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Regulation of Phosphatidylinositol 4-kinase III alpha Complex Formation in B Cells and its Role in Autoantibody Production

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Regulation of Phosphatidylinositol 4-kinase III alpha Complex Formation in B Cells and its Role in Autoantibody Production

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ABSTRACT

The plasma membrane protein Efr3b, the Tetratricopeptide domain repeat 7 (Ttc7), and the kinase phosphatidylinositol 4-kinase III alpha (PI4KIIIα) are found to form a complex at the plasma membrane. This complex is thought to play a role regulating the PI4P pools at the plasma membrane, which is imperative to membrane identity and vesicle transport. Efr3b is post transcriptionally palmitylated and is currently believed to be constitutively located at the plasma membrane. Ttc7 encodes for a protein with TPR domains that are involved in many intracellular interactions such as protein: protein interactions and vesicle transport. The mutant form of Ttc7 was discovered to be the genetic cause of the systemic autoimmune disorder found in Flaky skin mice (fsn). The fsn mice have since been used as a model for the human autoimmune disease Systemic Lupus Erythematosus (SLE). The kinase, PI4KIIIα is normally found in the endoplasmic reticulum (ER) and its activity impacts plasma membrane identity and is involved in trafficking vesicles to the plasma membrane Previous research has shown that Ttc7 is the bridge between Efr3b and PI4KIIIα. This study focused on the localization of Efr3b, and the changes in complex formation before and after stimulation with anti-IgM. Changes in complex formation was also assessed with both wild type and mutant Ttc7.

A possible link between Hepatitis C infection and onset of the autoimmune disease SLE has previously been found. The non-structural Hepatitis C protein NS5A has been shown in previous studies to interact with PI4KIIIα. The current understanding is that NS5A is reliant on PI4KIIIα activity for viral replication and therefore may sequester host PI4KIIIα. If so, this may render the kinase unavailable for complex formation and may lead to complex disruption that could impact PI4P regulation.

It was found that Ef3b is not constitutively localized to the plasma membrane in immature B cells. Co-localization of Efr3b and Ttc7 was found be increased after stimulation with anti-IgM. Complex formation also appeared more diffuse with the fsn Ttc7.

Transfection of immature B cells with NS5A lead to a new phenotype found in WEHI 231 cells. NS5A appeared to cause the presence of intracellular droplets post transfection, as well as induce a change in cellular morphology. Cells transfected with NS5A appear enlarged and elongated.

ABBREVIATIONS

Efr3b	Protein Efr3 homolog b
Ttc7	Tetratricopeptide repeat domain 7
PI4K	Phosphatidylinositol 4-kinase
PI4P	Phosphatidylinositol 4-phosphate
TPR	Tetratricopeptide repeat
fsn	Flaky Skin
SLE	Systemic Lupus Erythematosus
ER	Endoplasmic Reticulum
HCV	Hepatitis C Virus
NS5A	Non-structural protein 5a
ANA	Anti-Nuclear Antibodies
IgM	Immunoglobulin μ
IgG	Immunoglobulin γ
IgD	Immunoglobulin δ
T1	Transitional type 1
T2	Transitional type 2

T3	Transitional type 3
MZ	Marginal Zone
E2A	B Cell Transcription Factor
IL-7	Interleukin- 7
UPR	Unfolded Protein Response
XBP1	X-Box Binding Protein
HEAT	Huntington, Elongation factor 3, Regulatory subunit A of protein phosphatase 2A, and Target of rapamycin
PI	Phosphatidylinositol
ATF6	Activating Transcription Factor 6
ROS	Reactive Oxygen Species
PBR	PI4K Binding Region
PFIS	PI4K
BCR	B Cell Receptor

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BACKGROUND

Autoimmunity

The main role of the immune system is to protect the body against invading pathogens. To carry out this job, the immune system must be able to determine the difference between an invading pathogen that needs to be removed and a component of self. The ability to differentiate between self and non-self is a process called tolerance [1]. The outcome of the loss of this ability can lead to autoimmune disorders. Tolerance is a process that occurs in two stages, central tolerance and peripheral tolerance. An immature lymphocyte that responds strongly to self antigens will be given a death signal, or be signaled to inactivate, this is central tolerance. Central tolerance occurs in the thymus for T cells and in the bone marrow for B cells. Central tolerance can also be considered negative selection, as cells that react inappropriately to self antigens are signaled to die and hopefully removed from the repertoire. Peripheral tolerance occurs outside of the main lymphoid organs [1]. If a lymphocyte is found to be reactive to self antigens in circulation the mechanism to remove them usually results in anergy for B cells, peripheral tolerance can also lead to cell death and some autoreactive cells escaping into circulation.

The presence of autoreactive cells does not necessarily result in an autoimmune disease. Some autoreactive lymphocytes are needed for normal immune function. To effectively clear a pathogen lymphocytes are also required to remove cells infected with the pathogen thus some form of autoreactivity is normal and needed for proper function [1]. These autoreactive lymphocytes however need to be removed from circulation. Many lymphocytes that have low affinity for self antigens will typically ignore self, making them harmless. If however, the threshold for activation is lowered, like in the case of an infection the autoreactive lymphocyte

can become activated [1]. As previously mentioned the presence of autoreactive lymphocytes does not mean that an autoimmune disease is occurring.

When an autoimmune disease does arise it is categorized based on the major autoreactive cells involved in the disease, and the tissues that they impact. B cell mediated autoimmunity is caused by the formation of autoantibodies that target self antigens and cause severe tissue damage [1]. The autoantibodies can be targeted at components such as cell surface molecules or antigens from the extracellular matrix, intracellular proteins, or nucleic acids. These autoantibodies can cause cell death and also lead to the formation of immune complexes. These complexes are formed by cross-linked autoantibodies bound to self antigens. These immune complexes initiate a local inflammatory response that can also cause capillary and kidney damage such that is seen in Systemic Lupus Erythematous (SLE). T cell mediated autoimmunity is caused by T cells that target self antigens and cause cell death, which can cause the lack of pancreatic β-cell antigen as seen in Type I Diabetes [1]. During the process of an immune response to self antigen the inflammatory response is also triggered which causes the tissue damage, sometimes permanently. The other way in which autoimmune diseases can be classified is by the tissue that is impacted by the autoreactive lymphocyte. When autoreactive lymphocytes target molecules that are ubiquitous in the body, such as double stranded DNA, the disease is classified as a systemic autoimmune disease. Systemic autoimmune disorders have a large range of symptoms that result from a systemic autoimmune response. In organ specific autoimmune disorders generally the targeted organ is severely affected. In type I diabetes, the β-cell proteins are the self antigens, which are found only in the pancreas.

Autoantibody formation is currently understood to develop due to the breaking of tolerance. If a B lymphocyte skips any of the tolerance checkpoints and is allowed to circulate through the body

there is a chance it could become an autoantibody producing cell. The exact mechanism behind autoantibody formation is not fully understood. Some viruses have been linked to the presence of autoantibodies such as the Hepatitis C Virus. There is a connection between mass apoptosis and autoantibody formation; if the cellular debris is not properly cleared after apoptosis, the likelihood that antibodies against the cell debris (like DNA, nuclear proteins etc.) increases. If autoantibodies do form, it poses a threat that the autoreactive cell will proliferate and potentially cause an autoimmune disease.

Systemic Lupus Erythematosus is an autoimmune disease that causes a chronic inflammatory state with a number of varying symptoms, a vaguely understood mechanism of disease, and is found to be difficult to diagnose [2]. The cause of SLE is still under investigation. One of the hallmarks of SLE is the presence of antinuclear antibodies. These autoantibodies can be against molecules such as nuclear proteins, double stranded DNA, chromatin, or histone. Interestingly roughly 15% of people can test positive for antinuclear antibodies and not have an autoimmune disorder. One major concern with SLE is the chronic damage to the kidney which leads to lupus nephritis, one third of patients already have lupus nephritis upon time of diagnosis. There have been multiple possible genetic polymorphisms have been linked to the development of the disease. Polymorphisms in the major histocompatability complex (MHC), complement components, and genes encoding immune signaling molecules such as tumor necrosis factor α (TNF-α), and Interleukin 6 (IL-6) [3]. Possible environmental factors are also under investigation, exposures such as infections, sunlight, and certain drugs/toxins have been linked with disease development [3].

One particular virus has been linked to the onset of a SLE like clinical presentation: the Hepatitis C virus (HCV). HCV infection has been linked not only to SLE development but to multiple

autoimmune disorders. HCV serves as a possible environmental link to autoimmune disorder, it is not however, a known factor in autoimmune disease [4]. Up to 30% of HCV patients have been found to have antinuclear antibodies. The current treatment for HCV infection is with interferon α (IFN-α), which in some cases has been linked to the development of autoimmune disease [4]. The exact cause of SLE is likely a combination of environmental factors and genetic susceptibilities that are unique to each individual. This may also explain the diverse clinical presentation of the disease.

The flaky skin mouse model

When using inbred mice strains for research spontaneous mutations commonly arise [5]. This feature allows for the study of the clinical outcomes of the mutation before the genetic cause is determined [6]. The flaky skin (fsn) mutation is an autosomal recessive mutation that arose spontaneously on the A/J strain at the Jackson Laboratory, Bar Harbor Maine in 1984. Due to viability constraints the mutation was backcrossed onto the BALB/cByJ strain, and is currently maintained on the CByJ.A-fsn/fsn strain that allows for adequate study [5].

The fsn mutation was mapped to the distal end of the mouse chromosome 17 [6]. The mutation was found to be an insertion of an endogenous retrovirus, 57 base pairs large into the tetratricopeptide repeat domain 7 (Ttc7) gene [8]. The insertion occurs within intron 14, upstream of the splice acceptor site for exon 15. The retrotransposon includes a splice site that is preferentially used rather than the wild type splice site [7]. The incorrect splicing that occurs because of this insertion causes a reduction in the wild-type transcripts of Ttc7 [7]. The majority of Ttc7 transcripts found in fsn mice include 61 amino acids that were coded for by the

retrotransposon viral DNA into one of the tetratricopeptide repeats (TPR) [8]. This disruption in the coding sequence of the TPR domain may prevent proper functioning of the protein.

The flaky skin mice have a phenotype that resembles a systemic autoimmune disease. The term "flaky skin" was given due to the skin phenotype seen in the fsn mice that resembles the human autoimmune disorder psorias [6]. The fsn mice have multiple affected organ systems, most notably the skin, blood, thymus, kidney and spleen [5]. Levels of soluble immunoglobulin are also abnormal. The fsn mice remain smaller than the littermate controls and can only survive for about fifteen weeks, at this point the physiological damage is fatal.

The fsn mice have dermal inflammation, and increased infiltration of mast cells, eosinophils and polymorphonuclear leukocytes [5]. The stratum corneum is keratinized and the bellies of the fsn have a striated hair growth pattern [5]. The fsn mice appear pale at birth due to a hypochormic anemia that worsens with age [6]. The thymus of the fsn mice is smaller than normal littermate controls [5]. The spleen and lymph nodes however, are very much enlarged in the fsn mice. The fsn mice develop glomerulonephritis, characterized by immune complex deposition upon histological examination. Immune complex deposition is indicative of autoantibody circulation. The fsn mice develop autoantibodies against nuclear proteins and double stranded DNA. Serum IgE is vastly increased in fsn mice, as well as IgM, IgG_1, and IgG_2, while levels of IgG_3 are decreased [5]. Due to the multiple immune irregularities, the fsn mice have been used as a model of a systemic autoimmune disease. The phenotype can be most easily compared to the human diseases SLE and psorias, although the fsn mice are not an exact model of either disease.

The presence of autoantibodies found in the flaky skin mice make for an excellent model of the mechanism behind autoantibody formation. The B lymphocyte populations in the fsn mice have

been found to be abnormal [7]. This is most likely due to a block in the development of the B cells in the bone marrow of the fsn mice. This loss of pre- and immature B cells in the bone marrow is age related, by seven weeks of age the pre- B cell population is reduced and by ten weeks of age there is an absence of pre- B cells in the bone marrow. The B cells that are developing in the bone marrow eventually become the B cells that circulate in the periphery, expectedly the B cells in the periphery of the fsn mice are also abnormal. Flaky skin mice have an increased population of transitional B cell type I (T1) and marginal zone (MZ) B cells, and a decreased population of transitional B cell type 2 (T2), and mature B cells [7]. This leads to a B cell population with a marked increase of T1, MZ, and B1 cells. All three of these B cell subtypes have been found to likely to become autoreactive [7].

TPR domains were originally identified in yeast [9]. TPR domains have been found across most biological kingdoms of life [7]. The human Ttc7 gene includes seven TPR motifs, as does the mouse Ttc7 gene [7]. These motifs fold into two anti-parallel alpha-helical arrays that stack on top of each other creating a super helical structure [9]. Each TPR domain consists of 34 amino acids that make up nine or ten inexact tandem repeats, thus it was called the tetratricopeptide [9]. The retrotransposon insertion that is responsible for the fsn mutation occurs within the second TPR domain [7]. TPR domains have been found to be important in protein-protein interactions, making them important in multiple cellular interactions [9]. Upon immunoprecipitation Ttc7 was found to interact with both Efr3b, and P14KIIIα [10].

The Ttc7 gene is expressed in the liver, kidney, spleen, skin, heart, lung, thymus, and stomach [8]. Ttc7 expression was found to be expressed the most in B lymphocytes and hematopoietic stem cells, which is well in line with the phenotype of autoimmunity and anemia [8]. This

widespread expression pattern also is coherent with the systemic phenotype seen in the fsn mice that results due to a mutation in the Ttc7 gene.

B Lymphocyte Maturation

B lymphocytes are derived from hematopoietic stem cells found in the bone marrow [1]. The bone marrow contains a type of non-lymphoid cells called stromal cells that direct the maturation of the common lymphoid progenitor cells. Stromal cells not only adhere to the developing lymphocytes but also produce cytokines and chemokines that direct differentiation. The cellular environment and provided signals in the bone marrow determine which lineage the common lymphoid progenitor cell is committed to, the B lineage or T lineage.

Committing to the B cell lineage requires the transcription factor E2A, which induces the expression of the early B cell factor [1]. Maturing B-lineage cells expressing these two factors receive a survival signal via IL-7. This combination of protein expression marks the pro-B cell stage. Early pro-B cells will begin gene rearrangement of the immunoglobulin heavy chain locus. The immunoglobulin heavy chain locus will eventually become the pre-B cell receptor. Early pro-B cells differentiate further into late pro-B cells that continue gene rearrangement until a complete immunoglobulin heavy chain locus is expressed. Upon completion of the immunoglobulin heavy chain, the cell receives a stimulation signal that further differentiates into large pre-B cells that express the pre-B cell receptor transiently at the surface. Large pre- B cells proliferate, eventually ceasing to become small resting pre-B cells. Small pre-B cells rearrange the genes of the immunoglobulin light chain locus. Once gene rearrangement of the light chain is complete the cell expresses immunoglobulin μ. Expression of IgM at the cell surface indicates an immature B cell [1].

Immature B cells will be tested for autoreactivity before leaving the bone marrow [1]. Immature B cells that react strongly to self antigens will be eliminated via clonal deletion. Some autoreactive B cells may be "rescued" through receptor editing that will in theory alter the affinity of the B cell receptor to self antigen. Immature B cells that respond mildly to self antigens may become anergic, or be allowed to migrate to the periphery where they will most likely be immunologically ignorant to self [1]. Immature B cells that do not respond strongly to self antigen migrate to the "red pulp" of the spleen for further maturation [11]. Immature B cells that have made it to the spleen are T1 B cells. Self antigen is once again presented to the T1 B cells and further selection occurs. T1 B cells that pass this step of selection become T2 B cells and migrate further to the follicle of the spleen. T2 B cells also express IgD on the cell surface. T2 cells differentiate into MZ, B1, and naive B2 lymphocytes [11]. A third type of transitional B cells, the T3 B cells are an anergic self reactive subset of B cells. T2 B cells that are found to respond to self antigen will become anergic T3 B cells.

