically bind dsRNA in a sequence dependent manner although, recent studies have shown that the C-terminal dsRNA-binding domain can also indirectly inhibiting cellular responses due to single-stranded DNA. Through binding of

the dsRNA ECTV can avoid activation of multiple host immune mechanisms such as the activation of production of IFN, PKR, and RNase L (11).

During the course of my research preformed several experiments to analyze the functional role of E3L during virus infection. We examined the role by using the mutant virus, which lacks the function E3L gene, compared to the wild-type virus. The main assays preformed during my research were: staining of cells for dsRNA, examining the activation state of PKR with the mutant virus and wild-type virus, quantifying the levels on secreted mouse IFN- β using an ELISA, and examining the virus host range using multiple cell lines from different species. Our hypotheses for the assays are as follows: because the mutant virus lacked the E3L gene we expected the virus to have a higher detectable level of dsRNA. In continuation with that hypothesis we also expected their to be a higher level of PKR activation in the mutant virus compared to the wild-type virus. Using the ELISA our hypothesis was the mutant virus would elicit a higher level of secreted IFN- β. Finally, our hypothesis regarding the host range of the mutant virus was that do to the lack of E3L the virus would be easily detected by the host cell immune system and viral replication would be terminated, negating the spread of the virus. We were able to study the host range of ECTVΔE3L by staining for B5 protein at the host cell surface after infection. The B5 protein is a late viral protein that is indicative of a successful replication cycle.



The study of poxviruses continues to be an intense area of research even following the wake of the eradication of smallpox because of their ability to evade host defense mechanisms. Studying these viruses allows researchers to better understand the interplay of the host immune system to viral evaders, which one-day could lead to

immunotherapeutic treatments. The idea of using poxviruses, with specific genes sliced into their genomes, as a recombinant vaccine vector has also been proposed. Lastly, the fear of using smallpox as a biological weapon has increased over the last decade so the demand for new and improved vaccines has increased as well (12).

# MATERIALS AND METHODS

**Cell Maintenance:**

Monkey kidney cell line BSC1, Mouse L929, DC2.4, HeLa, Vero, and HFF cell lines, were maintained in a T-75 flask containing Dulbecco's Modified Eagle Medium (DMEM; Life Technologies), 5% fetal bovine serum (FBS; Gemini BioProducts). Cells were maintained in 37˚C incubators (5% $CO_2$). To guarantee a sterile environment, all work with live cells was performed in a laminar flow hood and all materials used were sterile.

**Subculturing:**

Cell cultures were subcultured occasionally to maintain the cell line. DMEM medium was removed with a sterile vacuum pipette to ensure that all media was removed because FBS contained in the medium has a natural trypsin inhibitor. Then 4 mL of trypsin was added to the flask. The flask was then placed into a 37˚C incubator for five minutes. The trypsin removes cells from each other and from the flask. 6 mL of DMEM medium was added to the flask to inactivate the trypsin. The cells were then washed.



**Cell Counting:**

In order to count the cells, 10 µL of cells was removed from the conical tube and loaded into one chamber of the hemocytometer. The cells were examined using a light microscope under 40x magnification, with a field of view of one square millimeter (mm).

Two of the nine square mm squares from each of the chambers were counted to determine the number of cells per 1 mL of medium. To obtain the number of cells per 1 mL, the number of cells were divided in half and then multiplied by the dilution factor and 2000. Cell count was utilized to quantify the amount of cells available to plate.

**B5 surface staining:**

50,000 of Vero, HFF, or BSC1 plain cells were plated onto a 4 well chamber slide. Approximately 24 hours later the wells were infected with the virus of choice. Approximately 24 hours post-infection the media was removed from the chambers and the slide was submerged in 10% formalin for 10 minutes, followed by a PBS rinse. The slide was submerged in blocking buffer for 30-45 minutes. After blocking 200uL per well of 1° mouse α-dsRNA (5ug/mL) antibody was added and incubated at room temperature for 30 minutes. The slide was rinsed in PBS and 200uL per well of 2° **goat** α-mouse antibody (2.5ug/mL) was added and incubated for 25 minutes in the dark. The slide was rinsed with PBS followed by deionized water and allowed to dry. The slide was then mounted using DAPI media and visualized using light microscopy.



