**Summary of proposal**

Innate immune responses are an essential first line of defense against human pathogens. Proper activation and maintenance of these responses is critical for the prevention or clearance of many diseases. Of equal importance is the appropriate regulation and timely termination of these responses in order to prevent cytotoxic effects and tissue damage caused by prolonged immune activation. This is achieved by the activity of several inhibitory proteins and feedback pathways that facilitate the negative regulation of immune signaling cascades. Many viruses have adapted strategies to usurp or manipulate both innate immune activation and negative feedback mechanisms in order to avoid immune activation. One key regulation pathway that functions in normal immune modulation and in viral infection involves a small ubiquitin-like modifier (SUMO). SUMO-modification of several immune factors leads to the down-regulation of immune responses. Mechanisms by which many of these factors are sumoylated remain to be elucidated.

Nup358 is a large protein located on the cytoplasmic side of the NPC, which has specific SUMO E3 ligase activity. Since all immune signaling cascades that alter host cell transcription must cross the nuclear barrier, this function of Nup358 may provide an important link to the regulation of general immune activation. Nup358 levels are altered in both cellular immune responses and Hepatitis C virus infection. Additionally, knockdown of Nup358 results in up-regulation of several immune activators, further suggesting that Nup358 may be involved in regulating immune responses. This grant proposal outlines a series of experiments to define and characterize a novel role for Nup358 in regulating immune responses and to specifically investigate a potential role for this function in Hepatitis C virus infection. This hypothesis will be examined by three specific aims:

**Specific Aim 1:** In section I will determine if Nup358 is functioning as a general immune regulator by examining of a range of immune signaling pathways. I will then expand on these observations by manipulating Nup358 levels or function and observing the effects on immune signaling. This Aim will serve to narrow down which immune pathways Nup358 is functioning in, and to give insight into how these pathways are affected by Nup358.

**Specific Aim 2:** A number of signaling proteins involved in the propagation of immune activation signals have been observed to be sumoylated. This section will expand on the results obtained in aim 1 by specifically looking at the ability of Nup358 to facilitate sumoylation of immune effector proteins both *in vivo* and *in vitro.* This aim will give insights into the function of Nup358-mediated sumoylation in immune responses.

**Specific Aim 3:** Experiments done in HCV-infected tissue culture cells have indicated that Nup358 is up-regulated at early time points in infection. Building on the results obtained in aim-1 and aim 2, this section will examine a role for Nup358 in an HCV-mediated immune evasion strategy. Through genetic manipulation, this aim will investigate the function of Nup358 in HCV infection, and specifically examine the role of Nup358-facilitated sumoylation during infection.

**Background**

In Eukaryotic cells, innate immune pathways constitute a vital line of defense against human pathogens. These cellular signaling pathways are highly regulated as misregulation of immune responses can result in serious diseases such as: immuno deficiency, cytotoxic inflammatory diseases and autoimmune diseases1. Many viruses have developed strategies to manipulate immune regulation pathways for the benefit of infection. Further understanding of innate immune regulation through the study of host and viral cellular responses will be critical for the development of preventative or curative measures for both inflammatory diseases and viral infections. This proposal outlines a strategy to investigate a novel role for the nuclear pore protein Nup358 in regulating innate immune signaling pathways and to examine a specific role for this function in Hepatitis virus infection.

**Cellular Innate Immune Responses**: At a basic level, activation of innate immune pathways involves ligand stimulation of receptors leading to downstream recruitment of transcription factors to the nucleus. This causes a transcriptional change leading to a cellular antiviral state.Immune signaling is initiated by ligand activation of pattern recognition receptors (PRRs); which are present on the cell surface, within vesicles and in the cytoplasm2. Relevant constituents of cell surface and vesicular immune PRRs include members of the toll-like receptor (TLR) family which are stimulated by a number of different viral and bacterial molecules (**Fig. 1)**2. Additionally, cytoplasmic PRRs including Mda-5 and RIG-I can initiate specific immune signaling cascades through recognition of non-host RNA molecules2. Immune signaling leads to phosphorylation and consequent activation of transcription factors such as Signal Transducers and Activator of Transcription (STAT) and Interferon Regulator Factors (IRFs)2. This activation enables formation of homo or heterodimers that can be transported into the nucleus to effectuate transcriptional changes2. Transcriptional changes include the production of cytokines including interferons (IFN) and Interleukins (IL), which further stimulate immune signaling pathways and establish an antiviral state within cells2. **Figure 1** summarizes different activation stimulants and signaling pathways for a number of immune receptors.

Termination of immune signaling pathways is essential as prolonged immune activation has cytotoxic effects on cells2. In order to achieve this, cells have a number of immune regulatory pathways in place for the termination of immune signaling responses. These pathways employ various immune inhibitory proteins including PIAS (Protein Inhibitor of Activated STAT) and SOCS (Suppressor Of Cytokine Signaling), which cause the repression or down-regulation of immune gene activation2, 3. Viruses often exploit these immune regulatory pathways to avoid the antiviral effects of host cell immune activation. One specific immune regulatory mechanism involves modification of signaling molecules by a small ubiquitin-like modifier (SUMO), leading to down-regulation of immune transcriptional activation4-7. As will be discussed in more detail later in this grant proposal, sumoylation of immune factors can be facilitated by SUMO specific E3 ligases including PIAS proteins and the nuclear pore protein Nup3588-10. Though PIAS proteins have been reported to cause down-regulation of immune responses, Nup358 has not yet been implicated in an immune regulatory process. In addition to its SUMO ligase function we have observed that Nup358 is up-regulated in IFNγ signaling pathways **(Fig. 2A).** This grant proposal will outline a hypothesis for Nup358’s involvement in negatively regulating immune responses and specifically examine how this function of Nup358 is impacting several immune activation pathways and Hepatitis C virus infection.

**Hepatitis C virus:** Hepatitis C virus (HCV) is a major cause of liver disease world wide and remains a leading indication of liver transplant in North America and Europe11. Approximately 3% of the world’s population is infected with HCV, which leads to end-stage liver disease in an estimated 30% of cases12. The ability of HCV to establish a chronic infection in 65-80% of infected individuals along with the high prevalence of the disease has lead to extensive study since its discovery in 1989. To date, treatments for HCV infection are expensive, difficult to tolerate and have limited sustained effects on infection. Increasing our understanding of how this virus interacts with and regulates the host immune response will be vital to the generation of new anti-viral therapies and the eventual development of a vaccine.

HCV is a positive strand RNA virus that encodes for 10 viral proteins and completes is replication cycle in the cytoplasm of the cell. HCV is recognized by several host PRR’s including TLR-2, -3, -7 and RIG-I13. As with many viruses, HCV has developed a number of evasion strategies to avoid or attenuate immune activation. Virally encoded proteases cleave constituents of host immune pathways leading to a block in signaling cascades14, 15. It has also been demonstrated that HCV can block STAT activation by both inhibiting signaling proteins and increasing levels of the STAT inhibitors SOCS and PIAS14. Finally, like all positive strand RNA viruses, HCV causes a rearrangement of host cell membranes to form enclosed cytoplasmic replication complexes16, 17. This rearrangement, facilitated by viral NS4A and B proteins, is postulated to have a role in hiding viral replication complexes from cytoplasmic PRR’s16. Recent findings by our group indicate that nuclear pore complex (NPC) constituents may be contributing to the formation or maintenance of these ‘membranous web-like’ structures. Indeed, nuclear pore proteins are recruited to structures adjacent to HCV core protein in infected tissue culture cells (data not shown). We have also found that the nuclear pore component Nup358 is specifically up-regulated at an early time point after HCV infection in tissue culture cells (**Fig. 2C and D)**. Taken together, these results indicate that nuclear pore proteins may play a role in HCV immune evasion, and that Nup358 may be specifically impacting viral infection. It is possible that HCV is utilizing one of Nup358’s specific functions as yet another immune evasion strategy. In order to investigate this question further we must first discuss the NPC and Nup358’s known functions within this structure.