Mature naive B cells will further differentiate into lymphoblasts after successfully responding to an antigen challenge [1]. Lymphoblasts will proliferate and form a subset of memory cells and plasma cells. Plasma cells produce significant amount of antibodies when responding to antigens and therefore are producing large amounts of proteins. B cell maturation also requires an increased amount of protein expression to pass through the various checkpoints. Protein mis-folding is common during times of high protein expression. The presence of mis-folded proteins initiates the unfolded protein response (UPR) pathway which is signaled during times of ER stress. The UPR induces the splicing of a protein called X-Box Binding Protein 1 (XBP1). Spliced XBP1 indicates that the cell is undergoing ER stress. The UPR is capable of rescuing a cell in ER stress before it receives an apoptosis signal.

The Efr3b/Ttc7/PI4K Complex

Previous research has shown that a complex is formed at the plasma membrane between Efr3b, Ttc7, and PI4KIIIα. There have been differences found in complex formation between wild type Ttc7 and Ttc7^{fsn}. The downstream effects of the fsn mutation on complex formation has not yet been elucidated. It has also been found that the Hepatitis C Virus NS5A protein interacts with PI4KIIIα whether or not this leads to complex disruption is not fully understood. Lack of any one of the complex proteins prevents the complex from forming.

The Efr3b protein is post transcriptionally palmitoylated that allows it to be anchored in the plasma membrane [10]. Nakatsu *et al* found that Efr3b was constitutively located at the plasma membrane. Efr3b is a comprised of multiple α helical HEAT (Huntington, Elongation factor 3, Regulatory subunit A of protein phosphatase 2A, and Target of rapamycin) repeats [10]. The N terminus of the Efr3b protein contains a Cys rich motif that was confirmed to be a site of palmitoylation. Efr3b mutants lacking the palmitoylation sites were localized in the cytosol rather than at the plasma membrane, these mutants also lacked the ability to target Ttc7, and PI4KIIIα [10]. The Efr3b protein is capable of recruiting Ttc7 to the plasma membrane, but was not able to recruit PI4KIIIα alone. The formation of the complex requires Ttc7, it is hypothesized that Ttc7 serves as a bridge between Efr3b and PI4KIIIα [10]. This three way complex assembles in a stoichiometric manner, over expression of one of the proteins does not lead to increased complex formation.

The phosphatidylinositol 4-kinases synthesize phosphatidylinositol (PI) 4-phosphate an important plasma membrane phospholipid [10]. PI 4-phosphate has many intracellular roles, PI 4-phosphate can be found in Golgi, the endosomes, and the plasma membrane. From PI 4-

phosphate, PI 4-5 bisphosphate can be produced with the help of a PI monophosphate kinase. There are four PI 4-kinase isoforms, PI4KIIIα, PI4KIIIβ, PI4KIIα, and PI4KIIβ [10]. The PI4KIIIα isoform has been found to interact with both Efr3b and Ttc7. PI4KIIIα is an ER associated protein that also dynamically interacts with the plasma membrane through recruitment of Efr3b and Ttc7 [10]. In a knock out study of PI4KIIIα it was found that plasma membrane pools of PI4KIIIα were depleted, as well as many plasma membrane proteins were found in intracellular vesicles rather than at the plasma membrane [10].

The Hepatitis C Protein NS5A

The Hepatitis C Virus (HCV) is an enveloped, positive sense, single stranded RNA virus of the *Hepacivirus* genus, part of the *Flaviviridae* family [12]. HCV infection causes acute hepatitis, in 70%-80% of patients HCV infection causes chronic hepatitis which leads to long term health concerns. Worldwide 170 million people (3% of world population) are infected with HCV [12]. The HCV genome encodes for ten complete proteins that are cleaved by proteases during viral replication [13]. The HCV genome encodes for the following structural proteins; core, E1, E2, p7, and NS2. The structural proteins are involved in producing infectious virions. The non-structural proteins, NS3, NS4A, NS4B, NS5A, and NS5B are required for viral RNA replication. Viral RNA replication occurs within a membranous web that is necessary for replication [13].

As previously mentioned HCV infection has been linked to the development of autoimmune diseases [4]. The immunological manifestations of chronic HCV infection can sometimes lead to development of autoantibodies including ANA's and anti-DNA antibodies [14]. Patients with chronic HCV infections experience a "lupus-like syndrome" categorized as the presence of autoantibodies, arthritis, neuropathy, hypocomplementemia, and cryoglobulinemia. Among

patients currently meeting the diagnostic criteria for SLE, 13% tested positive for anti HCV antibodies compared to 2% of the general population [14]. Ramos-Casals *et al* reported that patients with both SLE and anti-HCV antibodies experienced a slightly altered symptom profile than those without HCV infection. Patients with both HCV antibodies and SLE had decreased cutaneous symptoms and increased liver involvement, along with decreased anti-DNA antibodies, and increased hypocomplementemia and cyroglobulinemia [14]. The apparent overlap in symptoms caused by HCV and SLE, and link between the two diseases has lead Ramos-Casals *et al* to suggest that HCV testing be made part of the SLE diagnostic criteria.

Viral RNA replication of the HCV genome requires a membranous web that is derived from the ER [12]. It has been found that cells capable of supporting HCV RNA replication show signs of ER stress. Cells infected with HCV have active forms of ATF6, a transcription factor that is a marker of the unfolded protein response (UPR). ATF6 also initiates the cleavage cascade that results in the spliced version of XBP1, another marker of ER stress. It has been reported that cells with replicating HCV, XBP1 is repressed, the mechanism of this outcome is not well understood. HCV infection has also been shown to increase the amount of reactive oxygen species (ROS) leading to oxidative stress in infected cells. The HCV protein NS5A has been shown to induce oxidative stress in cultured cells [12].

The Hepatitis C Virus non structural protein 5 A (NS5A) is a phosphoprotein that is the replicase factor for NS5B, the HCV RNA dependent RNA polymerase [15]. Reiss *et al* have reported that PI4KIIIα is necessary for viral replication. HCV infection has been shown to cause an increase of the PI4KIIIα product, PI4P [15]. To determine this, the Reiss team screened the human kinome and found that the only protein that repeatedly interacted with NS5A was PI4KIIIα, this interaction occurred in every screen the team conducted. It was found that NS5A is made up of

three domains, domain 1 interacts with PI4KIIIα, and domain 3 is necessary virion assembly, the function of domain 2 is unknown [15]. Each domain is separated by two low complexity sequences and has an N-terminal amphipathic helix that is required for its association with the membrane [13]. Any mutations in the domain 1 of NS5A and it can no longer interact with PI4KIIIα. The phenotype that arises when domain 1 is mutated matches that caused by PI4KIIIα silencing [13]. Domain 1 of NS5A was further investigated for its interaction with PI4KIIIα. It was found that there is a PI4KIIIα binding region (PBR) at the C-terminal end of domain 1, from amino acids 187 to 213 [13]. The PI4KIIIα functional interaction site (PFIS) was found to exist between amino acids 202-210 within the PBR.

PI4KIIIα supports the formation of the membranous web that is required for viral RNA replication [15]. The co-localization of NS5A and PI4KIIIα appear as punctate clusters inside the cell. In cells with silenced PI4KIIIα, the clusters appear abnormal. It is suggested that PI4KIIIα is recruited to the membranous replication compartment by NS5A, NS5A then activates the kinase and an increase in the kinase product PI4P is generated which contributes to the membranous web [15]. Cellular PI4P plays a role in trafficking vesicles to the plasma membrane [13]. HCV infection causes alterations in the plasma membrane pool of PI4P, while the global level of PI4P remains the same through compensatory activity of other PI kinases [13].

NS5A has been found to exist within cells in two different phosphorylation states, a hypophosphorylated state (p56) and hyperphosphorylated state (p58) [13]. It is the ratio of these two phosphorylation states that regulates viral RNA replication. A low amount of p58 is required for RNA replication, which may indicate that p58 is a negative regulator of RNA replication, or it is a viral assembly necessity [13]. Reiss *et al* found that PI4KIIIα is responsible for the phosphorylation state of NS5A. Silencing of PI4KIIIα prevented HCV replication [13]. This

makes for a complex interaction between NS5A and PI4KIIIα. It is currently understood that NS5A and NS5B are required to activate PI4KIIIα, and active PI4KIIIα alters the phosphorylation state of NS5A. Phosphorylation states of NS5A determine viral RNA replication, and PI4KIIIα produces PI4P which forms the membranous web required for viral RNA replication [13, 15]. The complete mechanism behind the interaction between NS5A and PI4KIIIα and how it regulates viral RNA replication remains to be elucidated.

Rationale

This study aims to determine changes in Efr3b/Ttc7/PI4KIIIα complex formation. To do this the localization of Efr3b in immature B cells must first be determined. The $Ttc7^{fsn}$ protein has been found to be less stable than the wild type protein, changes in complex formation due to the genetic mutation are of interest. The use of a stimulatory step mimics the activation process of B cell maturation. Changes in complex formation after stimulation is also of interest.

Efr3b/Ttc7/PI4KIIIα complex formation was also studied in immature B cells after transfection with the HCV protein NS5A. Previous data has shown PI4KIIIα and NS5A interact in cells infected with HCV. Those studies used cells infected with the entire virus, in contrast to this study which used a single HCV protein. To determine possible complex disruption the phenotype induced by transfecting immature B cells with NS5A was characterized. The interaction between NS5A and PI4KIIIα was determined before and after stimulation.

Hypothesis: Disruption of the Efr3b/Ttc7/PI4KIIIα complex by either a genetic mutation in Ttc7, or by sequestering the PI4KIIIα with NS5A will lead to ER stress in immature B cells.

METHODS

WEHI 231 Culture

WEHI 231 cells are cultured at 37C, 10% CO_2, in DMEM (Lonza) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, and supplemented with 10% FBS (HyClone, Fisher Scientific), 100 units/mL Penicillin (Gibco), 100 ug/mL Streptomycin (Gibco), and 0.05 mM 2-mercaptoethanol (Sigma). Cell conditions were kept per ATCC instructions. Cell cultures were harvested at the exponential phase of growth for all experiments.

WEHI 231 Cell Transfection

WEHI 231 cells were transfected using the BTX T820 Electrosquarelectroporator (Harvard Apparatus). Cells were washed twice with PBS, resuspended in transfection buffer and added to transfection cuvettes with the plasmids. Cells were electroporated at 170V, 1 pulse, for 50 miliseconds. Post transfection cells were added to fresh DMEM (Lonza) and incubated until analyzed. Some experiments required a stimulation step at time=12 hours. To do this, 10 mg/mL of goat anti mouse F'(ab) anti-IgM (Southern Biotech) was added to the cells. WEHI 231 cells were transfected with the following plasmids; EFR-GFP, EFR-mCherry, Ttc7-wt-GFP, Ttc7-fsn-GFP, NS5A-mCherry, and P14K-GFP. For six million cells, 1.5 milligrams of plasmid was added, the exception being NS5A-mCherry. NS5A-mCherry required 3 milligrams of plasmid per six million cells. The NS5A timeline experiments were transfected as described above. Cells were imaged at the following time intervals; time=0, time=12, time=13, time=24, time=36, and time=48. At time=12 cells were stimulated with anti-IgM.

Cell Staining

Cells were stained with the following dyes; DAPI (Molecular Probes) to stain the nucleus, Cell Mask (Molecular Probes) to stain the plasma membrane, ER Tracker (Molecular Probes) to stain

the endoplasmic reticulum, and Bodipy (Invitrogen) which stains lipid droplets. WEHI cells were stained according to manufacturer instructions before being analyzed.

Confocal Microscopy

To prepare cells for confocal microscopy, cells were mounted onto a well slide. After transfection, 2-3 mL of cells were harvested, spun at 1000g for 5 minutes and washed twice with PBS. At this point if a live stain was used the cells were stained and fixed in a 2% paraformaldehyde- 0.1% sucrose solution for ten minutes. Cells were washed twice more with PBS and incubated with DAPI (Sigma) for twenty minutes. The stain was removed and cells were mounted using Prolong Gold Antifade (Molecular Probes). A glass coverslip was kept in place with clear nail polish and slides were left to cure at room temperature. Confocal microscopy was performed using an Olympus IX81 confocal microscope (software: FluoView version 4.3). Images were acquired through a 100x oil objective. At least five randomly chosen fields were analyzed for each treatment condition, containing a minimum of 25 cells.

Flow Cytometry

To prepare cells for flow cytometry, cells were harvested after transfection and washed twice with cold PBS. The cells were stained with ER Tracker for 15 minutes and washed again with cold PBS. Cells were then resuspended in cold PBS and added dropwise to cold 75% ethanol. Cells were stored in ethanol at 20 °C until analysis via flow cytometry. Flow cytometry was performed using the BD FACS Calibur (San Jose, CA) flow cytometer. A minimum of 10,000 events were collected and analyzed using WinList 7.0 (Topsham, ME).

RESULTS

This study investigated the co-localization of the Efr3b and Ttc7 proteins. Previous studies had found that there was co-localization of the PI4K and Ttc7 proteins at the plasma membrane. It had also been determined that Efr3b was anchored in the plasma membrane. One major goal of this study was to determine if Efr3b and Ttc7 colocalize at the plasma membrane in immature B cells. The WEHI 231 cells used in this study are naive, immature B lymphoblasts from the species *Mus musculus.* This study also focused on the previously unknown outcome of transfection of WEHI 231 cells with the Hepatitis C protein NS5A. Previous studies had found that NS5A protein interacts with the PI4K protein. To determine the possible impact of NS5A on PI4K, cells were transfected with NS5A. As this had not previously been investigated a new phenotype arose.

Efr3b is constitutively localized to the plasma membrane

Previous studies have indicated that when the Efr3b protein is in complex with Ttc7 and PI4K, it is associated with the plasma membrane. Prior to this study it was not well known if the Efr3b protein was transiently localized to the plasma membrane during complex formation or if the Efr3b protein was constitutively localized at the plasma membrane. The impact of stimulation on the localization of Efr3b was previously unknown. Immature B cells that have proven not to be auto reactive will eventually become mature B cells. The maturation process begins when the B cell is presented an antigen that it can respond to by having a fully functioning BCR on the cell surface. This response is the activation step that is part of the maturation process of B cells. To better study changes in WEHI 231 cells anti-IgM treatment was used to represent the natural process of activation. To determine the localization of the Efr3b protein WEHI 231 cells were transfected with an Efr3b-gfp vector, 24 hours post transfection the cells were harvested and the

plasma membrane was stained with Cell Mask, a fluorescent plasma membrane dye. Fluorescent microscopy was used to determine the possible localization of the Efr3b protein to the plasma membrane. Cells were treated with anti-IgM, to stimulate antigen engagement, for 1 hour prior to harvest. Unstimulated cells transfected with the Efr3b protein showed localization of Efr3b to the plasma membrane (Figure 1). However, after stimulation the distribution of the Efr3b protein is altered as seen in Figure 1, and is observed to reduce the localization of the Efr3b protein to the plasma membrane.