**dsRNA Staining:**

50,000 BSC1 plain cells were plated onto a 4 well chamber slide. Approximately 24 hours later the wells were infected with WT ECTV, ECTV**Δ**E3L, WTVACV, VACV**Δ**E3L**Δ**K3L. Approximately 24 hours post-infection the media was removed from the chambers and the slide was submerged in 10% formalin for 10 minutes, followed by a PBS rinse. The slide was then rinsed in acetone and submerged for 20 minutes at -20°C. The slide was rinsed with PBS and submerged in blocking buffer for 30-45 minutes.

After blocking 200uL per well of 1° α-dsRNA (5ug/mL) antibody was added and incubated at room temperature for 30 minutes. The slide was rinsed in PBS and 200uL per well of 2° alexa-568 α-mouse antibody (2.5ug/mL) was added and incubated for 25 minutes in the dark. The slide was rinsed with PBS followed by deionized water and allowed to dry. The slide was then mounted using DAPI media and visualized using light microscopy.

**ELISA assay:**

Supernatant from L929,DC2.4, and BMDC cells were infected with WT ECTV, ECTVΔE3L,VACVΔE3LΔK3L, or NDV virus strains was collected and filtered. Using a LEGEND MAX ELISA Kit with Pre-coated Plates the included procedure was followed using our samples. The quantitative amount of mouse IFN-B was calculated using the regression line from the standard curve.

**EIF2-α flow-cytometry staining:**

HeLa, HeLa$^{PKRKDsiRNA}$ BSC1, DC2.4, BMCD, and L929 cells infected with WT ECTV, ECTVΔE3L, and VACVΔE3LΔK3L were trypsinized and collected into 5 mL tubes. FAS buffer was added to the tubes at a level of one half inch from the top. The cells were spun at 1500 rpm for 6 minutes. The tubes were decanted and 350 uL of fixative buffer was added followed by a vortex. The cells were incubated for 15 minutes at 37°C. The tubes were then filled half way with phosphate perm wash buffer (PWB) and spun at 1800 rpm for 6 minutes. The tubes were decanted and 50 uL of the 1° antibody (α-EIF2α) was added, vortexed, and incubated for 45 minutes. The tubes were filled half way with PWB and spun at 1800 rpm for 6 minutes. The tubes were decanted and 50 uL of the 2° antibody (α-rabbit) was added, the tubes were vortexed, and allowed

to incubate for 45 minutes in the dark. The tubes were filled half way with PWB spun and decanted. 300 uL of 2% paraformaldehyde was added and then the tubes were analyzed using flow cytometry.

# RESULTS

**B5 surface staining:**

When Vero, HFF, or BSC1 plain cells were infected with WT ECTV the cells were positive for both GFP and B5 surface markers (figures 1, 2, and 3). On the other hand, when the same cells were infected with ECTVΔE3L the cells were absent of any detectable level of GFP or B5 surface staining (figures 1, 2, and 3). Interestingly, when BSC1+E3L cells are infected with the mutant virus the cells are positive for both GFP and B5 surface markers (figure 3).

**dsRNA staining:**

BSC1 plain cells infected with WT ECTV showed minimal presence of dsRNA throughout the time course maxing out at less than 5% of cells positive for dsRNA (figure 4, row 1). Upon infection with ECTVΔE3L the cells showed high levels of low intensity staining of dsRNA as early as the 8-hour time point. Approximately 75% of the cells were dsRNA positive at the 24-hour time point (figure 4, row 2). When infected with WT VACV dsRNA levels are negligible until the 12-hour time point when dsRNA levels jumps to nearly 100% (figure 4, row 3). Infection with VACVΔE3LΔK3L results in high levels of dsRNA as early as the 4-hour time point where nearly 100% of cells are positive for dsRNA (figure 4, row 4).