**The Nuclear Pore Complex:** The NPC is a massive and exceedingly complex macro molecular structure that dictates the flow of molecules between nuclear and cytoplasmic cellular compartments18. Structurally, the NPC is composed of approximately 30 distinct nucleoporins (nups), which coalesce into eight identical subunits giving the complex its characteristic octagonal symmetry (**Fig. 3**)18. Components of the NPC can be divided into three main subgroups based on function. The first is composed of integral membrane proteins, which function to anchor the NPC in the nuclear envelope19. The second group includes nups involved in forming and maintaining the structural scaffold of the NPC19. The third group, often containing Phenylalanine-Glycine (FG) repeats, functions mainly in transport of molecules across the nuclear envelope19.

The most well-characterized function of the NPC is the regulation of transport between nuclear and cytoplasmic compartments. Classical nuclear transport involves the utilization of soluble transport molecules termed karyopherins (kaps), which form complexes with cargo proteins through recognition of either nuclear localization/import signals (NLS) or nuclear export signals (NES)20. Formation of complexes between kap and cargo proteins facilitates movement through the channel of the NPC20. A RanGTP/RanGDP gradient across the nuclear envelope gives directionality to the transport process. At higher concentrations in the nucleus, RanGTP causes the dissociation of import complexes and facilitates the formation of export complexes20. In the cytoplasm RanGTP is hydrolyzed to RanGDP causing the dissociation of export complexes and facilitating formation of import complexes20. This Ran gradient is maintained by the presence of Ran guanine nucleotide exchange factor (RanGEF) in the nucleus and Ran GTPase activating protein (RanGAP) in the cytoplasm20.

Though nucleoplasmic transport is the NPC’s most well-defined function, several alternative roles have been discovered for the nups. A small but rapidly growing field of study over the last decade has involved the investigation of a role for nups in innate immune responses and viral infection21-24. Specific nups have been reported as interferon stimulated genes (ISG’s) and are observed to have positive effects on fighting viral-induced transport defects21, 25, 26. Included in this group is Nup98, which, upon up-regulation, has been demonstrated to reverse the mRNA export block caused by Influenza or Vesicular stomatitis virus (VSV) infection21. The role of Nup98 in influenza infection became increasingly complex when it was discovered that Nup98 is not only involved in reversing viral-induced mRNA export block, but is also required for efficient viral particle production27. This study by Karlas *et al.* (2010) gives the first evidence that NPC proteins may be involved in non-transport related viral processes27. Several other viruses, including VSV, hepatitis B and C viruses and HIV, have been implicated in altering or disrupting NPC function24. A cellular process linking both viral infection and immune responses to the NPC is sumoylation. As mentioned earlier, SUMO modification of immune factors can lead to down-regulation of immune responses. Additionally, several viruses have been demonstrated to alter sumoylation in infected cells28-30. Furthermore, Nup358 has SUMO-specific E3 ligase activity through which it may be able to regulate immune signaling4, 31.

**Nup358 and Sumoylation:** Nup358 is a large FG repeat nup located on the cytoplasmic side of the NPC and is the major component of the cytoplasmic filaments **(Fig. 3 and 4)**. In addition to nuclear transport, Nup358 is also involved in mitosis32, 33, microtubule stability34 and sumoylation31(**Fig. 4)**. Sumoylation is a post-translational modification that is analogous to ubiquitination and affects a number of cellular pathways. The SUMO protein is a small ubiquitin like modifier that can be covalently linked to target proteins leading a change in substrate function. Attachment of SUMO to substrates is facilitated by an enzymatic cascade involving an E1 activating enzyme (Aos2/Uba1), an E2 conjugating enzyme (Ubc9) and one of a number of SUMO specific E3 ligases (**Fig. 5)**35. Fine mapping of SUMO-modified proteins has revealed a Ψ-K-X-E/D consensus sequence for SUMO attachment (where Ψ represents a large hydrophobic residue and X represents any amino acid)36. SUMO-modification can lead to cellular changes in nuclear transport37, transcriptional regulation38, apoptosis39 and protein trafficking40. Attachment of SUMO to substrates can facilitate these changes through a number of different mechanisms: Sumoylation can alter protein interactions, modify protein conformation or change the capacity of substrates to be further modified by proteins such as ubiquitin41-44. The involvement of sumoylation in transcriptional regulation is one of its best-characterized and most significant cellular functions. Generally it has been observed that increases in SUMO modification lead to transcriptional repression38. SUMO modification can function to either inactivate transcription factors or activate transcriptional repressors5, 9, 45. This has been reported for a large number of transcription factors and pathways, several of which are involved in cellular innate immune responses. Sumoylation of numerous immune related signaling and transcription factors, including STAT1, IRF-1,-3,-7 and NF-κB pathway components, has been reported to have negative effects on down-stream immune activation5-7, 9, 45-47.

Nup358 functions in the SUMO pathway as a SUMO-specific E3 ligase that binds directly to Ubc9 (**Fig. 4)**48. Several substrates, including the PML body component SP100 and the histone deacetylase HDAC4, are SUMO-modified by Nup358. The role of Nup358-mediated sumoylation is not well understood, but is proposed to be involved in coupling of SUMO modification and nucleoplasmic transport49. As a large number of proteins traffic through the NPC, Nup358’s localization gives it the opportunity to regulate a considerable number of processes through SUMO modification. In addition to Nup358, The PIAS family of immune inhibitory proteins make up a group of typical sp-RING motif SUMO E3s capable of facilitating sumoylation of targets3.

Nup358-mediated SUMO modification of HDAC 4 leads to the down-regulation of immune transcripts46. Knockdown of Nup358 decreases the amount of SUMO-HDAC4 present within cells and causes the up-regulation of IRF-1 and TNF-α transcript levels46. Furthermore, the Nup358 SUMO substrate SP100 is an interferon-stimulated gene and has been implicated in downstream immune signaling events29. These results indicate that, through sumoylation, Nup358 is involved in immune responses and may be causing negative regulation immune activation. Further to these findings, we have found that Nup358 is modulated by IFN stimulation and viral infection (**Fig. 2)**. Mouse primary macrophage cells treated with IFNγ show an increase in Nup358 transcript levels (**Fig. 2A**). This up-regulation of Nup358 follows a similar pattern to that of IRF-1 (**Fig. 2B**). Nup358 levels are also increased at early time points in HCV infection of tissue culture cells further indicating that it might have a role in immune regulation (**Fig. 2 C and D**).