Co-localization of Efr3b and Ttc7 is impacted by stimulation and the fsn mutation

Co-localization studies have discovered differences in complex formation between wild type Ttc7 and mutant Ttc7^{fsn} when interacting with PI4K [16]. Complex formation is commonly described as PI4K patches which form puncta that appear as dense fluorescent intensity, representing the two proteins interacting. This study investigated whether or not the fsn mutation altered the Efr3b-Ttc7-PI4K complex formation. To investigate the difference between wild type Ttc7 and Ttc7^{fsn}, WEHI 231 cells were transfected with Efr3b-mCherry, and either Ttc7-gfp or Ttc7^{fsn}-gfp. Cells transfected with the Ttc7^{fsn} protein show a decrease in puncta formation when compared to the wild type controls (Figure 2a and b). The Ttc7^{fsn} protein appears more disperse than the wild type counterparts (Figure 2a and b). The Efr3b-Ttc7 complex formation is also impacted by stimulation. Cells treated with anti-IgM show an increase in Efr3b-Ttc7 complex formation (Figure 2a and b). The cells that were transfected with the Ttc7^{fsn} and stimulated also show an increase in puncta formation, however it does appear more diffuse than the wild type Ttc7. This pattern is consistent with the co-localization studies performed using Ttc7 and PI4K [16].

The Efr3b protein has been shown to be localized to the plasma membrane. Complex formation is altered by both the fsn mutation and treatment with anti-IgM. Cells transfected with the $Ttc7^{fsn}$ protein indicated the most significant decrease in puncta.

NS5A transfection alters cellular morphology

Hepatitis C Virus infection has been previously linked with autoantibody formation [14]. The Hepatitis C Virus is also known to rely on PI4K for viral replication [13]. These links prompted the study of NS5A-mCherry transfection of WEHI 231 immature B cells to determine the impact of NS5A on PI4K. Sequestering PI4K would render the kinase unavailable for complex formation, similar to the impact on complex formation by $Ttc7^{fsn}$. The possibility that complex formation may be impacted by a viral protein is a possible environmental link to the mechanism of autoantibody development and the consequences of disruption of the Efr3b-Ttc7-PI4K complex.

Previous studies have focused on the outcome of infection with the Hepatitis C Virus. Prior to this study the phenotype resulting from transfection of the HCV protein NS5A had not been determined. Another novelty to this system is that previous research used cells that remain in a steady state, rather than this system in which the cells are stimulated with anti-IgM at the 12 hour time point. This study focused on the outcome of transfecting WEHI 231 cells with NS5A-mCherry. To determine what phenotype would arise from NS5A transfection, WEHI 231 cells were transfected with NS5A-mCherry. Initial transfection studies indicated that there may be time dependent outcomes. This prompted a time dependent study in which cell cultures were monitored at the following time points: T=0 hours, T=12 hours, T=13 hours, T=24 hours, T=36 hours, and T=48 hours post transfection. The cells were also harvested and prepared for analysis

via confocal microscopy at T=24 hours, T=36 hours, and T=48 hours post transfection. The phenotype that arose from transfection of WEHI 231 B lymphoblasts with the HCV protein NS5A is characterized by the appearance of white droplets. Upon repeated trials of NS5A transfection the cells appear to produce white droplets (Figure 3b, d and e) by 12 hours post transfection. Stimulation appears to cause an elongated cellular morphology (Figure c, d and e). Cells transfected with NS5A and stimulated also appear to have an altered morphology. WEHI 231 cells are expected to have a small, round cell morphology. NS5A transfection, as well as stimulation appeared to have induced an elongated and enlarged cellular morhphology typically inconsistent with immature B cells, as well as the presence of small white droplets. Cells that were not transfected with the NS5A protein maintain their expected phenotype. Mock transfected cells that were stimulated begin to develop the elongated cellular morphology as well, indicating that this outcome may be related to the treatment with anti-IgM. Cells transfected with NS5A but not stimulated with anti-IgM do develop the elongated cellular morphology, at 48 hours post transfection the cells continue to develop an altered phenotype, consisting of an enlarged cell size and an increase in lipid droplet formation from the transfection (Figure 3f). Confocal analysis of this experiment confirmed the transfection of NS5A as can be seen by the presence of mCherry in the transfected cells (Figure 4).

NS5A and PI4K co-localization

Prior research has indicated that the Hepatitis C Virus NS5A protein binds to PI4K, the virus requires the PI4K for replication [13]. The effect of NS5A transfection on PI4K had not previously been studied in B lymphoblast cells, therefore the impact of stimulation had also not been investigated. In this study WEHI 231 cells were transfected with NS5A-mCherry and PI4K-gfp, a group of cells was also stimulated for one hour with anti-IgM before being harvested and

prepared for fluorescent microscopy. Unstimulated cells indicate that NS5A and PI4K do colocalize (Figure 5). Stimulated cells show an increase in the co-localization of NS5A and PI4K. These findings are consistent with the data indicating that NS5A binds PI4K in cells infected with the Hepatitis C Virus [15].

NS5A transfection alters ER size

Viral infection with the Hepatitis C Virus results in ER stress, the virus relies on the host cell for viral replication which causes the cell to upregulate the production of viral proteins in the ER [13, 15]. Mass production of protein is a known trigger of ER stress. ER stress is a naturally occurring phenomenon in maturing B cells and results from the mass production of antibodies that is required of a B cell [1]. Whether or not transfection of the HCV protein NS5A causes ER stress was currently unknown. ER stress is known to cause an enlargement of the endoplasmic reticulum, an expansion of ER is consistent with ER stress. To determine if transfection of WEHI 231 cells with the NS5A-mCherry protein lead to ER stress, the ER of the cells was stained with ER Tracker. The effect of stimulation with anti-IgM was also investigated. After transfection with the NS5A protein the ER of the WEHI 231 appears larger than the untransfected controls (Figure 6). After stimulation the transfected cells show less of an increase in ER size compared to the unstimulated controls.

To quantify the abundance of ER in the cells, flow cytometry analysis of the cells was performed. The flow cytometry data of the WEHI 231 cells stained with ER Tracker reflect the findings of the confocal microscopy analysis (Figure 7). The cells that are found to be ER Tracker positive have been successfully labeled with the ER Tracker dye. The cells that are found to be ER Tracker high have an increase in fluorescent intensity much more than the

positively dyed cells. This increase in intensity reflects an enlargement of the ER. The percentage of untransfected cells that were ER Tracker positive was slightly lower than the NS5A transfected cells, however the population of ER Tracker high was larger than that of the cells transfected with NS5A at time 0 hours. At time 36 hours the population of ER Tracker positive in the untransfected controls was slightly higher than the NS5A transfected cells, while the population of ER Tracker high cells were lower in the untransfected cells compared to the NS5A transfected cells. When comparing the cells at time 0 hours to the cells at time 36 hours, the overall population of cells ER Tracker positive and ER Tracker high increases in all samples. When comparing the untransfected cells to the untransfected stimulated cells at T=36 hours, the population of ER Tracker positive cells was higher in the unstimulated cells, while the population of ER Tracker high cells were lower in the untransfected and unstimulated cells. When comparing the NS5A transfected cells to those that were both transfected and stimulated, the population of ER Tracker positive cells was higher in the transfected and stimulated cells while the population of ER Tracker high cells were larger in the transfected, un-stimulated cells. The differences in populations of ER Tracker positive and ER Tracker high were not statistically significant, the trend described was qualitatively observed.

NS5A transfection alters lipid distribution

The appearance of the small white droplets prompted the investigation of the lipid distribution in NS5A transfected cells. It had been previously found that HCV infection may lead to small lipid droplet formation that may be used for viral packaging [13]. To investigate the lipid distribution of WEHI 231 cells transfected with the NS5A-mCherry protein, Bodipy, a lipid soluble dye was used to visualize the lipid distribution of the cells. Cells transfected with the NS5A protein do appear to have an altered lipid distribution that may be characterized by an increase in lipids at

the cell surface (Figure 8). Stimulated cells that were transfected with the NS5A protein do not appear to have the same lipid distribution as the unstimulated cells that were transfected with the NS5A protein. The combination of NS5A transfection and stimulation appears to induce an elongated phenotype as well as the increase in small white lipid droplets (indicated by arrows). Transfected and stimulated cells do not appear to have the same lipid distribution as the mock transfected cells. The mock transfected cells serve as a control that maintains the expected phenotype of WEHI 231 cells. Any changes in found in the mock transfected cells is a result of the transfection process and appears to resolve after the cells have were allowed to rest for 24 hours.

This study investigated the phenotypic changes in WEHI 231 cells upon transfection with the Hepatitis C Virus NS5A protein. The impact of stimulation on NS5A transfected cells was also studied. The phenotype that arose from NS5A transfection can be characterized by the presence of small white droplets that may be lipid droplets. Transfected cells also appear to be in ER stress. Treatment with anti-IgM appears to alter the lipid distribution of cells transfected with NS5A, stimulation also impacts cellular morphology causing an elongated phenotype as well as altering the size of the ER. Further studies are needed to fully investigate the phenotypic outcome of transfecting WEHI 231 cells with the HCV protein NS5A.

Figures

Figure 1. Changes in Efr3b localization upon stimulation with αIgM

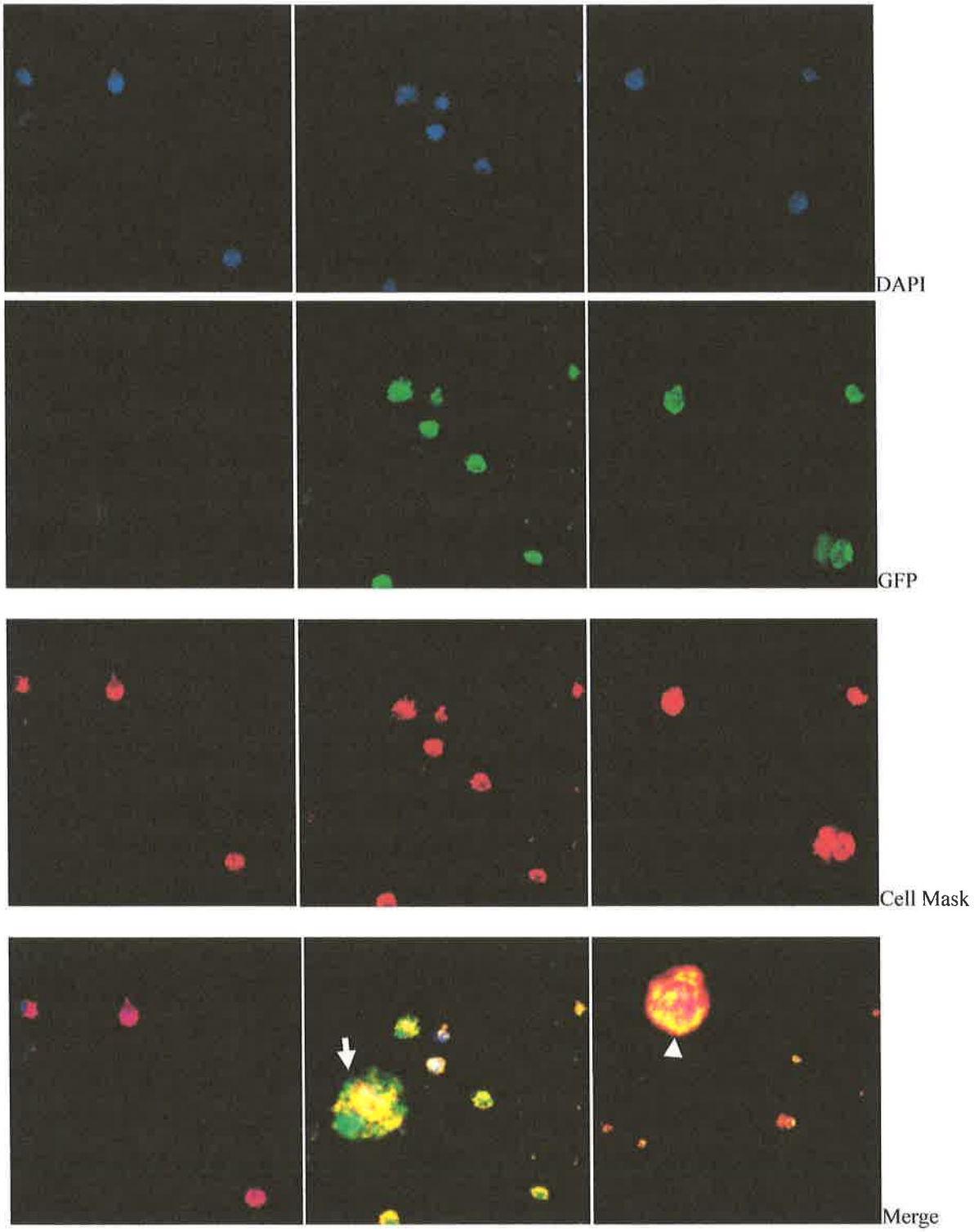
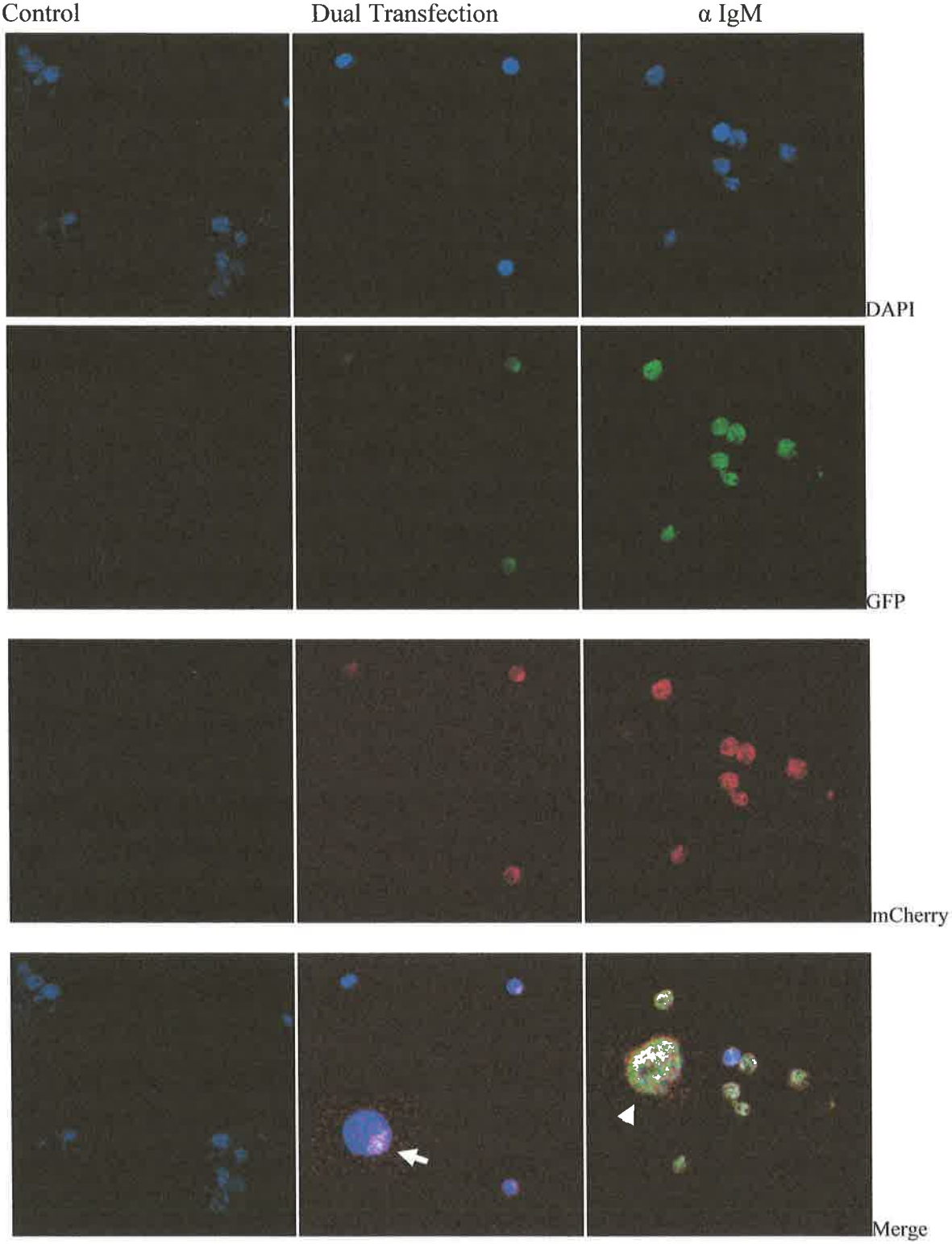


Figure 1: *Changes in Efr3b localization upon stimulation with αIgM*

Untreated control cells indicate that the Efr3b protein is localized to the plasma membrane. Comparisons between untreated control cells and treatment with αIgM indicate that stimulation decreases levels of Efr3b at the plasma membrane.