**ELISA Assay:**

From the standard regression line the amounts of IFN-beta present in the infected cell supernatant was calculated. Regardless of cell type secreted INF levels for WTECTV, ECTVΔE3L, and VACVΔE3LΔK3L were negligible. Cells infected with the NDV virus, used as a positive control, were above the highest concentration of IFN-B on the standard curve.

**EIF2-α flow-cytometry staining:**

The phosphorylation of eIF2- α was detectable in all cell lines for VACVΔE3LΔK3L except BMDCs and HeLa$^{PKRKDsiRNA}$ . WT ECTV did not cause the phosphorylation of eIF2- α in any of the cell lines. The E3L mutant virus showed significant phosphorylation in the normal HeLa, DC2.4, and BMDCs while showing slight activation in the L929 cells.

# DISCUSSION

*B5 surface staining:*

The results from the B5 surface-staining assay indicated that the E3L mutant virus's replication was being aborted during infection. Presence of GFP indicated that the mutant virus was gaining access to the cell line and beginning replication, but was then being terminated. Lack of B5 surface markers, a late viral event, shows that the host cell is successfully recognizing the viral pathogen and employing antiviral mechanisms to shut down its replication. This data suggests that the E3L protein has a critical role in host evasion of the immune system and without it the infection is abortive. Previous research has shown that the E3L protein is involved in sequestering dsRNA and "hiding" it from the host immune system (11). In conjunction with the B5 surface staining and assay was run to determine more precisely at what point dsRNA was formed in virus

infection. A drug called ArC, which inhibits DNA synthesis, was added to the supernatant of infected cells. Results from this assay showed that when DNA synthesis was halted dsRNA was not formed by any virus (data not shown). These results suggest that the formation of dsRNA is a late viral event, which would explain the mutant virus's ability to enter the cell and begin replication to eventually be halted in the absence of E3L. Because dsRNA is a potent activator of dsRNA this data seems to suggest that a main contributor to the abortive infection of ECTVΔE3L is the recognition by PKR and the subsequent termination of translation.

*dsRNA staining:*

As we would expect the ECTV mutant and VACV mutant produced more widespread levels of dsRNA earlier in the infections then the WT viruses. This makes sense because, as mentioned earlier, the mutants lack E3L (additionally K3L in VACV mutant) that is thought to be involved in masking dsRNA from the immune system. These proteins would block the binding of the dsRNA antibody resulting in less fluorescence. Interestingly, if we compare the relative amounts of dsRNA in WT ECTV to WT VACV, which both have the E3L or K3L proteins intact, we see that at the 24 hour time point WT ECTV has significantly less dsRNA than WT VACV which has nearly 100% positive for dsRNA. There are several explanations for why this could occur: unequal levels of E3L expression in WT ECTV vs. WT VACV, more precise termination of transcription in ECTV vs. VACV, or the fact that VACV infection is in general a much faster infection may lead to more formation of dsRNA and the virus doesn't need to hide it from the immune system because it has already moved on.

In order to determine if WTECTV produced more E3L staining using flow cytometry was done to measure the relative amounts of E3L in virus infected cells (data not shown). The results from this assay showed that levels of E3L in WTVACV infected cells were lower than levels in E3L. This data suggests that one potential reason for the difference is expression of dsRNA is the relative level of E3L expression between the two viruses. One potential reason for this is the fact that VACV has an addition protein, K3L that aids the virus in evasion of the immune system. This protein may compensate in other areas to pick up the slack from less E3L production.

It has been well documented that dsDNA viruses are capable of producing dsRNA through convergent transcription and read-through (18). Our hypothesis is that WT VACV is less efficient at termination of transcription late in infection, thus producing more overlapping sequences, which would lead to production of higher levels of dsRNA late in infection, as we see. In order to test this theory it would be necessary to isolate RNA from virus infected cells of both WTECTV and WTVACV and produce a primer that is specific for one of these overlapping sequences. Using reverse transcriptase, the overlapping segments would be made into a strand of DNA, which could then be run on a gel and quantified. This would give an indication of WTVACV is indeed producing more dsRNA read-through sequences in comparison to WTECTV.