Binding of Nup358 to Ubc9 also plays a role in increasing the efficiency of nuclear transport by forming a complex that sequesters RanGAP on the cytoplasmic face of the NPC50, 51. Structural analysis of Nup358-RanGAP complexes has revealed that SUMO modification of RanGAP is necessary for its interaction with Nup35851. Crystal structure analysis suggests that sumoylated RanGAP interacts with Ubc9, which interacts with the Internal Repeats (IR) domain of Nup358 creating the Nup358-Ubc9-SUMO-RanGAP complex48(**Fig. 4**). Formation of this complex localizes RanGAP to the cytoplasmic face of the NPC; where it has been proposed to promote hydrolysis of RanGTP enhancing the efficiency of both export complex dissociation and import complex formation50, 52. Binding of RanGAP to Ubc9 on Nup358 has also been reported to inhibit the E3 ligase activity of Nup35848.

**Formulation of Hypothesis**: Based on the above observations, I propose that Nup358 is involved in negatively modulating immune signaling. Upon immune stimulation there is an immediate up-regulation of several small transcription factors, which is rapidly followed by down-regulation of this early response. In our experiments Nup358 transcripts are elevated at early time points, closely following the pattern outlined by IRF-1 induction (**Fig. 2**). This up-regulation of Nup358 at early time points after IFN treatment may indicate a regulatory role as rapid induction of other immune regulatory proteins, including SOCS and PIAS, has been observed in response to IFN stimulation53. I hypothesize that this increase in Nup358 leads to a larger pool of Nup358 not bound to RanGAP and that, since RanGAP impedes Nup358 E3 activity, free Nup358 may be mediating down-regulation of immune signaling by increasing the sumoylation of immune factors either in the cytoplasm or at the NPC (**Fig. 6)**. Furthermore, I postulate that HCV is usurping this immune regulatory function by specifically increasing Nup358 levels at early time points in infection. The lack of immune response at the time of Nup358 up-regulation indicates that HCV may be specifically modulating Nup358 levels, leading to a decrease in immune activation. The experiments outlined below are designed to determine how Nup358 is functioning in immune responses and how HCV may be utilizing that function for the benefit of infection.

**Experimental Aims**

**Specific Aim 1: Determine if Nup358 is involved in immune signaling pathways:** *Observations outlined above lead to the proposal that Nup358 is involved in negative regulation of immune responses. My aim for this section will be to test if Nup358 protein levels mirror that of its transcript levels, and to expand this observation to include analysis of other immune effectors. A role for Nup358 in several pathways outlined in* ***Figure 1*** *will be examined to determine if Nup358 is generally regulating immune responses. To gain a better understanding of its involvement in immune regulation, I will manipulate the levels and function of Nup358, and evaluate the effects on immune signaling intensity and duration.*

**1-A: Regulation of Nup358 by innate immune responses**

We have previously shown that Nup358 transcript levels are elevated upon treatment of primary mouse macrophages with IFNγ (**Fig. 2)**. To determine if the up-regulation of Nup358 is a general immune response, I will expand this observation by examining a range of immune stimulatory molecules (**Table 1)**. As the timing of the Nup358 and downstream immune effector responses is essential to my model for how Nup358 is regulating innate immunity, I will do time course experiments, where the levels of Nup358 and downstream effectors of innate immunity will be monitored. Primary mouse macrophages will be treated with specific immune stimulants that lead to STAT, IRF or NF-κB activation and the effects on Nup358 and immune stimulated gene transcript and protein levels will be analyzed by qPCR and western blot. Immune stimulants, specific receptor activation and immune stimulated genes to be monitored are listed in **Table 1** and diagramed in **Figure 1**. I will also compare Nup358 levels to that of other immune regulatory proteins including SOCS and PIAS to determine if Nup358 induction is analogous in this system. Timing and level of change for each of these downstream effectors will be comparatively analyzed with respect to Nup358 levels to determine if Nup358 is involved in a general the immune response or specific pathways.Several other nups will be examined, composing an array that spans different NPC sub-complexes, to determine if this is a general up-regulation of NPC components or specific to Nup358. Reagents and protocols for qPCR assays are readily available, as these experiments have been done routinely in our lab. Also, antibodies for several nups including Nup358 are available are available in our lab for the western blot analysis. It expected that, if Nup358 is a general regulator of immune responses, then its levels will be altered by several immune stimulants. Alternatively, Nup358 may only be affected by a subset of immune pathways indicating a specific response. In the event that Nup358 is functioning in a subset of pathways, this first array will serve to narrow the range of immune stimulants to be used in the following experiments.

*Localization of Nup358:* In addition to examining the alterations in Nup358 levels, I will also look at changes in its localization. Up-regulation of Nup358 could result in an increase at the nuclear pore or an increase in cytoplasmic concentration. I will investigate this question by using the same protocol described above and observing protein localization through confocal immuno fluorescence (IF) microscopy. Specific antibodies obtained from the Dasso lab will be use to visualize Nup35854. These experiments might give insights into the cellular location where Nup358 is mediating its regulatory effects and into the specific targets of Nup358-mediated sumoylation to be investigated in aim 2. Since there is not expected to be additional binding sites for Nup358 at the NPC, it is likely that up-regulation will result in an increased cytoplasmic pool of Nup358. This could result in an increased cytoplasmic function for Nup358 or it could serve to sequester inhibitors of Nup358 function away from the NPC bound form.

*RanGAP regulation and localization:* It has been demonstrated that, at a stochiometric ratio, RanGAP can inhibit the SUMO E3 ligase activity of Nup35848. To date, levels of RanGAP have not been reported to change in response to immune stimulation. However, as our model predicts a change in ratio between Nup358 and RanGAP, examination of RanGAP levels will be important to both solidify past observations and give insights into the mechanism of Nup358’s function in immune responses. Transcript and protein levels will be observed through qPCR and western blot respectively. Should the levels of RanGAP remain constant while the levels of Nup358 increase it may indicate a greater amount of Nup358 available to facilitate sumoylation of substrates at certain time points after immune stimulation. Another possibility is that immune activation is changing the sumoylation state of RanGAP, releasing it from the RanGAP-SUMO-Ubc9-Nup358 complex. This would also result in the increased ability of Nup358 to facilitate target sumoylation. This will be tested by examining the SUMO state of RanGAP in cells treated with immune stimulants using techniques outlined in aim 2-A. It will also be important to look at the localization of RanGAP in cells containing increased levels of Nup358, as interactions with Nup358 might cause re-localization of RanGAP away from the nuclear periphery. Cells will be treated with immune stimulants and RanGAP localization monitored by IF microscopy using RanGAP specific antibodies (Santa Cruz). If RanGAP is localized to the cytoplasm upon Nup358 up-regulation in may indicate an increase in Nup358 E3 ligase activity at the NPC (**Fig. 6)**. However, sustained localization of RanGAP at the NPC in immune responses may indicate that Nup358 is functioning as a cytoplasmic regulator of immune signaling (**Fig. 6).**

**1-B: Effects of Nup358 expression levels on several immune pathways**

If Nup358 is acting as a negative regulator of immune responses then artificial manipulation of Nup358 levels should affect immune signaling pathways. To test this, I will examine immune stimulation in the presence of increased or attenuated Nup358 levels. The results from this section will be used to confirm the regulatory role of Nup358 in immune signaling pathways. Previous experiments (aim 1-A) were carried out in mouse primary macrophages; however, since these cells are short lived and relatively difficult to acquire they may not be the best system for experiments involving transfection and other gene manipulation techniques. Therefore, we will use one of several immortalized mouse and human macrophage tissue culture cell lines that are readily available and more amenable to gene manipulation experiments. Currently Raw264.7 mouse macrophages and U937 human macrophages are available in our lab.