Figure 2. Changes in Efr3b-mCherry and Ttc7-gfp co-localization upon stimulation with α IgM
A. Efr3b-mCherry and Ttc7-gfp-wt



B.Efr3b-mCherry and Ttc7^{fsn}-gfp

Control Dual Transfection α IgM

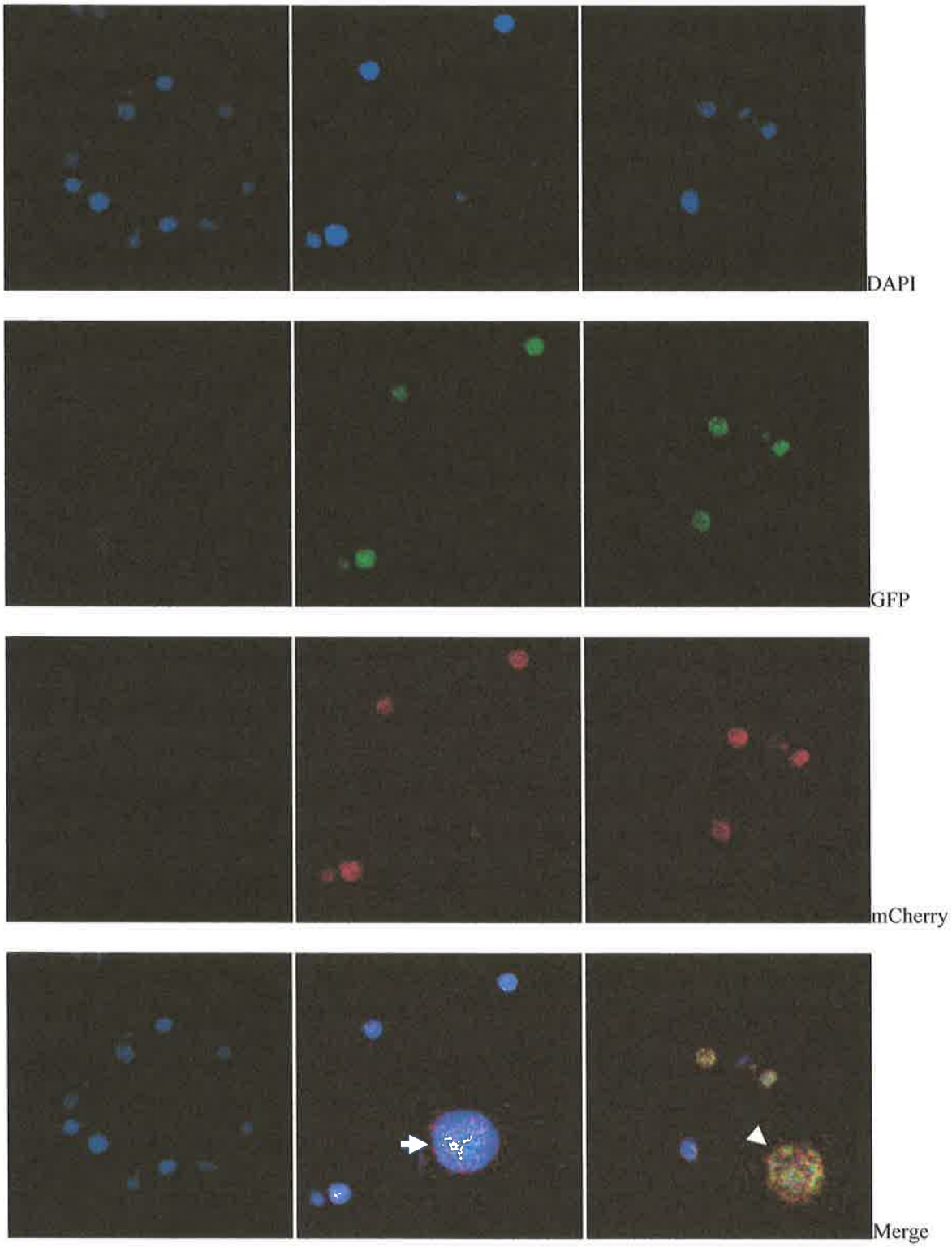


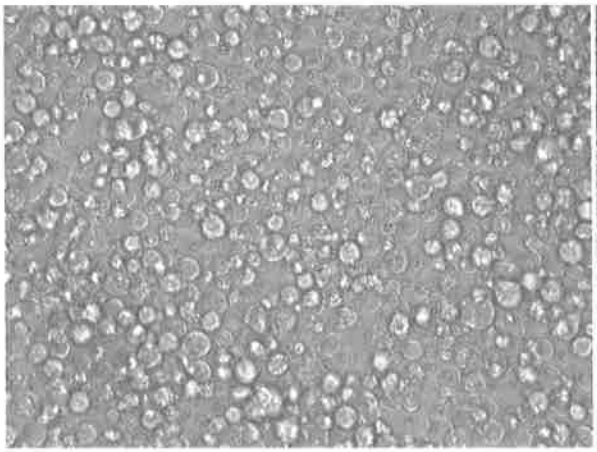
Figure 2: *Changes in Efr3b-mCherry and Ttc7-gfp co-localization upon stimulation with α IgM*

Cells dual transfected with the Efr3b and Ttc7 proteins indicate co-localization of Efr3b and Ttc7 at the plasma membrane (indicated by arrows). Comparison between untreated controls and treatment with αIgM indicate a decrease in the Efr3b and Ttc7 proteins at the plasma membrane (indicated by arrows). Comparisons between the fsn Ttc7 protein and the wild type Ttc7 protein indicate that the fsn mutation decreases the Ttc7 protein (indicated by arrows).

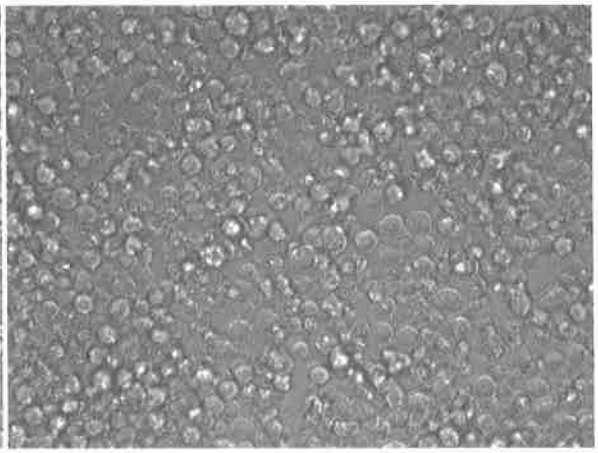
Figure 3. Changes in NS5A Transfected Cells upon stimulation with αIgM