*ELISA assay:*

Results from the ELISA assay suggests that there is no IFN-B being secreted, in any of the tested cell lines, by ECTVΔE3L, VACVΔE3LΔK3L, or WTECTV. This isn't surprising given the fact that previous research has shown that ECTV and VACV secrete soluble proteins to sequester IFN to halt its antiviral effects (19). Although IFN-beta isn't

secreted it is possible that the genes for IFN-beta are still being turned on in the host cells and transcribed into mRNA after detection of the virus. In order to test this RNA was isolated and sent to an independent laboratory for RT PCR analysis. Results indicated that mRNA was transcribed in the mutant viruses (data not shown). This suggests that detection of mutant virus, probably by dsRNA, activates the IFN pathway, which may result in an abortive infection of the mutant viruses in the absence of E3L.

*EIF2-α flow-cytometry staining:*

Data from the eIF2- α phosphorylation assay indicated that levels of phosphorylation for the viruses were dependent on cell line. It makes sense that WTECTV showed no increase in the amount of phosphorylated PKR in any cells lines because previous research has shown that E3L sequesters dsRNA. dsRNA is a potent activator of the PKR pathway so no activation in WT ECTV was expected. The data suggests that PKR phosphorylation may be more predominant in one cell line versus another and based on what cells line you infect determines the relative levels of phosphorylation. In the mutant ECTV infections the effects of the PKR mechanism may play an important role in the abortive infection in normal HeLa, DC2.4, and BMDCs and L929 cells, but doesn't appear to play a significant role in the other cells lines tested.

Our data has shown that the E3L protein plays a significant role in masking a potent pathogen associated molecular pattern that results in an abortive infection when absent. The accumulation of dsRNA in the absence of E3L appears to be a key contributor in the activation of host immune mechanisms such as PKR and possibly RNase L. Because E3L plays such a significant role in host evasion it provides a target

for vaccination in other poxviruses with a homologous protein and may possible open avenues of research for drug delivery for other diseases.

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Figures

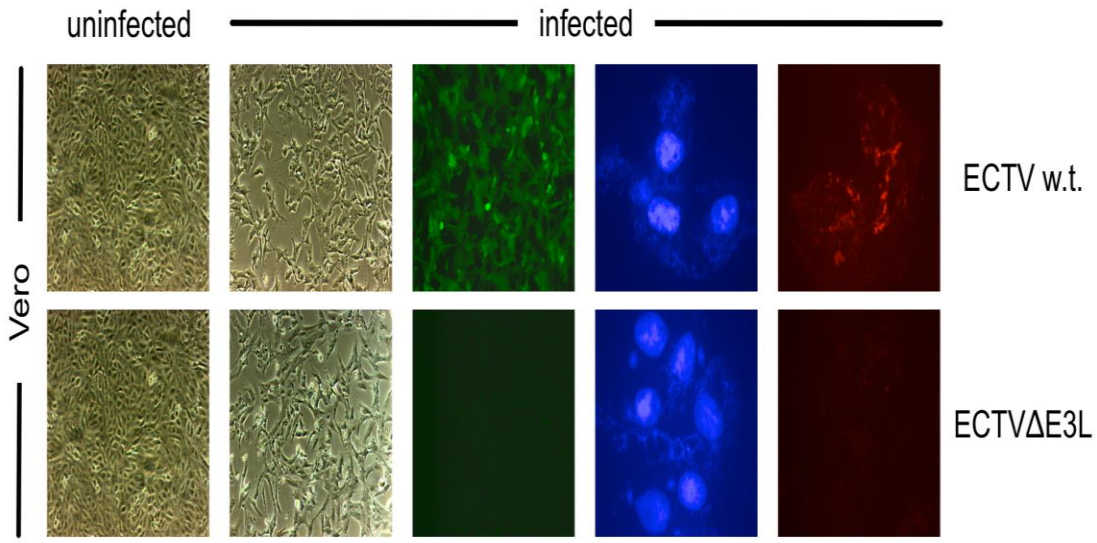


Figure 1: B5 surface staining in Vero cell line.