*Nup358 Knock down:* Knockdowns of Nup358 will be mediated by a lenti viral delivery system containing a pTRIPZ plasmid (open biosystems) encoding for an inducible Nup358 directed shRNAmir. This system gives the option of two different techniques for Nup358 knock down: an efficient lenti viral mediated transient knock down, or the production of an inducible stable line by transfection and selection for the pTRIPZ plasmid. Post transfection, transcription of the shRNA will be induced through treatment of cells with doxycycline. Efficiency of knockdown will be determined by Nup358 directed qPCR and western blot. Following the induction of Nup358 knockdown, cells will be treated with the immune stimulants determined in aim 1-A, and the affects will be analyzed through the methods described below.

*Nup358 overexpression:* A second set of experiments will examine the effects of Nup358 overexpression on immune signaling pathways. Full length Nup358 will be cloned into the doxycycline inducible pcDNATM4/TO/myc-His vector (invitrogen), which will allow for the production of cell lines that can be induced to overexpress Nup358 constructs. Immune stimulants will be added to cells with elevated Nup358 levels and the outcome analyzed by the methods explained below. Localization of Nup358 upon up-regulation will also be observed through IF microscopy.

*Immune response assays:* Results of these experiments will be analyzed by three different techniques. The first method, which will be the predominant method, will utilize a qPCR array established by our collaborators in Dr. Lorne Tyrrell’s group. This array, developed for use in Hepatitis infection, examines transcript levels of a large number of immune related genes, representing both the initial response to PPR stimulation and the down stream effectors of the immune response (**Table 2**). Several genes will need to be added to tailor the array for my experiments, including the addition of several nups as controls. If the results of the qPCR array indicate Nup358 is acting as a general regulator of innate immune responses, then microarray analysis will be used to expand on these observations. This technique, though less sensitive, will give important additional information into the overall transcriptional changes in immune stimulated cells expressing altered levels of Nup358. Finally, given that antibodies are available for specific targets identified by the qPCR analysis or microarray, protein levels will be examined by western blot analysis. Western blots will serve to confirm that the up-regulation of transcripts is resulting in an increase in protein levels. Protein levels will be quantitated using Li-cor technology. Results of knock down and overexpression experiments will be compared to determine the extent to which Nup358 is involved in specific immune pathways. I will also look at the effects of Nup98 knockdown or overexpression on the immune signaling pathways. As discussed earlier and seen in **Figure 2**, Nup98 is increased in response to IFN. However, it does not have E3 SUMO ligase activity and is predicted to be a positive regulator of immune responses21. Using Nup98 as a control will help to demonstrate that the function of Nup358 in immune regulation is separate from that of other nuclear pore proteins that are also up-regulated by immune signaling.

*Transport analysis:* To determine if the effects of Nup358 up-regulation or knockdown are due to general transport defects as opposed to the predicted sumoylation effects, I will examine nucleocytoplasmic transport under these conditions. It may be that up-regulation or down-regulation of Nup358 is leading to general transport defects resulting in altered gene expression. To answer this question I will co-transfect NLS-GFP or NES-GFP reporter plasmids into cells containing the Nup358 constructs mentioned above. The dynamics of nuclear transport will be monitored by fluorescence microscopy, examining how the ratios between nuclear and cytoplasmic fluorescence intensities correlate in control, Nup358 knockdown and Nup358 overexpression conditions.

*RanGAP and Nup358 sumoylation:* SUMO-modified RanGAP has been demonstrated to form a complex with Nup358, blocking Nup358’s E3 ligase activity48. This observation has led to my proposal that increases in Nup358 protein levels change the ratio between Nup358 and RanGAP allowing for increased sumoylation of substrates (**Fig. 6)**. To test this hypothesis I will overexpress both Nup358 and RanGAP, treat with immune stimulants and compare the effects to those seen in Nup358 overexpression. If up regulation of Nup358 is increasing sumoylation of immune proteins by altering the ratio between Nup358 and RanGAP then this experiment should result in the subsequent increase in immune gene activation. RanGAP will also be independently over expressed in cells followed by immune stimulation. This is also expected to result in increased or prolonged immune responses.

**1-C: Specific mapping of regions of Nup358 involved in immune responses**

Since the previous experiments are based on the knockdown of full length Nup358, they do not confirm if the observed results are due to sumoylation defects or one of the many other functions attributed Nup358. As we have predicted that the E3 SUMO ligase activity of Nup358 is important for its role in immune modulation, I will further characterize results obtained in aim 1-A and B by specifically deleting Nup358’s E3 ligase domain. This can be achieved in a number of ways. The simplest way to examine effects of E3 ligase deficient Nup358 will be to overexpress mutant forms of the protein. Overexpression plasmids, encoding for Nup358 truncations behind a CMV promoter, will be transfected in cells followed by analysis by the previously determined methods (Aim 1-B *Immune response assays*). Mapping of Nup358 E3 ligase activity has demonstrated that the IR1/M/IR2 region facilitates binding to Ubc9 and subsequent SUMO modification of substrates (**Fig. 4)**. Therefore specific mutations deleting this region (Nup358Δ2503-2893)will be used in these experiments. I will also do the reverse experiments in which the levels of the specific SUMO domain of Nup358 are elevated. Past studies have identified Nup358 fragment BP2ΔFG as sufficient to facilitate sumoylation *in vitro31***(Fig. 4)**. Cells transfected with plasmids encoding for Nup358Δ2503-2893 or BP2ΔFGbehind CMV promoters, will be subjected to the immune stimulants determined in aim 1-A and effects will be analyzed as in aim 1-B. Localization of specific mutants will also be monitored by IF microscopy. As the vector to be used encodes for a myc epitope, Nup358 truncation localization will be observed using a myc specific antibody (Roche Scientific).

*Depletion of endogenous Nup358:* One caveat of the experiments described above is the sustained presence of the endogenous Nup358 protein, as it may be rescuing cells from the expected mutant phenotype. Consequently, I will also perform a set of experiments where the endogenous protein is depleted leaving only the mutant Nup358. This will be attempted in two ways. The first will involve the production of a stable line expressing the inducible Nup358Δ2503-2893 mutant construct. Stable lines will then be treated with siRNA directed against the IR region of Nup358, effectively targeting just the endogenous Nup358. As siRNA is not completely efficient, there may still be some endogenous protein remaining, which will be monitored by qPCR. Analysis of the IR1/2 region of Nup358, by the ambion siRNA target finder tool (www.ambion.com/techlib/misc/siRNA\_-finder.html), reveals several suitable mRNA sequences for siRNA targeting.