A. Time= 0 hours

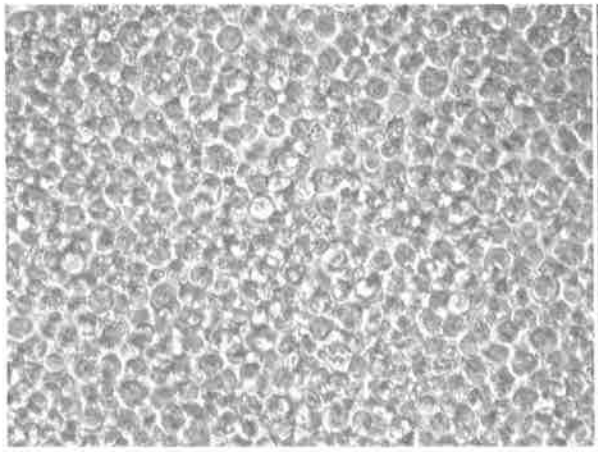
Mock



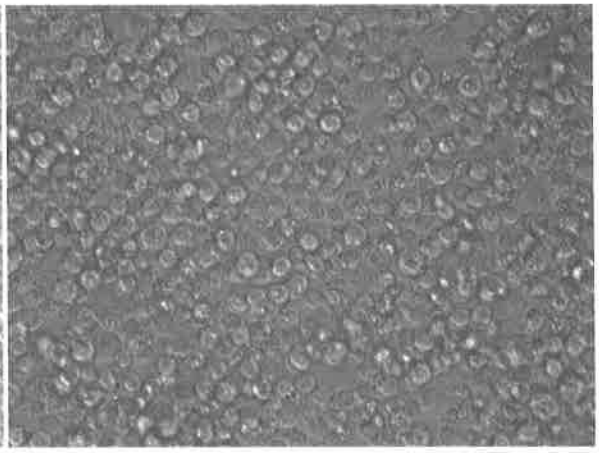
Mock Stimulated



NS5A

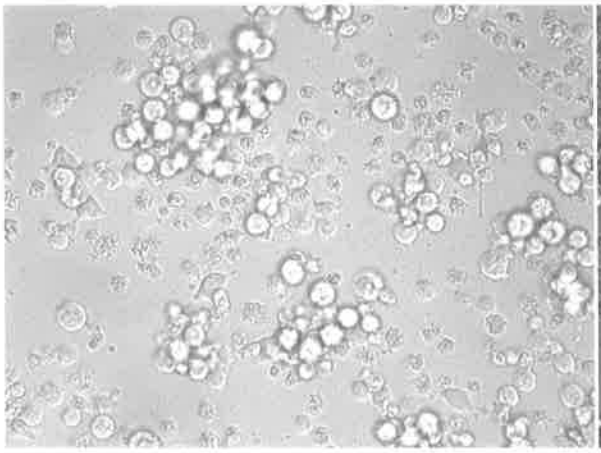


NS5A Stimulated

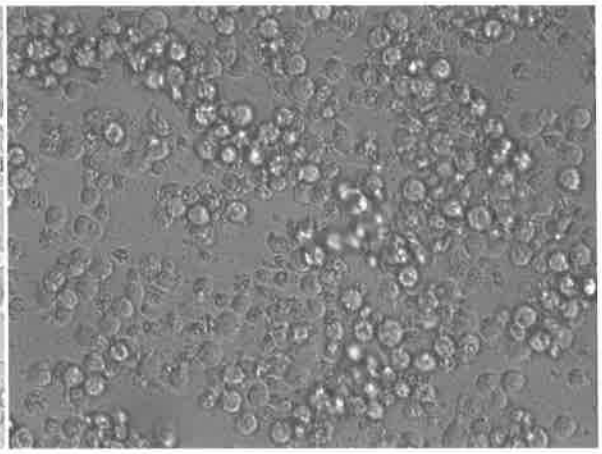


B. Time=12 hours

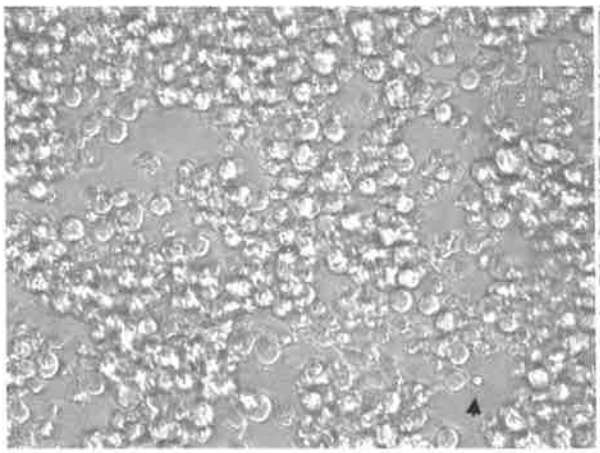
Mock



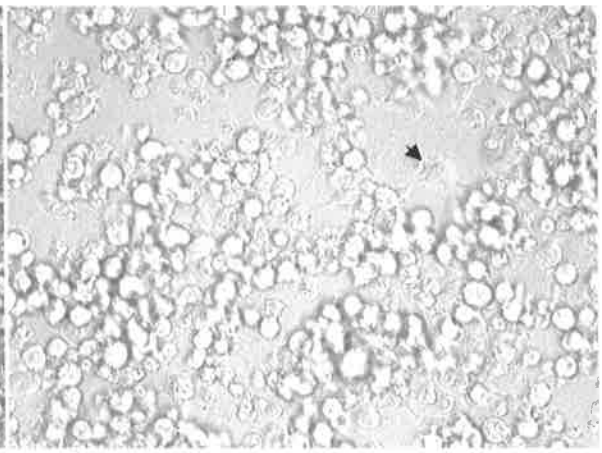
Mock Stimulated



NS5A

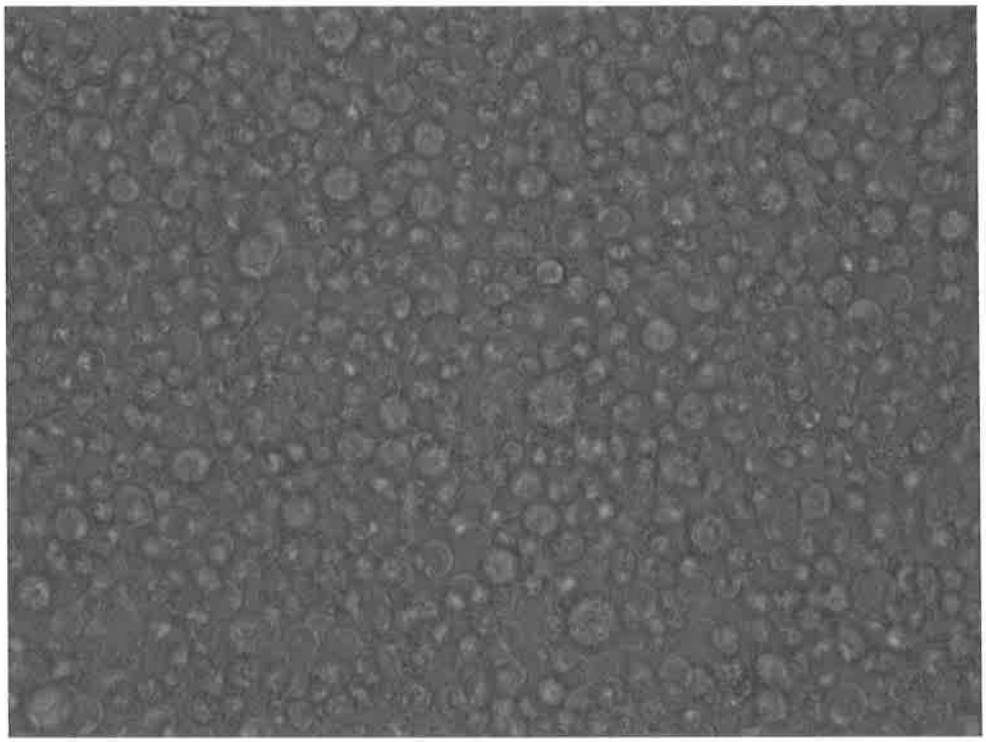


NS5A Stimulated

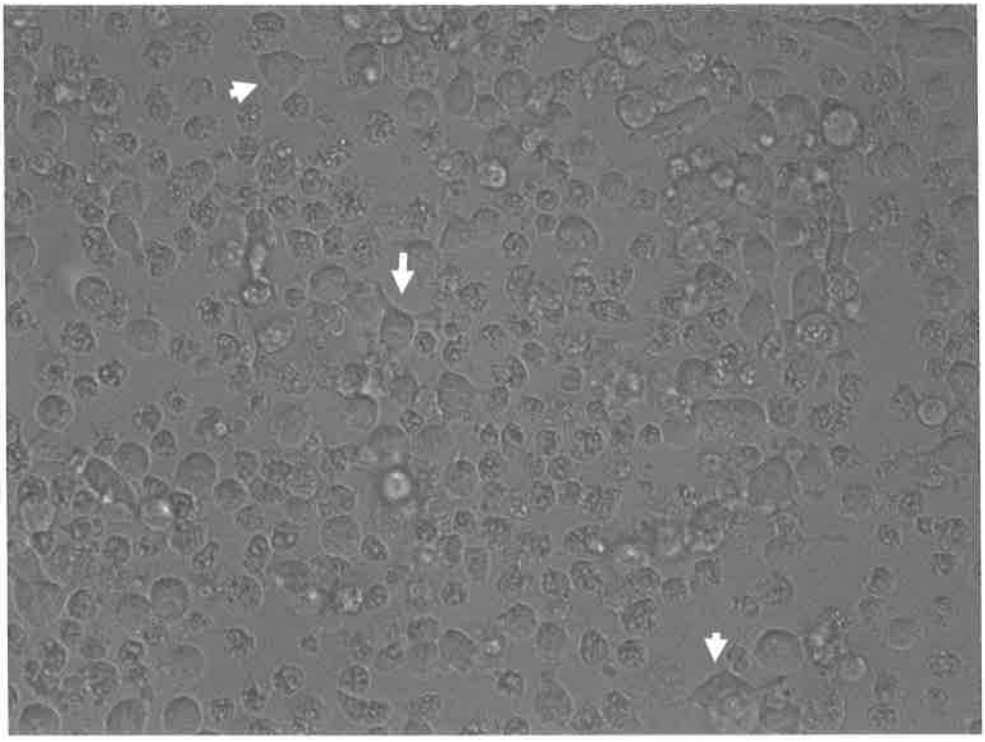


C. Mock Stimulated and NS5A Stimulated after 1 hour treatment with α IgM

Mock Stimulated

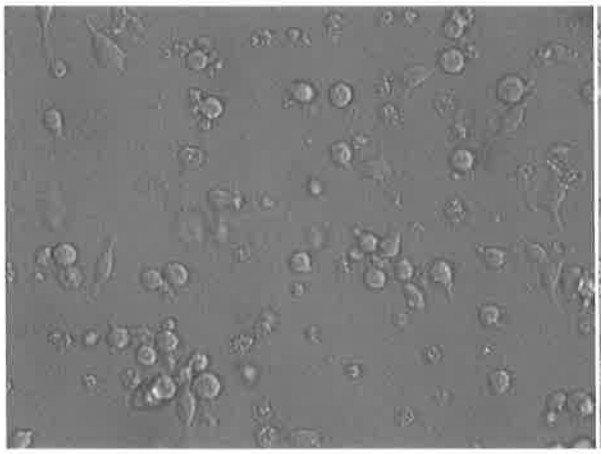


NS5A Stimulated

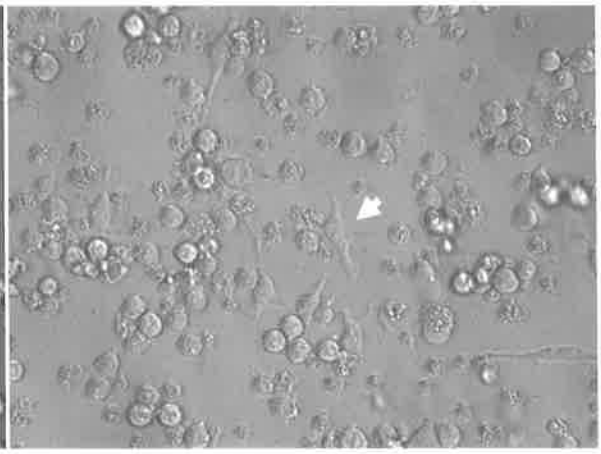


D. Time=24 hours

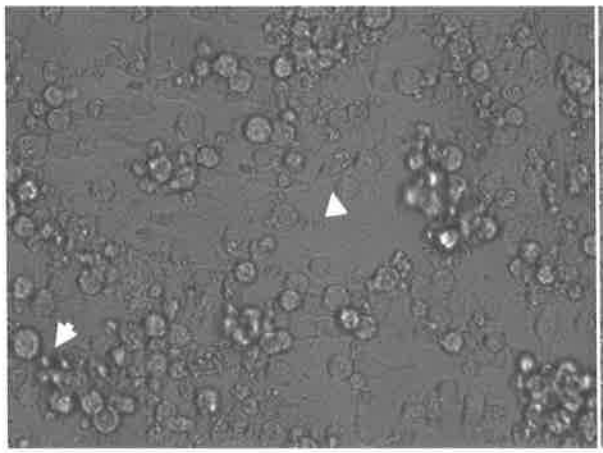
Mock



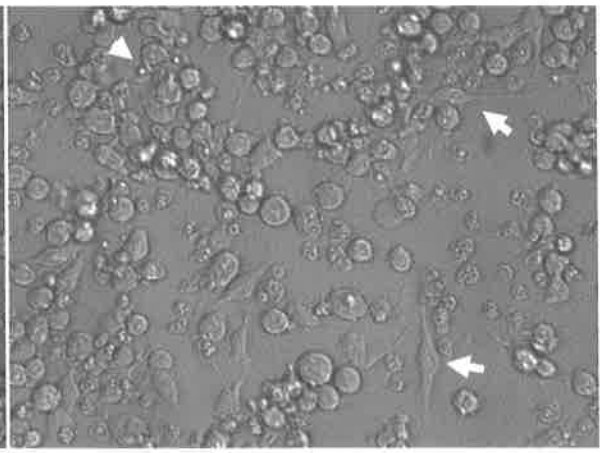
Mock Stimulated



NS5A

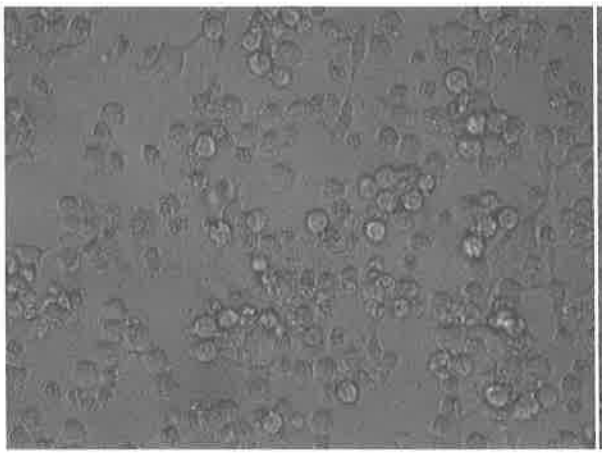


NS5A Stimulated

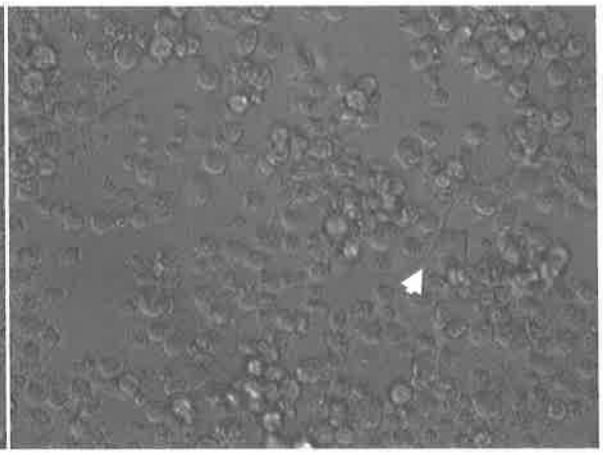


E. Time= 36 hours

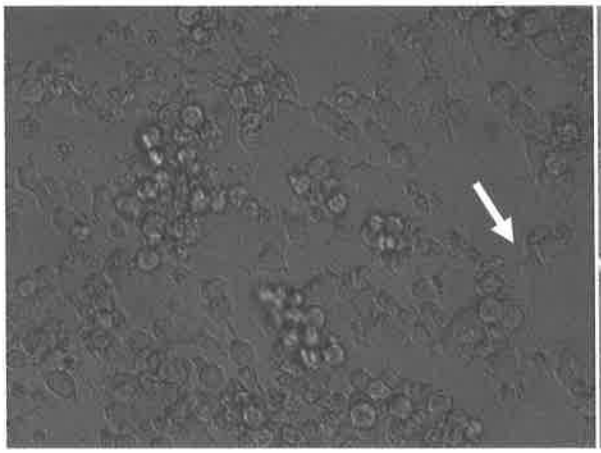
Mock



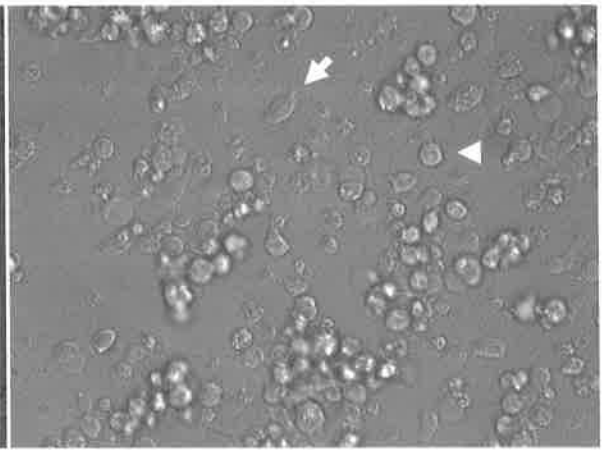
Mock Stimulated



NS5A

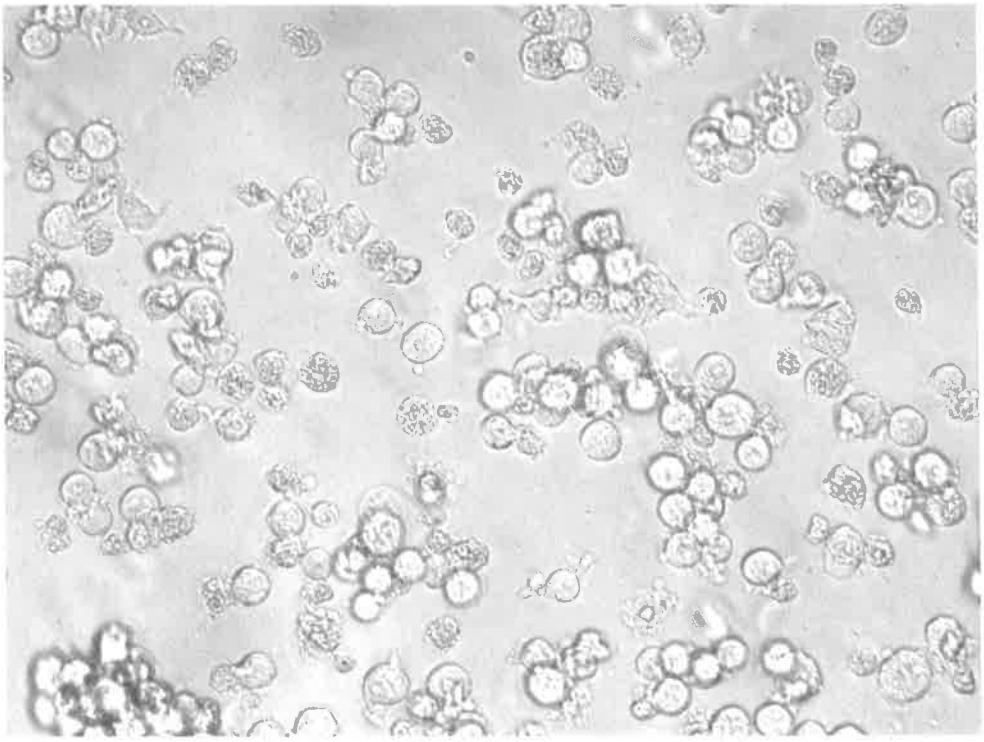


NS5A Stimulated



F. Mock and NS5A Transfected at T=48 hours

Mock



NS5A

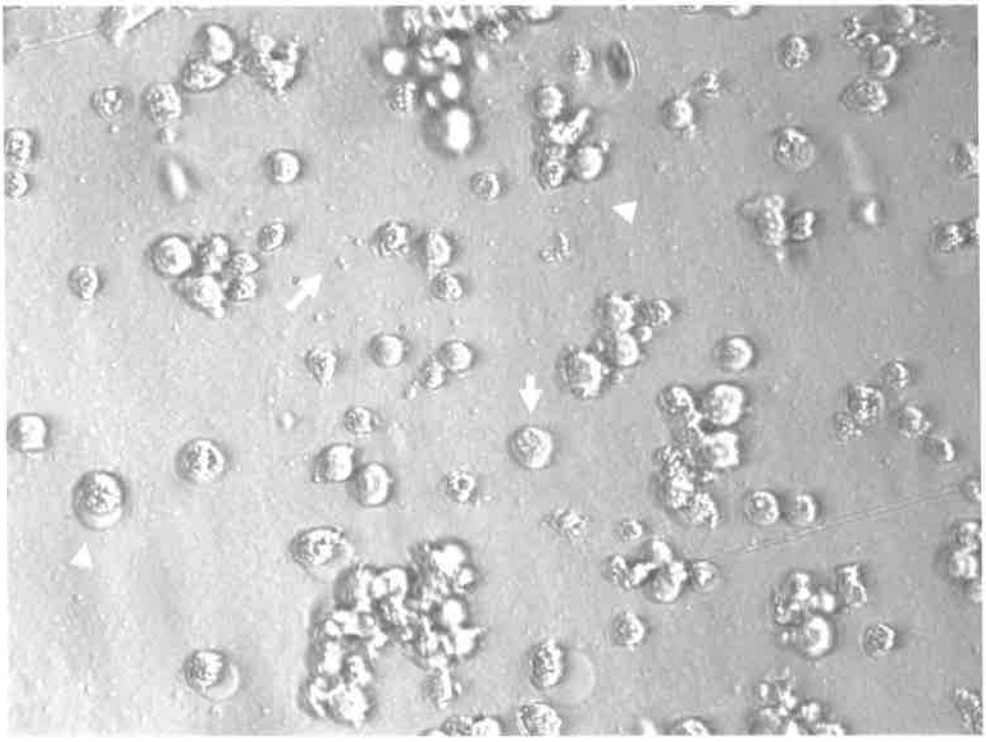


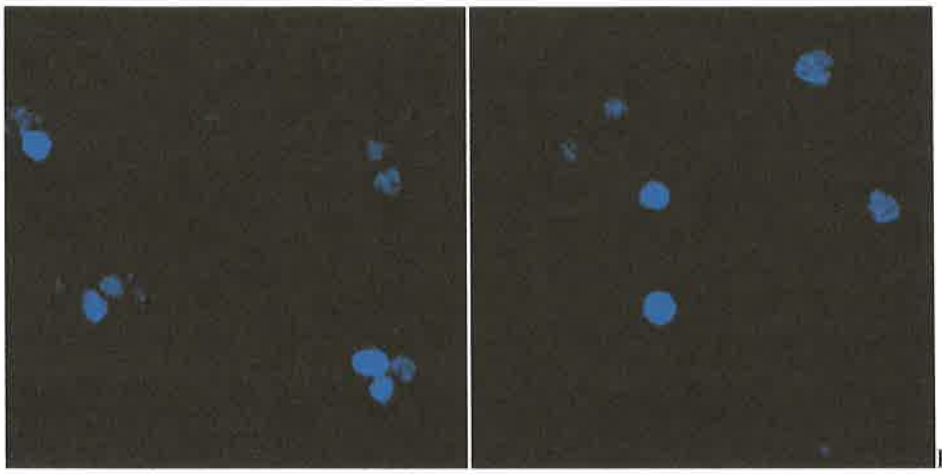
Figure 3. *Changes in NS5A Transfected Cells upon stimulation with αIgM*

Comparison between untransfected controls and cells transfected with the NS5A protein indicate an undetermined phenotype characterized by the presence of small white droplets. Comparison between untreated controls and treatment with αIgM indicate an increase in an elongated cellular morphology. Cells transfected with NS5A and stimulated indicate a morphology influenced by both the transfection as well as treatment with αIgM.

