

# Investigating the Molecular Basis of Rubella Virus-Induced Teratogenesis: A Literature Review

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## Abstract

Rubella virus (RV) is the etiologic agent of rubella, a disease more commonly known as German measles. The 1940 rubella epidemic in Australia allowed for the identification of RV as a teratogenic agent: infection early in pregnancy causes a variety of birth defects collectively referred to as congenital rubella syndrome (CRS). Although rigorous immunization policies have dramatically reduced the incidence of CRS, it is still estimated that around 100,000 infants are born with CRS every year. Furthermore, in light of the recent Zika virus epidemic which is now known to be a causative agent of microcephaly and other birth defects, a deeper understanding of RV may help elucidate the paradigm of viral teratogenesis and aid in the development of therapeutic agents to prevent the development of birth defects in fetuses after maternal infection. This review aims to give a summary of the current knowledge regarding the molecular biology of the virus followed by an overview of potential mechanisms of RV-induced teratogenesis as well as suggestions for possible future directions for research.

**Keywords:** rubella, teratogen, German measles, congenital rubella syndrome

## Introduction

Rubella virus (RV) is the pathogenic agent of rubella which is more commonly known as German measles, a mild disease characterized by fever and rashes. The disease was first identified in the 1700s in Germany by physicians de Bergan and Orlow and was considered to be a mild childhood illness until its teratogenic potential was discovered in 1941 (1,2). If a woman is infected with RV during pregnancy, the virus can also infect the fetus and cause congenital rubella syndrome (CRS). The risk and severity of congenital defects associated with CRS depend

on the time of gestation at which infection occurs and can include deafness, eye abnormalities such as cataracts, and congenital heart disease (3). Rubella virus can have such a devastating effect that a single rubella epidemic in the United States caused more birth defects in one year than thalidomide did in its entire time on the market worldwide (4). This prompted the development of a vaccine and widespread vaccination policies which have led to a dramatic decrease in the incidence of CRS (5). Yet it is estimated that more than 100,000 infants are born with CRS every year so it is critical to have a better understanding of the molecular mechanisms of rubella-

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induced teratogenesis in order to develop therapeutic agents to prevent CRS in those countries where a rubella vaccine is not available (6).

## Clinical Features of Rubella and Congenital Rubella Syndrome

Rubella is generally a mild self-limited illness with a fever and rash in children and adults. The true public health risk of rubella was identified in 1941 following a rubella epidemic in Australia, when ophthalmic surgeon Gregg Norman noticed an unusually high incidence of congenital cataracts (along with other birth defects) in newborns. He carefully studied histories of the mothers and identified the link between birth defects and rubella infection early in pregnancy, introducing the idea of viruses as teratogens (1).

Maternal rubella infection starts in the upper respiratory tract and nasopharyngeal tissue then progresses to generalized viremia. During viremia, the virus can infect the placenta and the fetus where it establishes a chronic nonlytic infection and causes birth defects (7). RV is capable of infecting every organ in the fetus with microscopic analyses of aborted fetuses showing cellular damage and non-inflammatory necrotic lesions in structures of the eyes, heart, brain, and ears (8). The risk and severity of congenital defects associated with CRS depends on the time at which infection occurs during gestation. The fetus is particularly susceptible to infection and defects if infection occurs in the first 8 weeks of pregnancy when nearly 100% of fetuses become infected and almost all develop severe congenital defects. This risk significantly decreases after the first trimester and particularly after 17 weeks of gestation (3).

The worldwide rubella pandemic of 1962-1965 was particularly devastating. It caused 11 000 fetal deaths and 20 000 cases of infants born with CRS in the United States alone which paved the way for vaccine development (9) (Orenstein *et al.*, 1984). A live-attenuated RV vaccine is now part of the MMR vaccine which is generally part of childhood immunization schedules and immunizes children against mumps, measles and rubella. Vaccination policies have greatly reduced the incidence of rubella and CRS, with the Americas being declared free of endemic rubella transmission in 2009 by the World Health Organization, yet it is estimated that 100 000 infants are born with CRS globally every year (10).