A second method for the replacement of endogenous Nup358 with the truncated Nup358Δ2503-2893 will employ a novel technique for mutating endogenous genes in higher eukaryotic cells through directed homologous recombination mediated by zinc-finger nucleases (ZFNs)55, 56(**Fig. 7)**. This system utilizes a gene cassette that encodes for a tandem array of specific zinc-finger motifs followed by the non-specific DNA cleavage domain of the type II restriction enzyme *FokI* 55, 57. Each of the zinc-finger motifs recognizes a precise 3 nucleotide sequence giving the tandem array specificity for a particular region of the genome58.The resulting protein will mediate a double strand break in genomic DNA at a site specified by the zinc-finger domains. This double strand break recruits cellular homologous recombination repair machinery, which can facilitate the homologous recombination of DNA sequences from a donor plasmid into the genomic DNA. The process of ZFN recognition and directed homologous recombination is reviewed in **Figure 7.** Sigma CompoZr® Zinc Finger Nuclease Technology will be used to design and produce ZFN proteins.In my experiments I will design a ZFN to specifically target the region of Nup358 encoding the IR domains (amino acids 2503-2893) and insert (off a donor plasmid) a portion of the Nup358 gene that is both: deficient in the SUMO E3 ligase domain and linked to an eGFP-encoding construct. Fluorescent activate cell sorting (FACS) will be used to select for mutants expressing GFP, and mutations will be confirmed by qPCR and western blot analysis. Procedures for designing ZFN’s and for inserting genes in to specific loci have been previously demonstrated to be powerful tools for gene manipulation in mammalian cell systems56, 57. Experiments with immune stimulants as described above (aim2-B) will then be implemented on the cells containing E3-deficient Nup358Δ2503-2893 mutants. An increase in immune activation is expected in this system, as Nup358 will no longer be able to negatively regulate immune responses through sumoylation.

Experiments outlined in aim 1 will demonstrate if Nup358 is involved in regulating immune responses and give insight into whether its E3 ligase domain has a specific function in immune modulation.

**Specific Aim 2: Identification of Nup358 sumoylation targets**

*Many different immune transcription factors have been reported to be sumoylated both in vivo and in vitro6, 7, 46, 47. All of these transcription factors undergo nucleocytoplasmic transport upon activation. The localization of Nup358 at the NPC as well as its SUMO E3 ligase activity make it an excellent candidate for facilitating the sumoylation of immune transcriptions factors. Alternatively, if up-regulation is observed to increase the cytoplasmic pool of Nup358, it could also lead to increased sumoylation of cytoplasmic proteins that are causing the negative regulation of immune activation. Indeed, SUMO attachment to the NF-κB inhibitor IκB has been demonstrated to prevent the activation of NF-κB43. Expanding on the results in Specific aim 1, this section outlines experiments to test the ability of Nup358 to facilitate the SUMO modification of specific immune transcription factors or cytoplasmic regulators both in vitro and in vivo.*

**2-A) Nup358 effects on sumoylation of immune targets *in vivo*:** Since we have postulated that Nup358 mediated sumoylation is important for its role in regulating immune responses, I will examine effects of Nup358 regulation on the sumoylation of specific targets *in vivo*59*.* This section will test the capacity of Nup358 to facilitate sumoylation of specific immune cytoplasmic and transcription factors, as determined by the pathways established in aim 1-A. I will focus on proteins that have previously been described to be SUMO-modified which will likely include IRFs, STATs, NF-κB subunits and IκB (**Table 1 *transcription factors*)**.

To test this, Nup358 will be depleted or overexpressed by the methods described above (aim 1-B), and sumoylation will be tested by one of the following techniques. The first analysis will be through IP of either SUMO or specific targets and examination of the SUMO state by immunoblotting. SUMO specific antibodies and many of the potential substrate antibodies are commercially available from Santa Cruz, Sigma or Abcam. A caveat of this experiment is that, due to the low steady state levels of sumoylation and the abundance of SUMO specific cysteine proteases (SENPs), the study of SUMO modification of endogenous proteins *in vivo* is dificult60. I will address this by altering the expression of Nup358 in a previously described cell line that stably overexpresses polyhistidine-tagged SUMO60. This will be followed by isolation of SUMO by pull down on a nickel column. Western blot analysis will be used to determine the sumoylation state of substrates. Sumoylation of proteins can be detected by either molecular mass shift of the target proteins or by staining blots with SUMO specific antibodies. Polyhistidine-tagged SUMO proteins will also be transfected and overexpressed in cells containing E3 ligase deficient Nup358 truncations (aim 1-C), followed by analysis of sumoylation for specific substrates. If Nup358 is responsible for sumoylation of specific substrates, then there is expected to be a marked decrease in sumoylation upon Nup358 knockdown or mutation. Alternatively, overexpression of Nup358 should result in increased SUMO-modification of targets *in vivo*.

I will also look at the effects of PIAS protein knockdown or up-regulation on the sumoylation of specific substrates and comparatively examine them with respect to Nup358 experiments. This will be used to determine if Nup358 and PIAS proteins are affecting similar immune pathways through sumoylation. It has been suggested that SUMO E3 ligases may be somewhat non-specific for their targets; thus it will be important to see if Nup358 is able to increase the sumoylation efficiency of the same substrates as PIAS proteins. It may be that Nup358 and PIAS mediated sumoylation is part of an analogous negative feed back mechanism, differing only in their specific cellular locations. Alternatively, comparative analysis may reveal that Nup358 dependant sumoylation has additional or divergent functions in the modulation of immune signaling. Though these experiments will determine if Nup358 truncation is affecting sumoylation of specific immune substrates, they do not address the question of Nup358’s ability directly facilitate SUMO-modification of those substrates.

**2-B) *In Vitro* Sumoylation using recombinant proteins:** To determine if Nup358 can specifically mediate sumoylation of targets examined in aim 2-A, I will use a previously described *in vitro* sumoylation assay59. Substrates and specific Nup358 constructs will be added to a sumoylation reaction mixture consisting of recombinant: Uab2/Aos1 (SAE1/SAE2), Ubc9 and mature forms of SUMO1 or SUMO2/3. Specific constituents of the *in vitro* assay (i.e. SUMO, E1 and E2 recombinant constructs) are readily available from the Melchior lab, as well as specific protocols for optimal reaction conditions59.

In addition to reagents acquired from the Melchior lab, specific SUMO E3 ligases (Nup358 truncations and PIAS proteins) and the substrates (IRF and STAT proteins) will be cloned and expressed as recombinant proteins for use in this assay. Production of recombinant proteins and assembly of the reaction mixture will be done as previously described59. Substrates will be chosen by combining the results obtained in aim 1 with the specific SUMO targets determined in aim 2-A. Sp100 and HDCA4 have previously been described as SUMO substrates of Nup358, and will provide good positive substrate controls for this assay8, 31. As full length Nup358 may be difficult to express as a recombinant protein, I will also use the SUMO active BP2ΔFG fragment of Nup358 that has been previously used in similar *in vitro* assays (**Fig. 4)**31, 59. Nup358 recombinant proteins to be examined will include, full length Nup358, SUMO active BP2ΔFG 31, 61, and SUMO inactive Nup358Δ2503-2893. In additional experiments, RanGAP will be added to reaction mixtures to further test our hypothesis that increases in Nup358 mediated sumoylation are due to changes in Nup358-RanGAP ratios. Addition of RanGAP will be expected to attenuate the accumulation of SUMO-modified substrates in this assay. Again, I will also look at PIAS proteins as positive controls for enhanced sumoylation of specific substrates, as they have been implicated in the sumoylation of IRF-1 and STAT proteins 10, 47.

Following the identification of Nup358 sumoylation targets, future studies will include looking mechanistically at the result of SUMO modification for those specific proteins. The effect of sumoylation on nuclear transport, protein interactions or degradation of substrates will constitute a large number of future experiments for specific immune SUMO substrates.