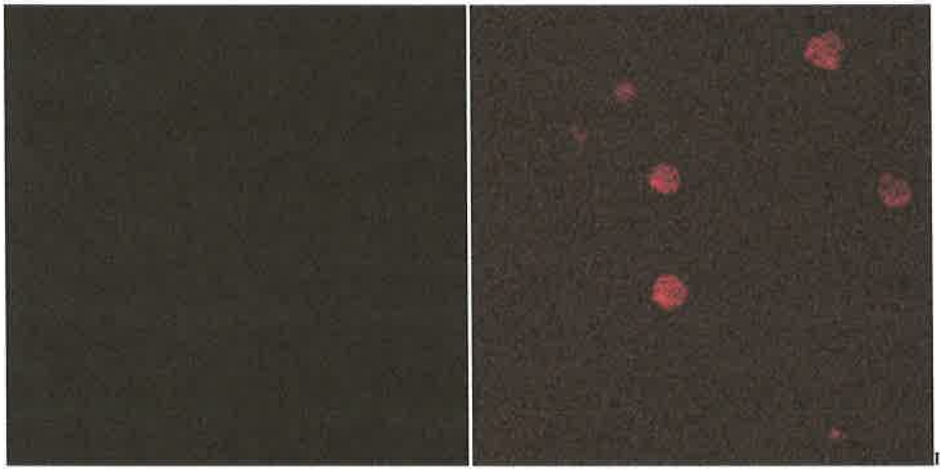
Figure 4. NS5A Time Trial

A. 24 hour post transfection

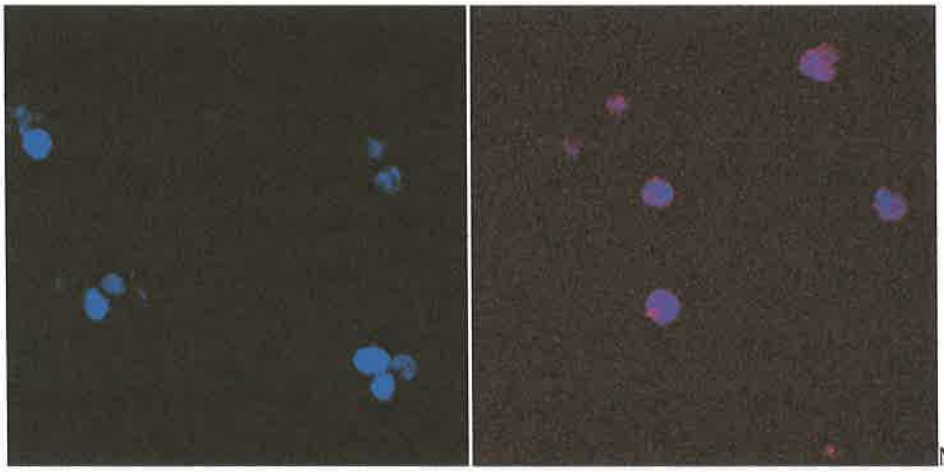
Mock NS5A



DAPI



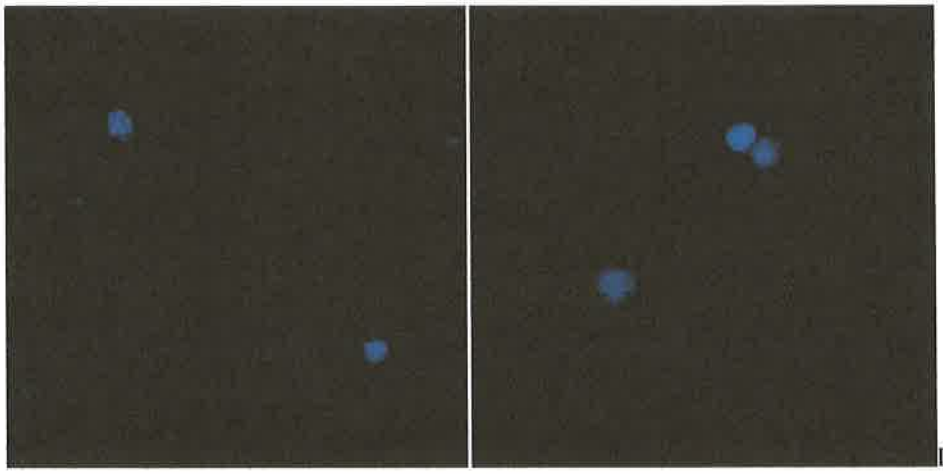
mCherry



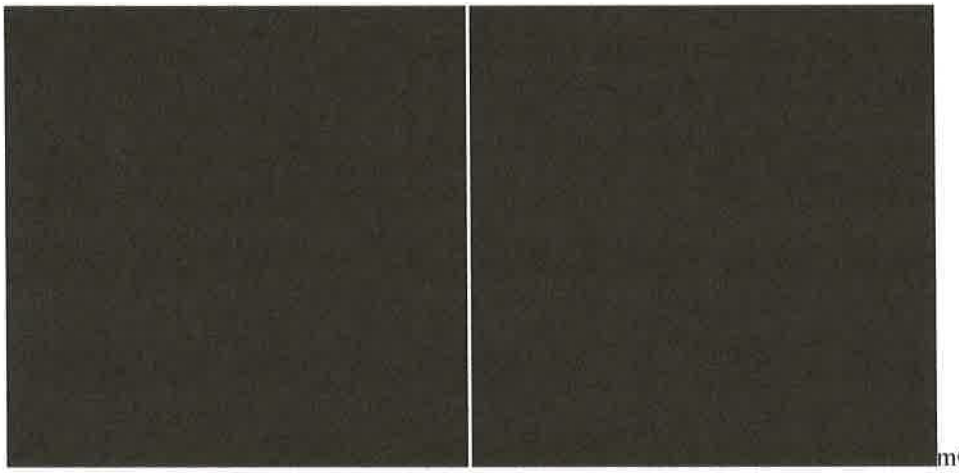
Merge

B. 36 hours post transfection Mock and Mock Stimulated

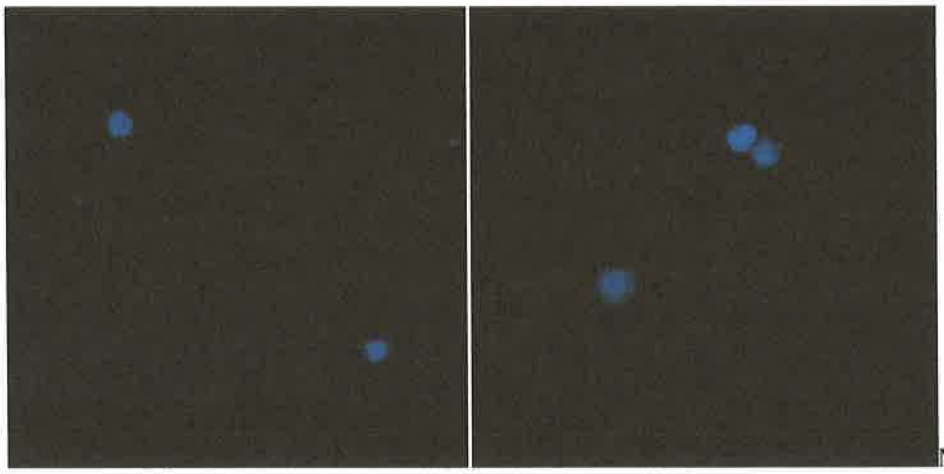
Mock Mock Stimulated



DAPI



mCherry



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C. 36 hour post transfection NS5A and NS5A Stimulated

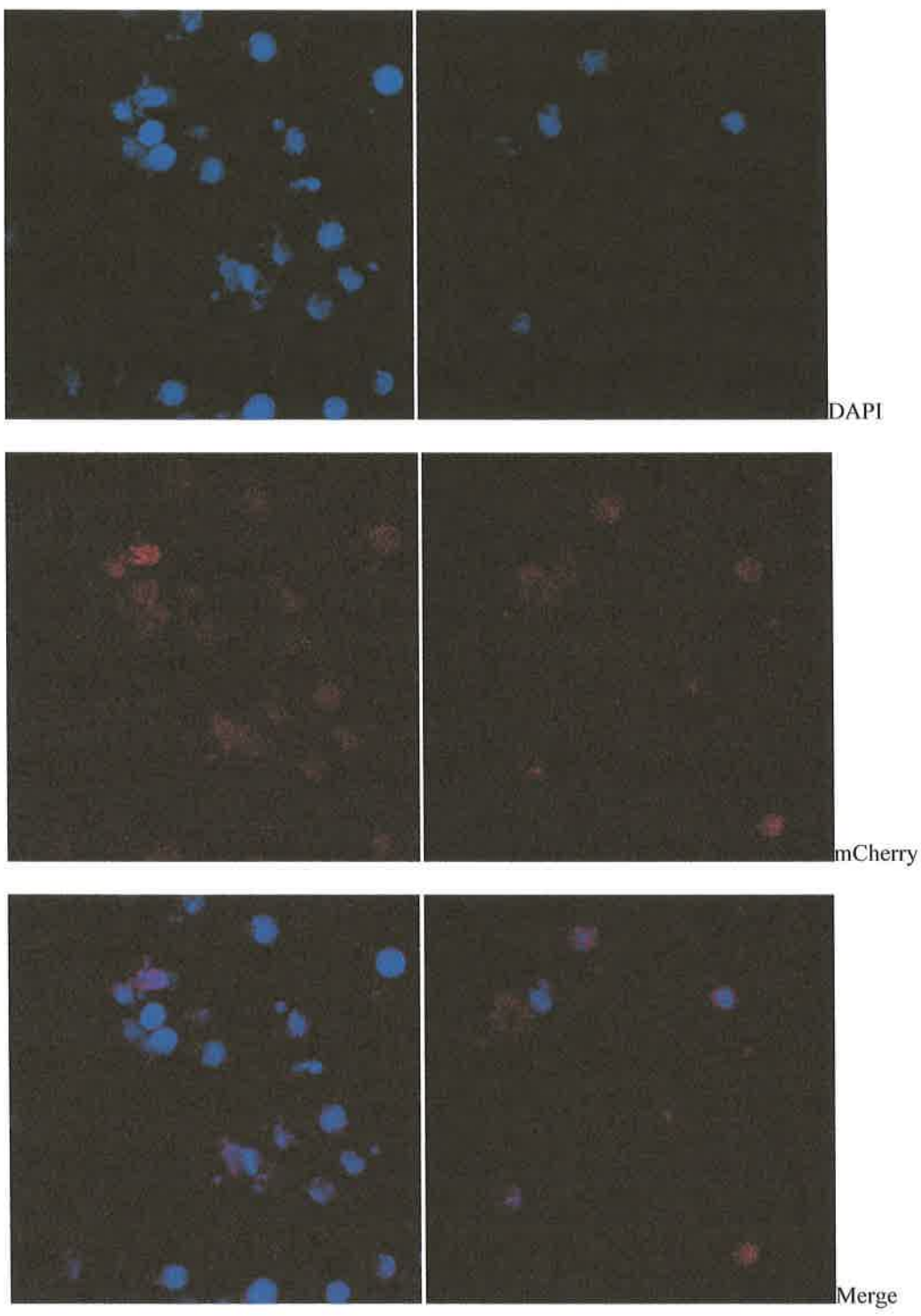
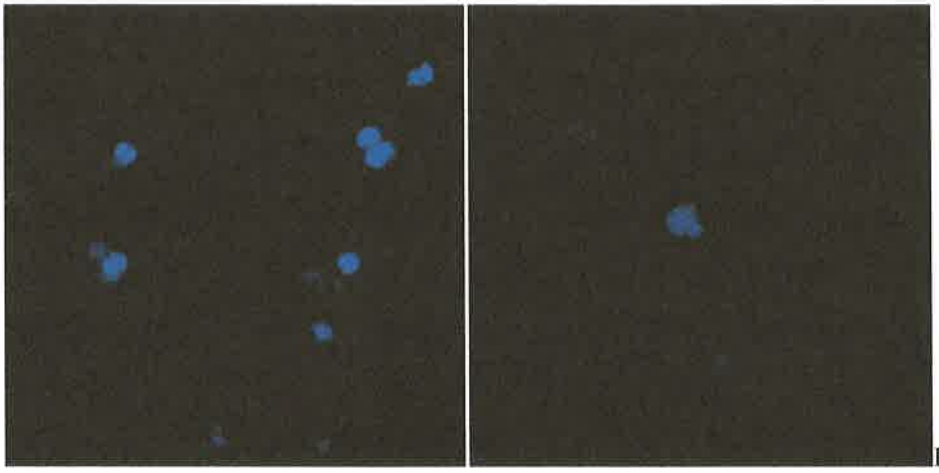
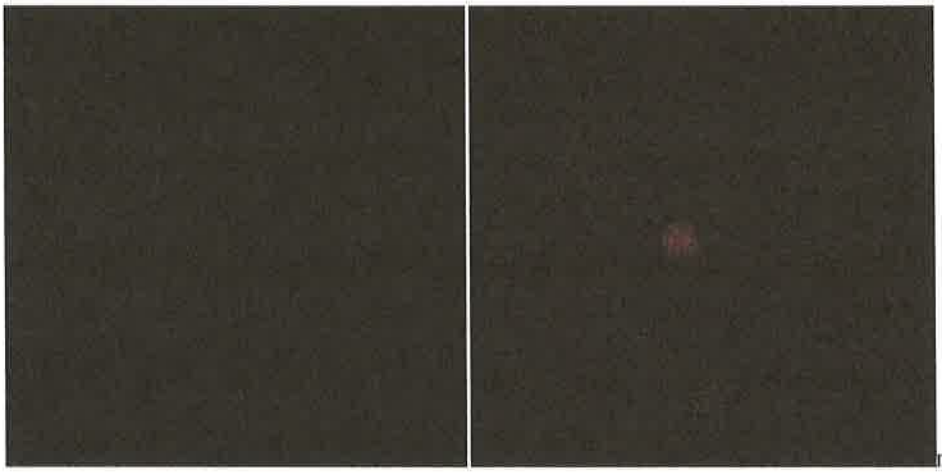


Figure D. 48 hours post transfection

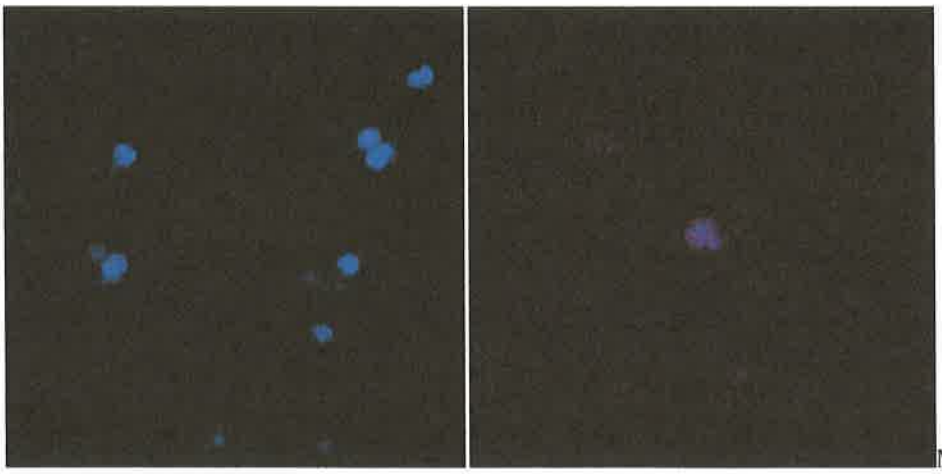
Mock NS5A



DAPI



mCherry



Merge

Figure 4. *NS5A Time Trial*

Comparison between untransfected controls and cells transfected with the NS5A-mcherry indicate the presence of the NS5A protein. Comparison between untreated controls and treatment with αIgM do not indicate any apparent differences in morphology.