## Rubella Virus Genome and Proteins

### Overview and Classification

RV particles are spherical and have a diameter of 60 to 80nm. The virus has an icosahedral capsid surrounded

by a host-derived lipid membrane which contains two viral glycoproteins: E1 and E2 (11).

RV is the only member of the *Rubivirus* genus in the *Togaviridae* family which also includes the genus *Alphavirus*. Alphaviruses such as Sindbis virus have been extensively studied and findings in this genus often help elucidate pathways in RV since they are closely related. There are, however, significant differences between the two. For example, all alphaviruses are arboviruses: their transmission occurs via arthropod vectors, but RV can only replicate in humans and spreads directly from person to person via respiratory aerosols (7,12).

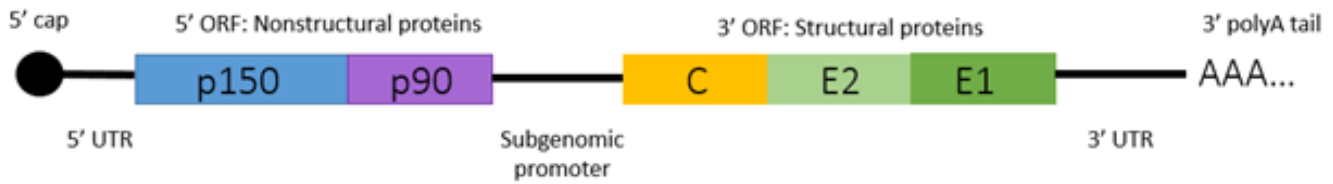
### Genome

RV is a group IV virus according to the Baltimore classification: it has a positive sense single-stranded RNA genome that can be immediately translated by host machinery once it enters the cell. Its genome is 9762 nucleotides in length and has a 5' methylguanosine cap and 3' polyA tail. Interestingly, the RV genome has a 70% GC content which is the highest of any known RNA virus (13). The genome contains two open reading frames (ORFs): the 5' proximal ORF which encodes the non-structural proteins including the viral RNA-dependent RNA polymerase and the 3' proximal ORF which codes for the viral structural proteins, including the capsid protein and surface glycoproteins E1 and E2 (13).

The viral genome also contains three untranslated regions (UTRs). The 5'UTR contains AA dinucleotides which are essential for viability as well as a stem-loop region which is a cis-acting regulatory region for the production of non-structural proteins. While mutagenesis in the 5'UTR is generally tolerated, most of the 3'UTR is required for viability since it is necessary for optimal replication. A stable stem-loop structure forms in the 3'UTR, about 58 nucleotides from the 3' polyA tail. Both the positive-polarity of this sequence and its complement negative-polarity resemble a eukaryotic TATA promoter sequence and are therefore thought to exhibit promoter activity. The 118-nt intragenic region between the ORFs is thought to act as a subgenomic promoter to initiate synthesis of the subgenomic RNA which is used to make the structural proteins (14).

### Structural Proteins

RV has three structural proteins which are encoded in the 3'ORF: Capsid protein, E1, and E2. Capsid protein is a 33 to 38kDa disulfide-linked homodimer which interacts with the viral RNA to form the nucleocapsid. The N-terminal half of the protein is suspected to interact with viral RNA because it is highly hydrophilic and contains clusters of proline and arginine residues. It also interacts with E1 glycoprotein which may be involved in budding. The C-



**Figure 1: Organization of the rubella virus genome.** RV has a (+)ssRNA genome which has two ORFs: one encodes the non-structural proteins and the other encodes the structural proteins. The genome is capped and polyadenylated and has three untranslated regions which act as regulatory sequences.

terminal half is hydrophobic and is anchored in the viral envelope. This suggests that membrane fusion and nucleocapsid uncoating may not be distinct events. Similarly, the processes of nucleocapsid assembly and budding may be linked. A 29-nt region in the RV genome has been found to interact directly with capsid protein but it is unclear if this is sufficient for genome packaging (14).