**Specific Aim 3: Determine if Nup358 functions to regulate immune responses in Hepatitis C virus infection**: *In addition to the increase in response to IFNγ we have also shown that Nup358 levels are elevated at early time points following infection of tissue culture cells with HCV (****Fig. 2C)****. This induction does not parallel increases seen in other immune stimulated proteins indicating that the virus may specifically facilitate Nup358 up-regulation (****Fig. 2D)****. If Nup358 is a negative regulator of immune activation, this up-regulation early in infection might serve to impair cellular immune responses, allowing the virus to gain an advantage. This section will outline experiments to characterize a function for Nup358 mediated immune regulation in HCV infection*.

**3-A) Nup358 impact on HCV infection:** To examine the question of Nup358’s role as a negative regulator of immune responses in HCV infection, I will test the effects of attenuated or elevated Nup358 levels in infected cells. As in aim 1-B, cells will be transfected with constructs to either knock down or over express Nup358. These experiments will be done in HUH7.5 cells, as this is the best-known tissue culture model for HCV infection. Since past experience has demonstrated that stable line production in these cells is exceedingly difficult, I will perform these experiments transiently with a lentiviral delivery system, which I have observed to be effective in HUH7.5 cells. Cells will be infected with HCV followed by transduction of the previously mentioned Nup358 overexpression of knockdown constructs by lentiviral infection. Following induction of Nup358 constructs transcription; effects over a time-course of infection will be tested in a number of different ways. Both viral RNA levels and immune stimulated gene mRNA levels will be analyzed by qPCR. The same Tyrrell lab qPCR array used in aim 1 will be utilized in these experiments. Additionally, viral particle production will be measured by a TCID50 viral titer assay13.

The effects of up/down-regulating Nup358 may be observed at a specific stage in the HCV life cycle. Decreases in RNA levels and viral titers when Nup358 is depleted would indicate that Nup358 is involved in the replication of HCV. This will be further analyzed through qPCR analysis of immune genes, which will reveal if Nup358 is involved in a HCV immune evasion strategy. It is expected that knock down of Nup358 will result in increased immune stimulation that will cause decreases viral replication and particle production. One alternative to this is that Nup358 has a structural role in HCV virion production, which would be indicated by normal viral RNA levels and decreased viral titers. Following this result, the role of Nup358 in HCV infection would have to be reassessed and further experiments designed to test this function.

**3-B) Sumoylation profile in HCV infection:** Several viruses have been reported to affect sumoylation of substrates in infected cells. Members of the Herpesvirus family including Herpes Simplex Virus, Cytomegalovirus and Human Herpesvirus 6 have proteins that specifically down regulate sumoylation, resulting in transcription modifications in infected cells28-30. To date, no alterations in sumoylation have been reported for HCV infection. However, increases in Nup358 may indicate a change in the cellular sumoylation state of infected cells. To test this, lysates from HCV infected or uninfected cells will be examined by western blot using SUMO specific antibodies to observed general changes in the cellular sumoylation state. Infected cell lysates will be harvested on day 2 after infection, since this is the time point when Nup358 levels are elevated. Following a positive result, these experiments will also be done in the presence or absence of Nup358 targeting siRNA. Results will indicate if Nup358 is mediating the change in sumoylation state. In addition to changes in overall sumoylation profiles, it will also be important to look at the sumoylation of specific substrates. Proteins identified in aim 2 as targets for Nup358 mediated sumoylation will be tested for changes in their SUMO state upon infection with HCV. As in aim 2-A cells will be transfected to overproduce his-tagged SUMO proteins. The SUMO modification of specific targets will then be compared between infected and uninfected cells by pull down of SUMO on a nickel column and subsequent immunoblotting for specific proteins. These experiments will give insight into the function of Nup358 in HCV infection.

**Concluding remarks**

Understanding mechanisms behind the regulation of cellular immune responses is critical to preventing and fighting many human pathogens and diseases. As all immune signaling cascades that alter host cell transcription must cross the nuclear barrier, the study of how NPC components affect these pathways may provide an essential link to the regulation of general immune activation. This proposal outlines a number of experiments to identify and characterize a potentially novel mechanism that viruses may use to negatively modulating immune signaling pathways. Results from these experiments may give significant insights into how nuclear pore proteins are involved in the interplay between host immune activation and viral immune evasion. If indeed Nup358 is generally regulating immune responses, then understanding of this mechanism may lead to novel treatments for chronic inflammatory diseases and pathogenic infections. Additionally, investigation of Nup358’s role in HCV infection may give important insights into a novel mechanism for viral immune evasion.

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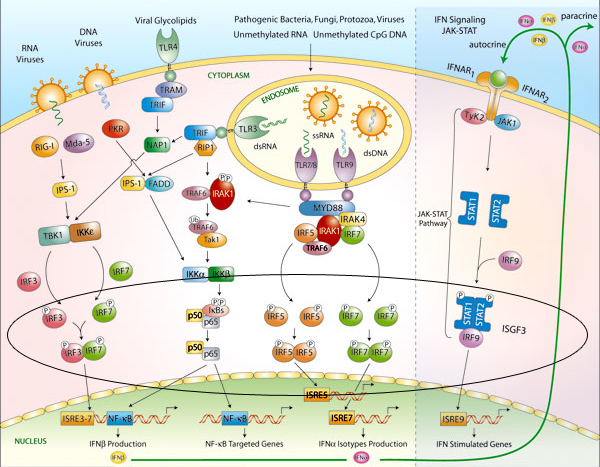
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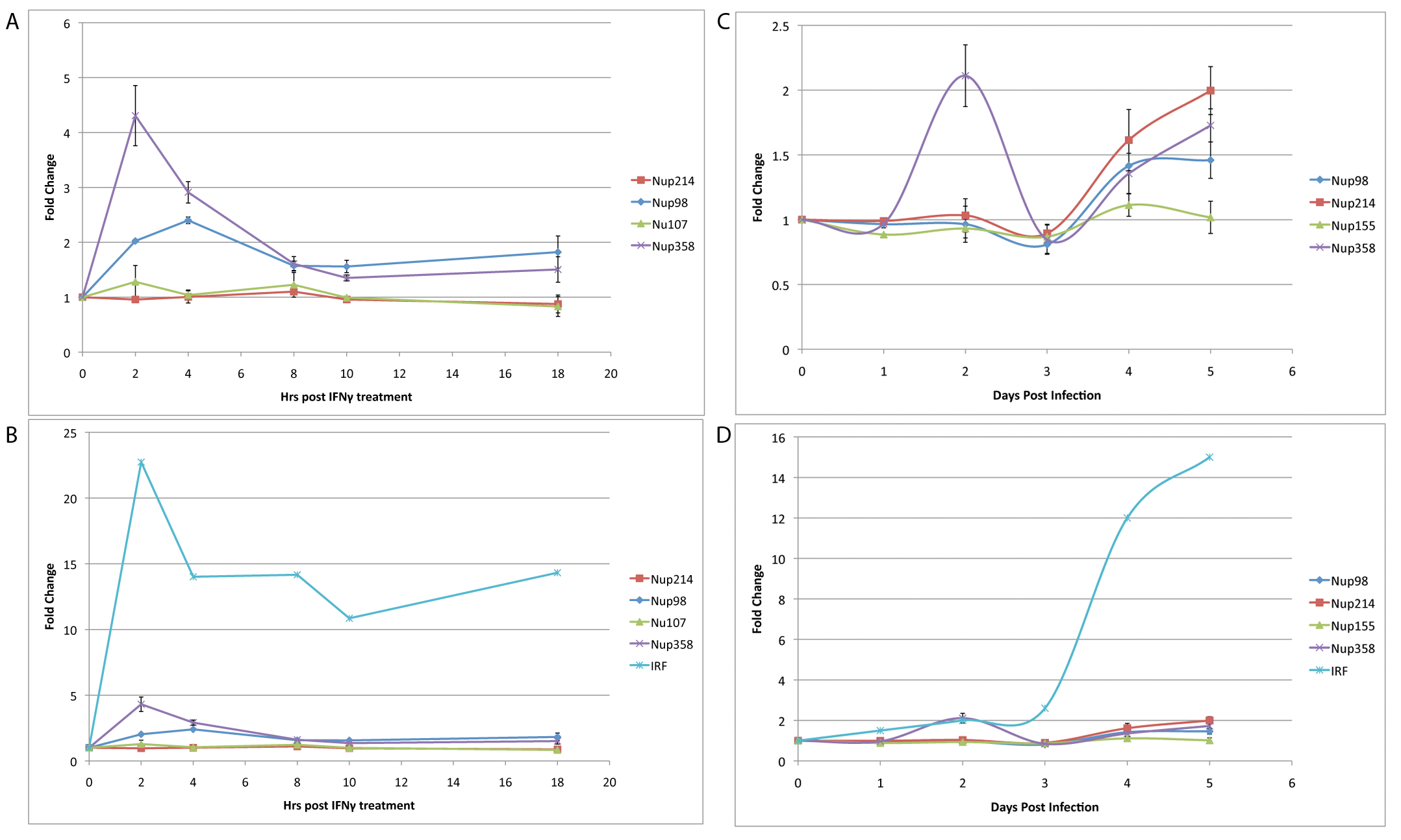
Figures