Figure 5.Changes in NS5A and PI4K co-localization upon stimulation with α IgM

Control NS5A/PI4K α IgM

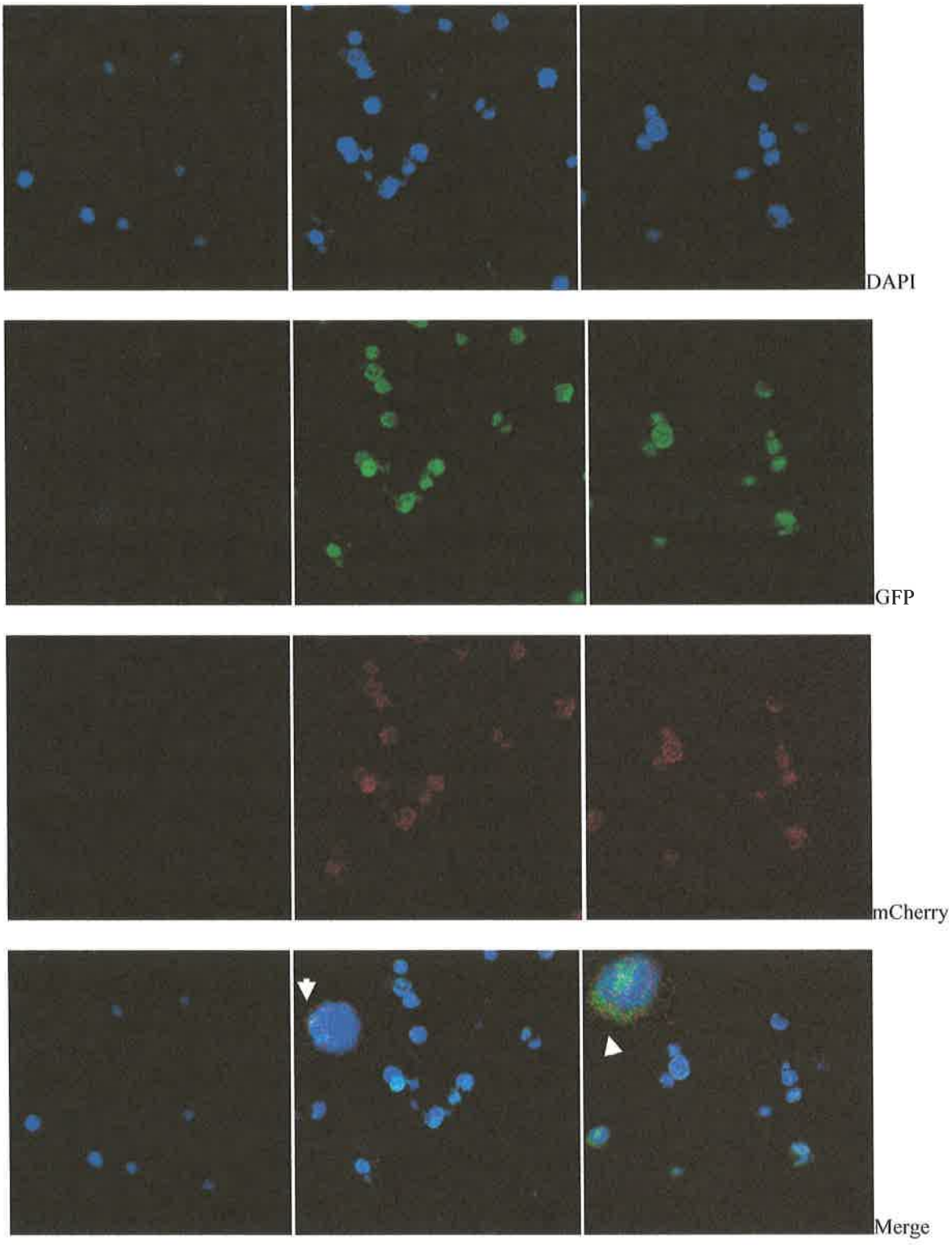


Figure 5: Changes *in NS5A and PI4k co-localization upon stimulation with α IgM*

Cells dual transfected with NS5A-mCherry and PI4K-gfp indicate a co-localization of the NS5A protein and the PI4K protein in the cytosol. Comparison between untreated controls and treatment with αIgM indicate a decrease in co-localization of the NS5A protein and the PI4K protein after stimulation.

Figure 6. Changes in ER size and NS5A localization upon stimulation with α IgM

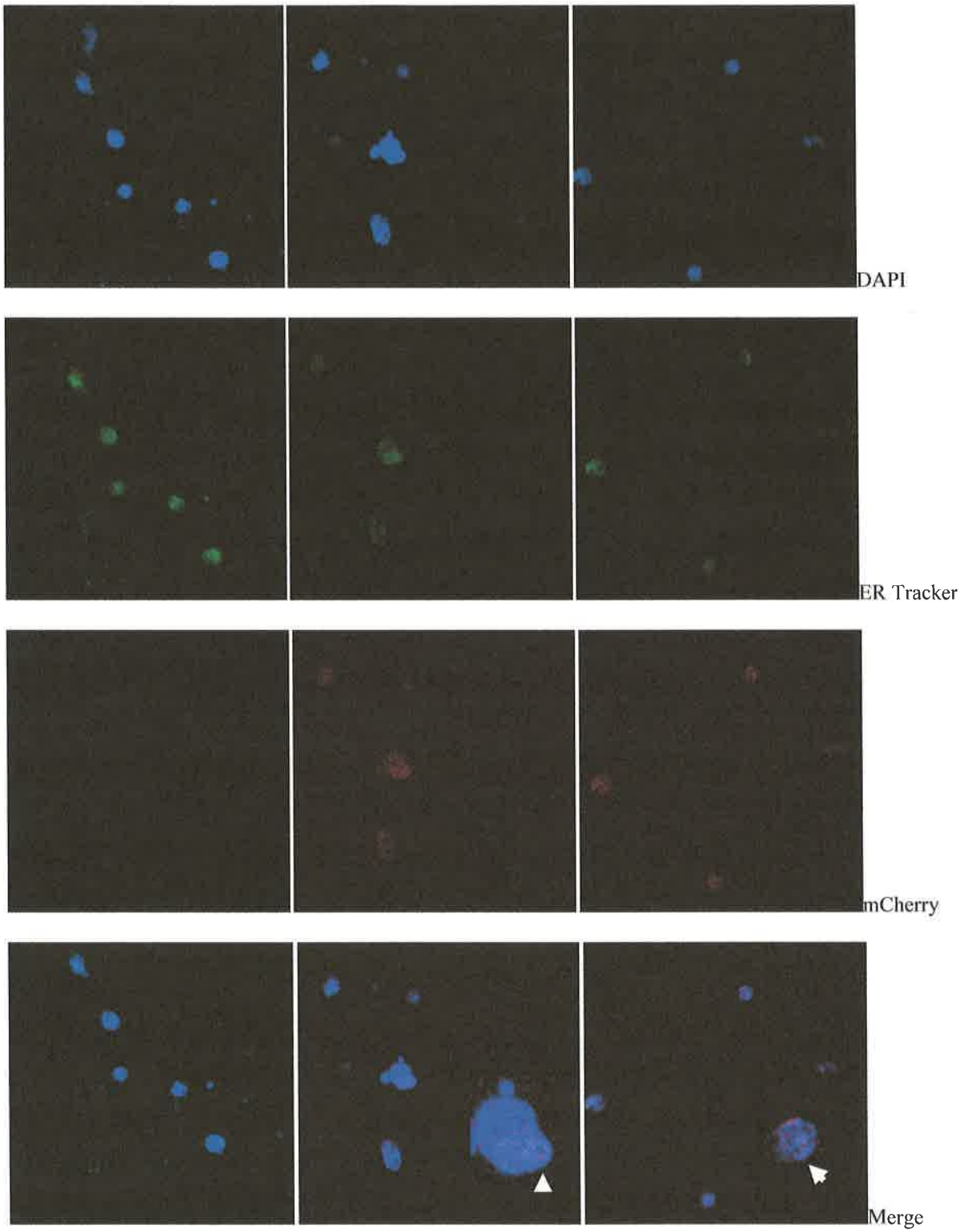


Figure 6: *Changes in ER size and NS5A localization upon stimulation with αIgM*

Comparison between untransfected controls and cells transfected with the NS5A protein indicate an increase in ER size. Comparison between untreated controls and treatment with αIgM indicate a decrease in ER size after stimulation.

Figure 7. Percentage of ER Tracker + WEHI 231 Cells

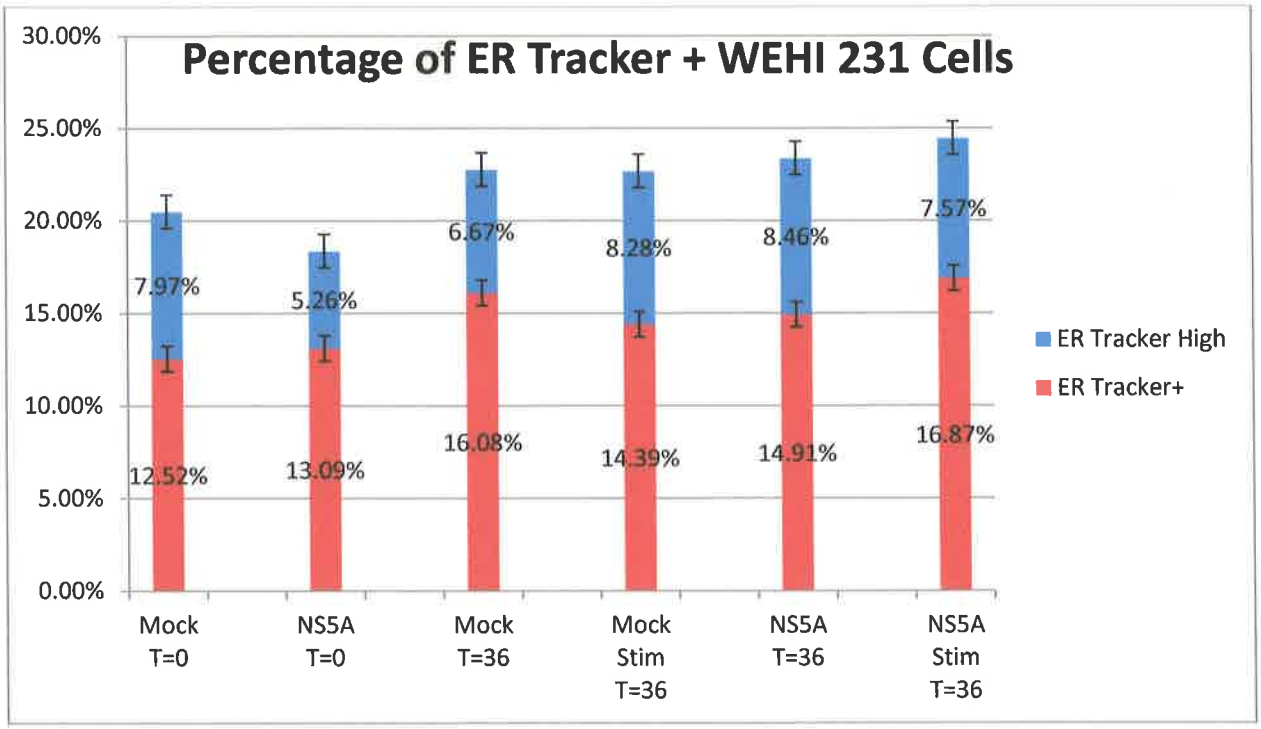


Figure 7. *Percentage of ER Tracker + WEHI 231 Cells*

WEHI 231 cells stained with ER Tracker were sorted using flow cytometry. ER Tracker + and ER Tracker High populations were distinguished by fluorescent intensity.

Figure 8. Changes in lipid distribution in NS5A transfected cells upon stimulation with α IgM

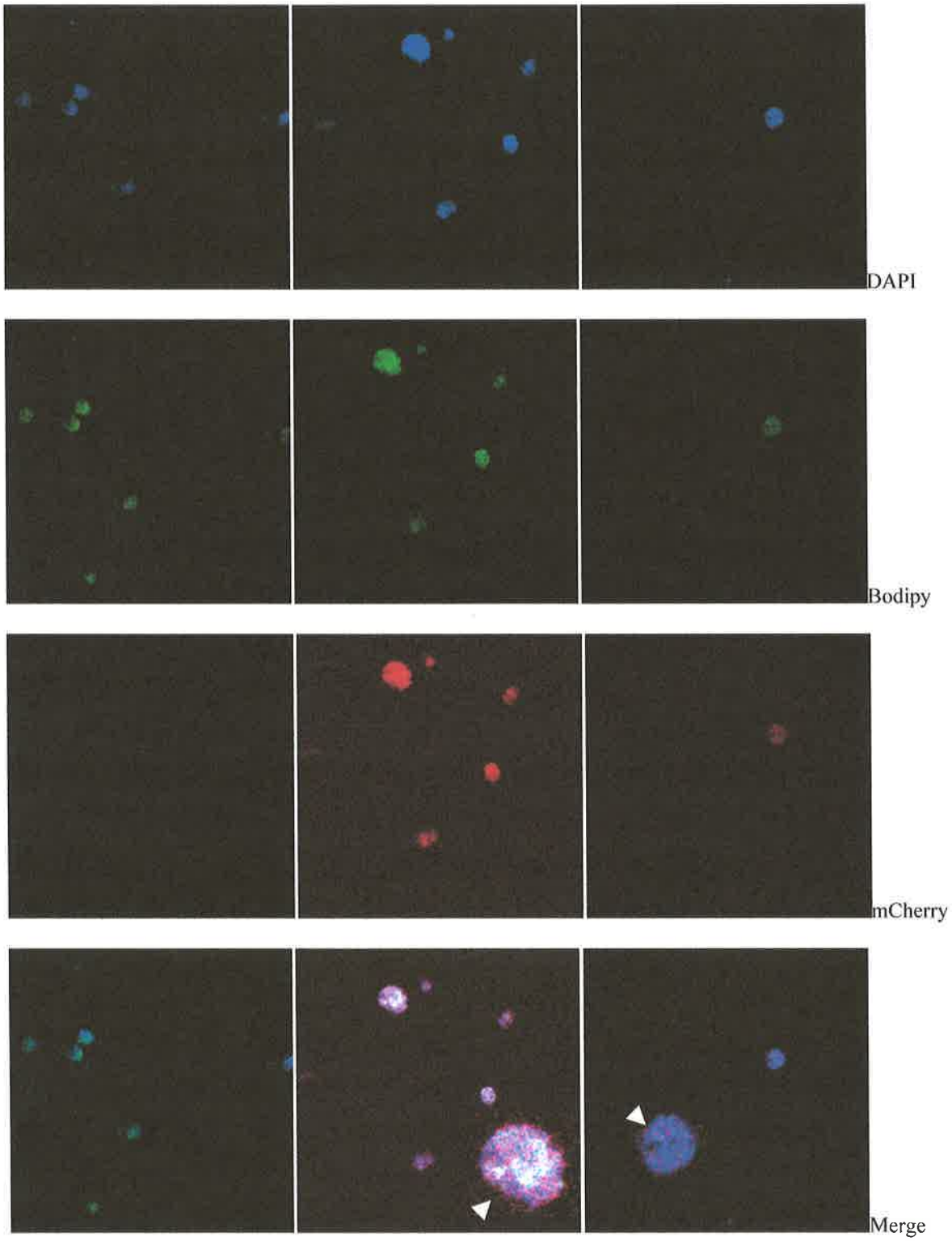


Figure 8*: Changes in lipid distribution in NS5A transfected cells upon stimulation with α IgM*

Comparison between untransfected controls and cells transfected indicate a change in lipid distribution. Comparison between untreated controls and treatment with αIgM indicate a change in lipid distribution as well.