The RV envelope also contains two glycoproteins, E1 and E2. They form heterodimers which are then organized into trimers at the virion surface. E1 is a class 1 transmembrane protein and contains three N-linked glycosylation sites which are required for proper protein folding and formation of infectious particles. Amino acids 81-109 of E1 form the fusion peptide responsible for fusion of the viral envelope with host membranes and are also responsible for interactions with E2. E1 glycoprotein is also critical for antigenicity of RV as it contains antigenic sites as well as a neutralization epitope at amino acids 208-239 (15).

Glycoprotein E2 is also a class 1 transmembrane protein although its role is not as well defined as E1. It contains many sites for both N- and O-linked glycosylation but their number varies between strains. E2 is disulfide-linked to E1 but it is poorly exposed on the surface although it does contain hemagglutination and neutralization epitopes (16). E2 glycoprotein of the related Sindbis virus is known to be involved in receptor binding of the virus but no such activity could be mapped to the RV E2 protein (17). Both E1 and E2 contain N-terminal signal sequences for translocation into the ER which is important for post-translational processing of the glycoproteins (18).

### Nonstructural Proteins

The 5'ORF encodes the RV non-structural proteins: p150 and p90. p150 contains a protease domain as well as a methyltransferase domain. Additionally, it has a region of unknown function (motif X) that is also found in the alphavirus non-structural protein nsP3 and hepatitis E virus 5'ORF product. p90 is the viral RNA-dependent RNA polymerase essential for replication of viral RNA and also contains a domain with helicase activity (13).

## Viral Lifecycle

### Attachment, Entry and Uncoating

RV attachment is not well understood but it is known that RV can establish infection in a variety of cell lines and can be recovered from almost every organ in infected fetuses (11). Recent studies showed that the viral E1 glycoprotein can specifically interact with myelin oligodendrocyte glycoprotein (MOG) on the cell surface and an antibody targeting MOG prevents infection by RV (19). Furthermore, a nonpermissive cell line could be rendered permissive by ectopic expression of MOG, further confirming this glycoprotein as the cellular receptor for RV (19).

Fusion of the viral envelope is well characterized in the related Sindbis virus: E1 undergoes conformational changes in low pH to expose the fusion peptide and induce viral envelope fusion (20). Although the same has not been directly shown for RV, their E1 proteins are highly homologous so the RV E1 likely undergoes the same changes to induce fusion (21). Furthermore, it has been shown that between pH 5.0 and 5.5, RV capsid protein changes conformation from having hydrophilic to hydrophobic properties (22). Finally, studies in Vero cells using various inhibitory drugs for endocytic pathways have shown that RV relies on clathrin-mediated endocytosis to enter the cell (23). Overall, those observations suggest that RV uses clathrin-mediated endocytosis to get into endosomes in which low pH triggers both envelope fusion and nucleocapsid uncoating. This model also supports the hypothesis that capsid uncoating and envelope fusion are not distinct events due to capsid protein being anchored in the viral envelope through a hydrophobic domain.