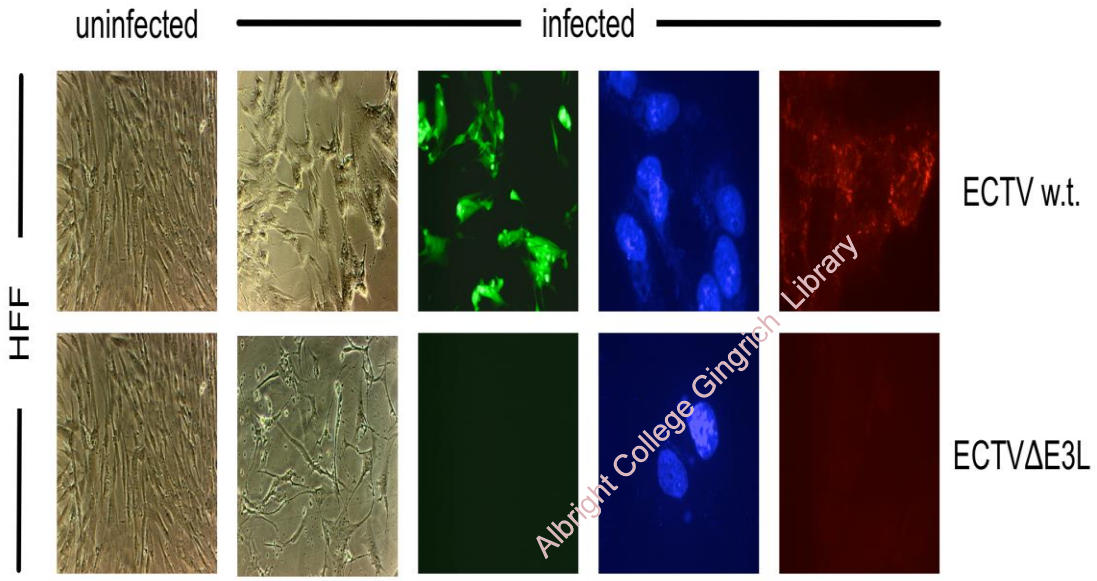




Figure 2: B5 surface staining in HFF cell line.