DISCUSSION

The aim of this study was to further investigate the formation of the Efr3b/Ttc7/PI4K complex, and the impact of complex disruption on B cells. To do this, the localization of the Efr3b protein was determined. The formation of the Efr3b/Ttc7/PI4K complex and its regulation was investigated before and after stimulation. Stimulation with anti-IgM mimics the outcome of B cell activation, in contrast to the previous research that focused on cells kept in a steady state [10]. Previous research had found that NS5A interacts with PI4K, possibly preventing the kinase from forming the Efr3b/Ttc7/PI4K complex. Prior to this study there is no data on the outcome of transfecting B lymphocytes with the NS5A protein, therefore there is also no data on the outcome of activating cells transfected with NS5A. The possible phenotype that would arise from transfection of B lymphocytes with the Hepatitis C Virus protein NS5A was determined.

Efr3b co-localization studies

It was found that the Efr3b protein is localized to the plasma membrane. After stimulation the protein is more diffuse and appears to have been reorganized (Figure 1). This may be due to the mechanism behind B cell activation, stimulation of the BCR initiates receptor mediated endocytosis. If this process is occurring then, the Efr3b localized to the plasma membrane involved in the endocytosis will no longer be localized to the plasma membrane. It is likely that after endocytosis is complete the Efr3b protein would be relocalized to the plasma membrane. Nakatsu *et al* found that Efr3b is localized to the plasma membrane constitutively, however, this group was using HeLa cells [10]. HeLa cells are kept at a steady state environment that differs from that of a WEHI 231 cell. The WEHI 231 cells used in this experiment were stimulated to induce a change in the cell, the impact of this change was monitored. The HeLa cells used by

Nakatsu *et al* were not treated in any manner to induce a change in the cell physiology. In this study the stimulation treatment represented the activation of the B cells, meaning they are no longer in a steady state. This finding that Efr3b is not constitutively at the plasma membrane after stimulation is a novel finding. No previous research has been conducted investigating the regulation of Efr3b at the plasma membrane in a non steady state until now. The results of this study indicate that Efr3b is constitutively associated with the plasma membrane it is not however, constitutively localized to the plasma membrane.

Stimulation appears to have dispersed the Efr3b protein from the plasma membrane. It is not clear if the Efr3b returns to the plasma membrane or is degraded and requires replacement. It is speculated that after the cell is allowed to rest following stimulation the Efr3b would again localize to the plasma membrane. The timing of how Efr3b is restored to the plasma membrane is also currently unknown.

The mechanism behind regulation of the Efr3b/Ttc7/PI4K complex formation is also currently unknown. Previous research in the Pelsue lab has found that treatment with anti-IgM changes the intensity of Ttc7/PI4K co-localization [16]. This study investigated the possible impact that stimulation may have on Efr3b/Ttc7 co-localization. Co-localization of Ttc7/PI4K was less intense when the $Ttc7^{fsn}$ protein was studied, compared to its wild type counterpart [16]. This study focused on the co-localization of Efr3b and Ttc7/$Ttc7^{fsn}$ before and after stimulation.

The co-localization of Efr3b and Ttc7 appear as puncta located at the plasma membrane. Stimulation appears to have increased the number of puncta found (Figure 2a). It was found that Efr3b and Ttc7 do colocalize at the plasma membrane during a resting state of the cell, as anticipated. The mechanism behind the regulation of the formation of the Efr3b/Ttc7/PI4K is

unknown, whether or not activation upregulates complex formation is yet to be elucidated. Stimulation of WEHI 231 cells increases the co-localization of Efr3b and Ttc7.

The co-localization of Efr3b and Ttc7^{fsn} also appear as puncta in the cell, however, the impact of the fsn mutation decreases the intensity of the co-localization. The puncta are more diffuse when the complex is formed by the Ttc7^{fsn} protein. After stimulation the puncta are once again increased.

It is also possible that the complex is forming at the same rate as the wildytpe Ttc7, however, it is less stable as a complex and is dissociating much more rapidly than the wild type. Protein stability studies have shown that the Ttc7^{fsn} protein is far less stable than the wild type counterpart [16]. The stability of this protein is crucial to complex formation, the Ttc7 is the bridge between the Efr3b and PI4K protein [10]. If the "bridge" between the two other proteins of the complex is significantly less stable than it should be, the complex itself will be far more transient. The fsn mutation lies within the second TPR domain, TPR domains are crucial to protein: protein interactions. This mutation appears to decrease the ability of the Ttc7^{fsn} protein to interact with other proteins [16]. The data supports this, the Ttc7^{fsn} protein was capable of forming the complex, and was also increased by stimulation. However, the puncta were less intense and more diffuse when the Ttc7^{fsn} protein was used compared to its wild type counterpart, indicating a decrease in stability of the Ttc7^{fsn} protein. It has also been found that the fsn mutation alters the protein structure of Ttc7, which also impedes protein: protein interaction [16].

The formation of the Efr3b/Ttc7/PI4K complex and what may regulate this complex formation is currently unknown. It has been found here that co-localization between Efr3b and Ttc7 is

increased after stimulation. It appears that co-localization between Efr3b and Ttc7^{fsn} is decreased compared to the wild type. Furthermore, co-localization between Efr3b and Ttc7^{fsn} is increased after stimulation with anti-IgM. These findings are consistent with the findings that co-localization between Ttc7 and PI4K are increased after stimulation.

The NS5A transfected phenotype

The Hepatitis C Virus protein NS5A has been found to interact with PI4K [13]. In fact, the virus relies on PI4K for viral replication [15]. This, along with the clinical findings that patients chronically infected with HCV have been found to produce autoantibodies, and suffer from a "lupus-like" syndrome prompted the investigation of NS5A in this study. When the NS5A transfections began, WEHI 231 cells were transfected with the NS5A protein and monitored 48 hours post transfection, at this point it became apparent that changes were occurring in the transfected cells and the cells were then monitored at time 0 hours, 24 hours, and 48 hours. It was again decided to further investigate more time points post transfection to better investigate the timing of the NS5A transfected phenotype. Curiosity also lead to the stimulation of the WEHI 231 cells and the cells were monitored at time 0 hours, 12 hours, 13 hours (1 hour post stimulation), 24 hours, 36 hours, and 48 hours.

The initial changes in WEHI 231 cells transfected with NS5A happens at the 12 hour post transfection time point. In Figure 3b the cells transfected with NS5A show an increase in small white droplets that were quite intriguing. It was contemplated that they were vesicles or perhaps they were lipid droplets. Both findings seemed logical with the transfection of this viral protein. For this reason it was decided to use a lipid stain that would fluorescently tag the lipid droplets for study with confocal microscopy and flow cytometry. It was also found at this time point that

the cells transfected with the NS5A protein appeared larger and elongated (Figure 3b). This elongated morphology appeared to be increased by the stimulation (Figure 3c). The mock cells not transfected with the NS5A protein also took on an elongated phenotype and for this reason ER Tracker was used to tag the ER for study under confocal microscopy and flow cytometry.

At the 24 hour time point the elongated phenotype persists in the stimulated cells. The cells transfected with NS5A appear larger in diameter at this time point. It was speculated that this may be due to an enlargement of the ER. The cells that were transfected and stimulated have an elongated phenotype, appear larger, and have an increase in small white droplets. These phenotypes persist for the remainder of the study, consistent at 36 and 48 hours post transfection.

It is quite possible that the transfection of NS5A is sequestering the PI4K that is endogenous to the WEHI 231 cells. This would lead to ER stress, by rendering the kinase unavailable to the WEHI 231 cells. It is also possible that this sequestering disrupts vesicle transport in the cells. This too could lead to ER stress. It is known that Hepatitis C infection causes ER stress. Hepatitis C infection causes an increase in viral protein production, which is orchestrated through the host cell ER, this drastic increase in protein production leads to ER stress. The findings in this study that show an enlarged ER after NS5A transfection indicate that the NS5A protein is causing ER stress in the WEHI 231 cells through a different mechanism than viral protein production. The most likely reason behind ER stress induced by the NS5A protein is due to its ability to sequester PI4K.

Confocal microscopy study of the NS5A transfection time trials ensured that the NS5A was indeed transfected and any changes in cells transfected was due to the NS5A protein. It was found that NS5A was still present in the cell 48 hours post transfection (Figure 4d). Any changes

induced by the stimulation were not visible by confocal microscopy at this point as only the nucleus and the NS5A protein were fluorescently labeled.

Confocal microscopy analysis of cells transfected with NS5A and stained with ER Tracker appear to show an enlargement of the ER (Figure 6). The combination of transfection with NS5A and stimulation with anti-IgM results in the largest population of cells that were ER Tracker positive as well as ER Tracker high (Figure 7). It is also clear that this finding is more drastic at time 36 hours than at time 0 hours. This is consistent with the theory that NS5A sequesters PI4K, which may lead to disruption of the formation of the Efr3b/Ttc7/PI4K complex which may lead to ER stress. If this is indeed what is occurring than it is likely that B cells infected with the HCV virus would be in ER stress and may be more likely to produce autoantibodies because of the signal from the stressed ER. The combination of the findings that chronic HCV infection has been associated with the production of autoantibodies, and that B cell maturation requires ER stress as a survival signal makes this assumption plausible [14, Jennifer Walker, Thesis]. It is also possible that transfection of NS5A is causing ER stress for more than one reason. If the NS5A protein is disrupting vesicle transport in the cell than ER stress may also be triggered. The cells that were stimulated with anti-IgM appear to have an enlarged ER as well.

When the aforementioned analysis was conducted with the lipid stain rather than ER Tracker it was found that NS5A transfection does alter the lipid distribution in WEHI 231 cells (Figure 7). It appears that the NS5A is localized with lipids that are stained. It seems logical that if the NS5A that was transfected into the cells is utilizing the endogenous PI4K to create the membranous web, that is formed at the ER (that was found to be enlarged) that the co-localization of the lipid stain and NS5A is actually co-localization of NS5A and the membranous web. After stimulation this localization appears different, perhaps less associated with the lipids.

Cells that were stimulated appear to have less intensely stained lipid droplets. This is a novel finding that at this point in time has no clear explanation.

NS5A and PI4K co-localization studies

Previous research had found that the Hepatitis C Virus as reliant on the PI4K for its viral replication [15]. It had been found that the NS5A protein directly interacted with the PI4K and sequestered the kinase for viral use [13]. Until this study, the outcome of transfecting a cell with solely the NS5A protein had not yet been determined. In this study the WEHI 231 cells were dual transfected with the NS5A and PI4K proteins in an attempt to delineate any interaction between the two proteins. If NS5A was indeed capable of sequestering the PI4K, that would render the kinase incapable of forming the Efr3b/Ttc7/PI4K complex. This would lead to complex disruption. If this did indeed lead to complex disruption than a link between environmental factors (HCV infection) and autoimmune disorders may be plausible.

It was found that the NS5A and PI4K protein do indeed colocalize (Figure 5). The co-localization appears to occur in the cytosol, or at least not at the plasma membrane where the Efr3b/Ttc7/PI4K complex is formed. After stimulation it appears that the co-localization is decreased.

This study focused on the possible co-localization of NS5A and PI4K. The finding that NS5A and PI4K do interact in B lymphocytes had not previously been studied. The impact of activation indicates that the stimulation initiated a change in the interaction between the two proteins. Until this study research regarding NS5A had been done with cells infected with HCV. The impact of stimulation on cells currently infected with HCV has not yet been elucidated. The possibility that NS5A is capable of sequestering PI4K and preventing the kinase from forming the

Efr3b/Ttc7/PI4K is a novel finding that may implicate HCV infection as capable of disrupting the Efr3b/Ttc7/PI4K complex. Disruption of this complex may lead to ER stress which may lead to autoantibody formation. This may be a link between environmental factors such as HCV infection and autoimmune disorders. This is consistent with the current epidemiological research that has found a link between chronic HCV infection and patients suffering from SLE.

It may not be feasible but it would be very interesting to further study the patient population that is diagnosed with SLE and also currently infected with HCV. Determining which came first, the infection or the autoimmune disease would be imperative to elucidating the link between HCV and autoimmune disorders. If it were possible to determine if the NS5A of the HCV that the patients are infected with was interfering with the formation of the Efr3b/Ttc7/PI4K complex, a more direct link could be made. The implications of such a finding would solidify the concept that environmental factors can induce autoimmune disorders.

Future Studies

It is currently unclear how the Efr3b protein returns to the plasma membrane after treatment with anti-IgM. One way in which to determine this would be to repeat the experiment carried out in this study with multiple collection points. After the stimulation, if the anti-IgM could be removed and the cells let to rest for another 24 hours before a second collection. This may help to determine what long term impacts stimulation may have on Efr3b at the plasma membrane. It may also be necessary to collect cells on a time schedule after stimulation to determine the timing of restoring Efr3b to the plasma membrane.

It would be helpful to determine if cells transfected with the $Ttc7^{fsn}$ were in ER stress through PCR. If the $Ttc7^{fsn}$ was causing a disruption of the complex, it would be likely that this would

cause ER stress. To determine if the Ttc7^{fsn} was causing ER stress through disruption of the complex would be helpful in determining the full impact of the fsn mutation. As previously mentioned ER stress is a natural step in B cell maturation. If the fsn mutation is causing ER stress through disruption of the complex, this may provide an activation signal that pushes the cell into antibody production. If the antigens available to the maturing B cell are self antigens (as is plausible) it is possible that this combination between presence of self antigen and trigger of ER stress is causing the B cell to produce autoantibodies. This theory of autoantibody formation challenges the accepted theory of breaking tolerance [16].

This study focused on two of the proteins involved in the Efr3b/Ttc7/PI4K complex, Efr3b and Ttc7. Previous research studied Ttc7 and PI4K. To completely visualize complex formation it would be interesting to try transfecting all three proteins of the complex. This type of experiment was considered for this study but has many technical complications and therefore was not attempted. However, if it were possible this would be helpful in visualizing complex formation with all three proteins.

The NS5A studies left many questions unanswered. There are a plethora of further experiments to be done regarding the impact of NS5A transfection. It was attempted, though never completed in this study to determine if cells transfected with NS5A were expressing a cleaved version of XBP1. The PCR experiment was attempted many times but was not successful. Therefore, the most important next step would be to use PCR to determine if the cleaved version of the XBP1 gene product was being expressed in cells transfected with NS5A or not. There are other markers of ER stress that may be more feasible to study the ER stress pathway other than XBP1.

The impact of the NS5A protein on the Efr3b/Ttc7/PI4K complex and the downstream impact of ER stress is very unclear at this point. It would be interesting to study cells currently infected with HCV, which would by default have the NS5A protein as well, and transfect them with Efr3b and Ttc7 to determine if there is any impact on complex formation. Comparison of the Ttc7 and Ttc7^{fsn} in this system would also be interesting.

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