**Figure 1**. *Pattern Recognition Receptor and Interferon Signaling*. Activation of PRRs by viral constituents leads to signaling cascades that cause the eventual phosphorylation and consequent dimerization of specific IRF transcription factors (Circle). Activated IRFs are then translocated into the nucleus through the NPC where they initiate the transcription of immune-related genes. Stimulation of certain PRRs also causes the phosphorylation and subsequent ubiquitin mediated degradation of the inhibitor IκB, leading to NF-κB transcriptional activation. Initial response to PRRs causes the production of IFN proteins, which feed back to further increase immune activation. Binding of IFN proteins to IFN receptors causes initiation of JAK/STAT signaling, leading to induction of IFN stimulated genes.

Figure adapted from invitrogen (www.invivogen.com/ressource.php?ID=20).

**Table 1.** *Immune stimulating molecules to be test in Aim1-A.* The table shows specific immune stimulants, the receptors that are stimulated by each stimulant, the corresponding transcription factor activation and the genes that will be monitored by qPCR or western blot in Aim1-A. Each of the genes to be monitored is a rapidly induced, positive regulator of immune activation. STAT phosphroylation will be monitored by western blot with specific phospho-STAT antibodies (Sigma). Up-regulation of the remaining genes will be monitored by qPCR.

|  |  |  |  |
| --- | --- | --- | --- |
| Immune Stimulant | Receptor Activated | Transcription factor activated | Genes to be monitored |
| LPS | TLR-4 | IRF-3/7 | IFNα/β gene activation |
| poly I:C | TLR-3, RIG-I and Mda5 | IRF-3/7 and NF-κB | IFNα/β gene activation |
| IFNα/β | IFNα/β receptors | STAT1/2 and IRF-9 | STAT phosphorylation and IRF-1 gene activation |
| IFNγ | IFNγ receptor | STAT1 and IRF-9 | STAT phosphorylation and IRF-1 gene activation |
| Herpes simplex virus | TLR-9 | IRF-5, IRF-7 and NF-κB | IFNα/β gene activation |
| Vesicular stomatitis virus | TLR-7/8 | IRF-5, IRF-7 and NF-κB | IFNα/β gene activation |
| TNF-α | TNF-α receptor | NF- κB | Inflammatory cytokine gene activation **(**IL-1α and IL-1β) |



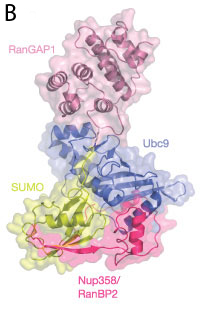
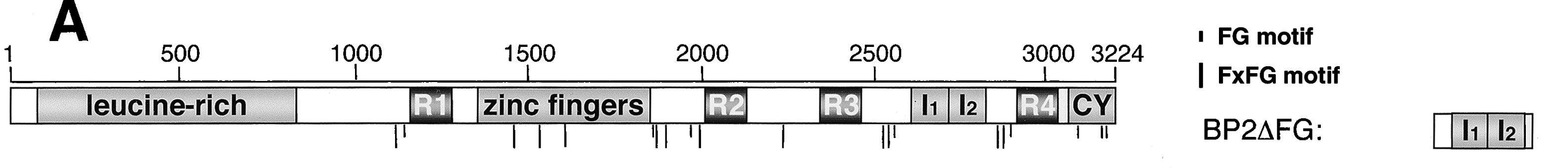
**Figure 2.** *Transcriptional regulation of Nup358 in immune response and in HCV infection:* **A**) Nup transcript levels in mouse primary peritoneal macrophages treated with IFNγ. Mouse peritoneal cells were harvested from mice and macrophages were isolated by attachment to tissue culture-treated plates. Macrophages were treated with 1000U/mL mouse IFNγ for a time course of 18 hr and lysed with TRIzol reagent (invitrogen). Total RNA was isolated and transcript levels were determined by real-time qPCR. All samples were normalized to HPRT. **B**) Graph same as A but with the addition of IRF-1 transcript. **C**) Nup transcript levels in HUH7.5 cells over a time-course of HCV infection. HUH7.5 cells infected with HCV for a time-course of 5 days. On each day post infection cells were lysed in TRIzol and total RNA isolated. Individual transcript levels were determined by real-time qPCR and normalized to HPRT. **D**) Graph same as C but with the addition of IRF-1 transcript.



**Figure 3.** *Schematic representation of one subunit of the nuclear pore complex*. Nups in green are the integral membrane nups involved in anchoring the NPC into the nuclear envelope. Nups in blue represent scaffold proteins required for the formation and maintenance of the NPC structure. Nups depicted in red represent those involved in transport and more peripheral functions of the NPC.