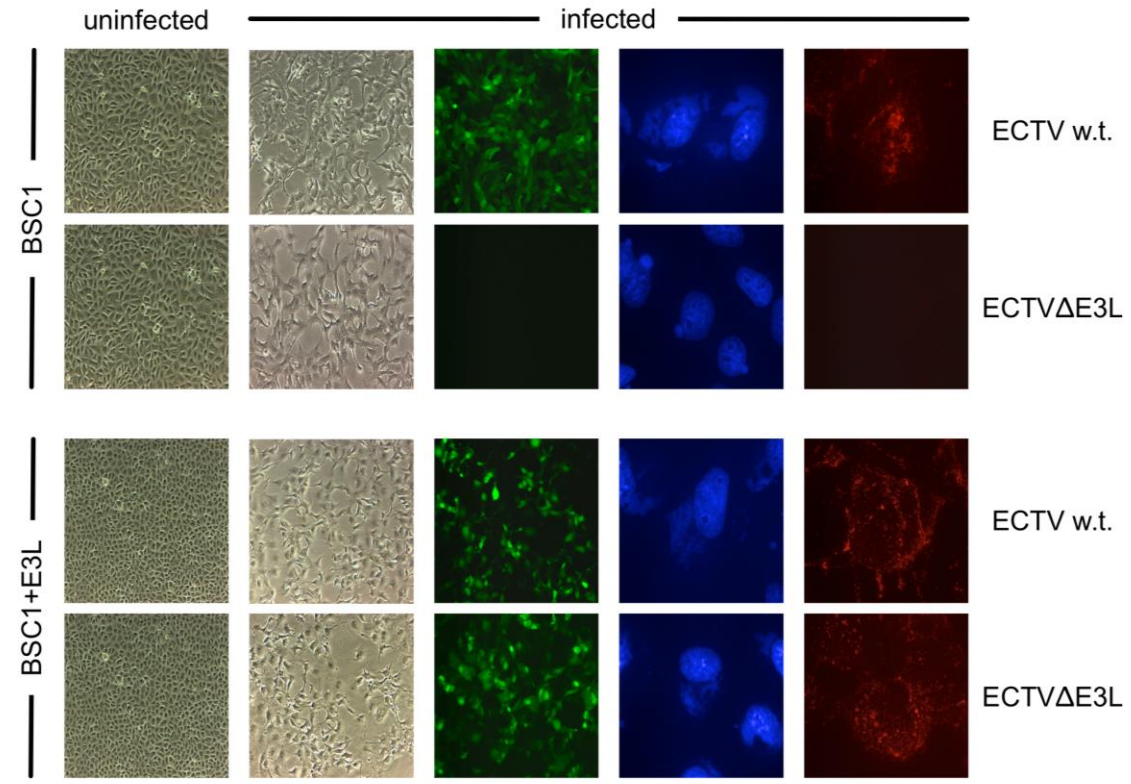


Figure 3: B5 surface staining in BSC1 plain and BSC1+E3L cell lines.

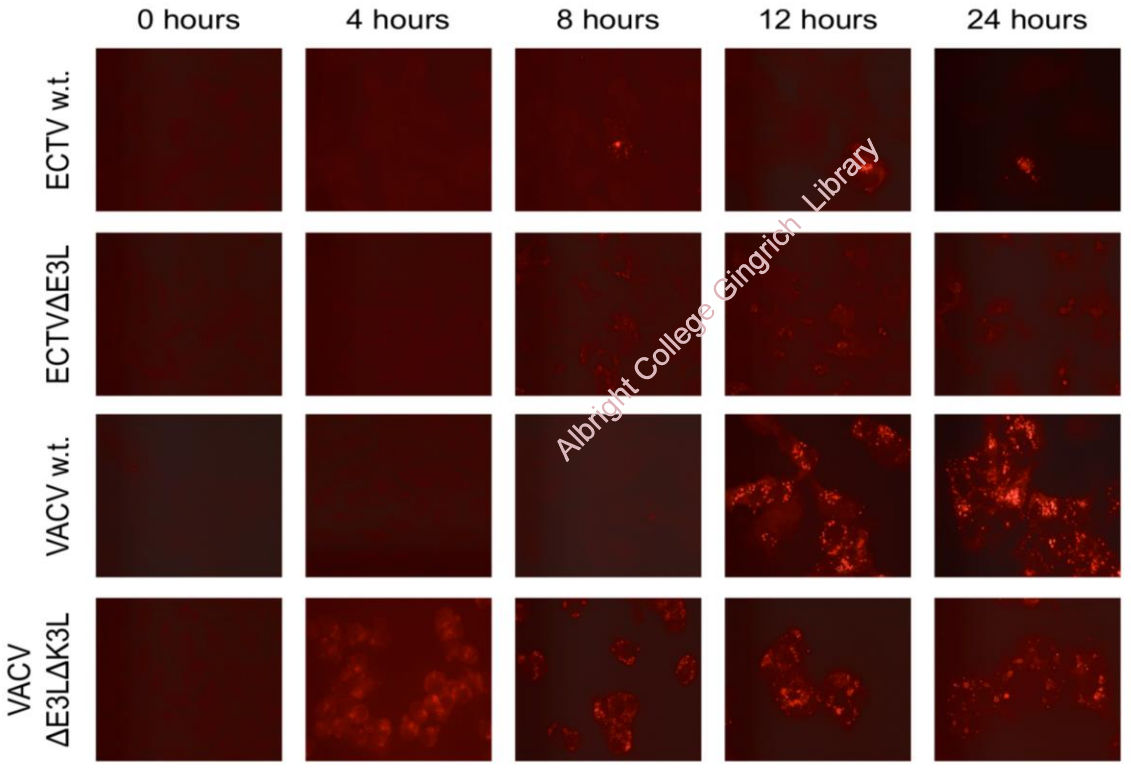

Figure 4: dsRNA staining in BSC1 plain cells.