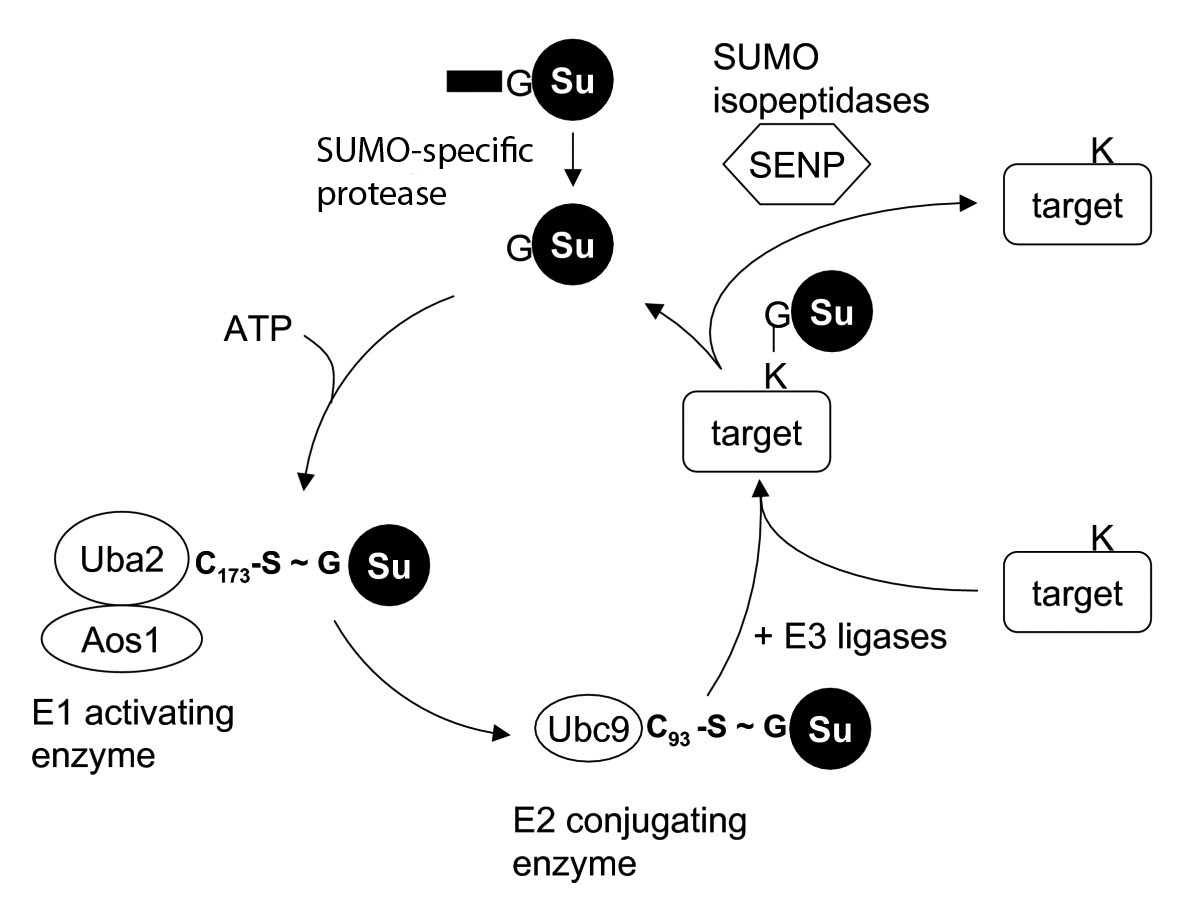
Cytoplasm

Nucleus



**Figure 4.** *Nup358 and Nup358-RanGAP complex*. A) Schematic representation of Nup358. Nup358 is 358 kDa nuclear pore protein found on the cytoplasmic face of the NPC. It contains several FG and FxFG repeat domains as well as several other binding domains. Of particular importance is the Internal Repeats (IR) region of Nup358 located near the C terminal region of the protein. This region binds to Ubc9 and RanGAP1 and give Nup358 its SUMO E3 ligase activity. The Nup358 Fragment BP2ΔFG is shown on the left, and contains only the IR1-M-IR2 regions of the protein. B) Structural representation of the complex formation between Nup358/RanBP2-SUMO-Ubc9-RanGAP1. Figures adapted from Pichler *et al.*, (2002)31: and Reverter & Lima, (2005)48.

Figures

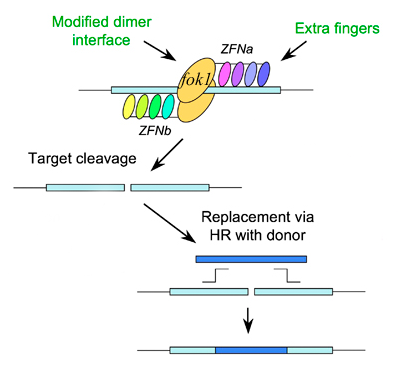


**Figure 5.** *The SUMO pathway*. SUMO precursors are processed to the mature form by a SUMO-specific protease. A thioester bond at the C-terminal glycine residue of mature SUMO links it to the E1 activating enzyme Uba2/Aos1 (Sae1/2). SUMO is then shifted to the active cysteine residue of the E2 enzyme Ubc9. Facilitated by SUMO-specific E3 enzymes, SUMO is then transferred to the ε-amine group of a target protein lysine residue, forming an isopeptide bond. Several SENPs (SUMO-specific Proteases) mediate the de-conjugation of SUMO from target proteins, maintaining the highly dynamic nature of the SUMO pathway. Figure adapted from Bosis and Melchior, (2006)62.

**Table 2.** *Genes present in the Tyrrell qPCR array*. Genes are grouped by functions and timing of response. Initial response genes (immune activators and repressors) are rapidly induced upon immune stimulation and function in further activating down stream effectors or negatively regulating the initial response. Down stream effectors and cytokines/chemokines/HLA’s are induced at later time points and have various functions in inflammatory and immune responses. Markers and my specific gene additions will be used as controls in these experiments.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Initial Response Genes** | | **Down stream effectors** | **Cytokines Chemokines HLA's** | **Markers and Gene additions to the array** |
| **Activators** | **Regulators** |  |  |  |
| RELA (p65) | SOCS1 | 1FI44 | CXCL9 | PCNA |
| IFIH1 (Mda5) | SOCS3 | MX1 | CXCL10 | HSPA5 |
| TICAM1 (TRIFF) | STAT1 | OASL | CXCL11 | ALB |
| VISA (IPS1) | STAT2 | OAS3 | HLA-A | HPRT |
| IRF-7 | PDLIM2 | ISG20 | HLA-C | Nup155 |
| IRF-9 | OTUD5-DubA | IFIT2 | HLA-DRA | Nup107 |
| IRF-3 | NLRX1 | IFIT3 | HLA-G | Nup214 |
| IFNA2 |  | MX1 | IL18 | Nup153 |
| IFNB1 |  | PCNA | IL15 | Nup62 |
| STAT1 |  | HSPA5 | ICAM1 | Nup98 |
| STAT2 |  |  | ULBP2 |  |
| IFNG |  |  | CCL8 |  |
|  |  |  | MICA |  |
|  |  |  | CLEC2D |  |

**Figure 6.** *Hypothetical model for the effect of Nup358 up regulation.* In un-stimulated cells, Nup358 is located at the nuclear pore and is in a complex with sumoylated RanGAP and Ubc9. This complex inhibits the SUMO E3 ligase activity of Nup358. As levels of Nup358 in the cell increase (either through immune stimulation or HCV infection) the ratio between Nup358 and RanGAP is altered. This leads to an increase in the fraction of Nup358 with the ability to facilitate sumoylation of substrates either in the cytoplasm or at the nuclear pore. Sumoylation of substrates causes the down regulation of immune signaling and immune responses in the cell. Depending on the localization of Nup358 upon up-regulation, there may be increases in sumoylation at the NPC or in the cytoplasm that are mediating the immune regulatory effects.



**Figure 7.** *ZFN mediated gene replacement*. ZFN proteins bind to a specific genomic site dictated by the tandem zinc-finger motifs. The non-specific DNA nuclease *fok1* then mediates a double strand break. This double strand break initiates the homologous recombination repair system that inserts DNA from a donor plasmid into the genomic break site. Figure adapted from Carroll, 200858.