### Replication, Translation and Post-Translational Processing

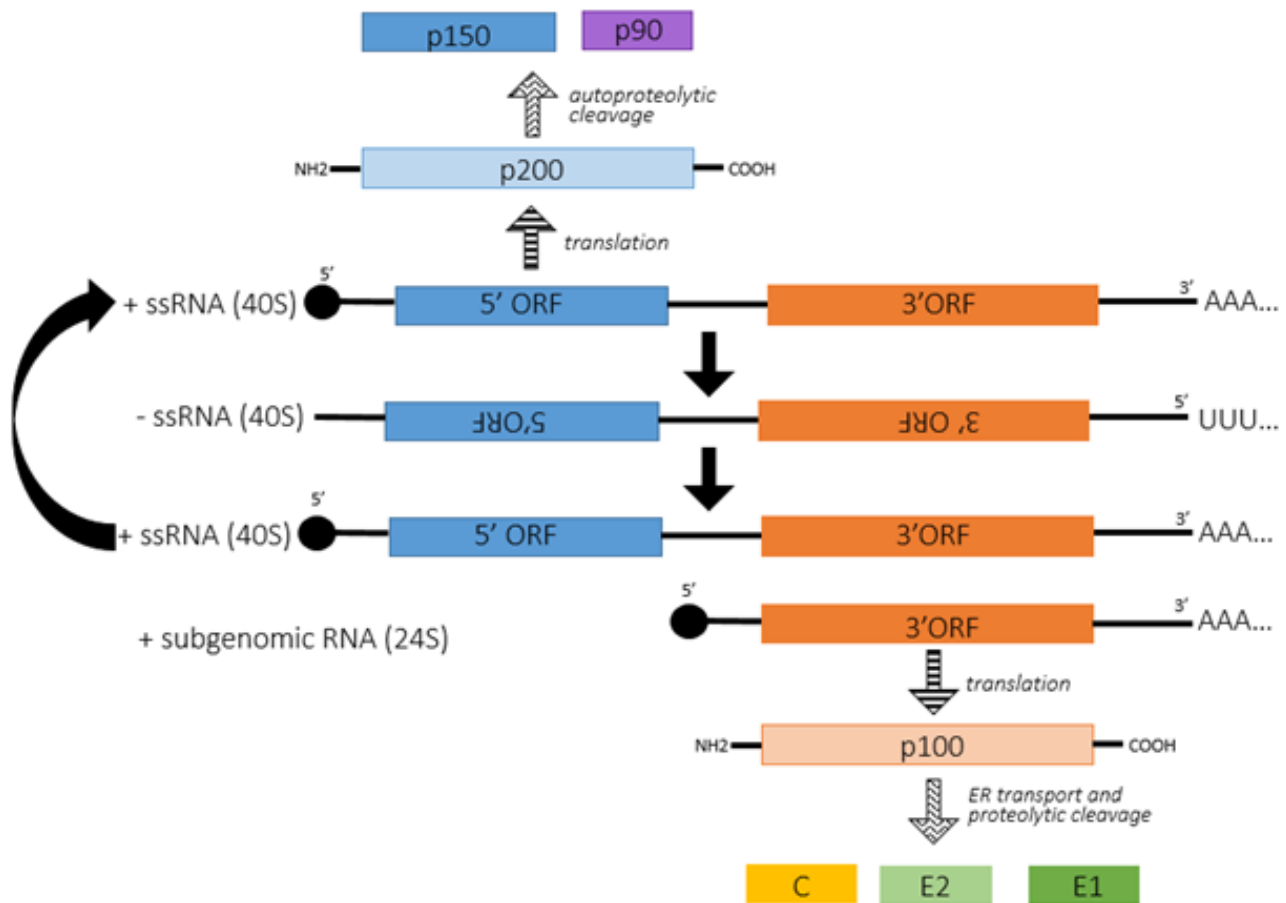
Since the 40S viral genome is a positive sense RNA, the 5'ORF can be directly translated by host machinery following nucleocapsid uncoating. Its product is a polyprotein, p200, which contains a protease domain and

can undergo autoproteolytic cleavage to produce the viral non-structural proteins p150 and p90 (24). Following cleavage, p90, which has RNA-dependent RNA polymerase activity, replicates the viral RNA to make the viral anti-genome from which two RNA species are produced: the full-length 40S genome as well as a subgenomic RNA (24S) (11). The negative-polarity RNA is detected in infected cells only in double-stranded form. dsRNA species in infected cells can be either fully double-stranded (termed replicative forms) or partially double-stranded (termed replicative intermediates). The replicative intermediates are believed to be double-stranded complexes undergoing active transcription (25).

Cellular proteins are essential to viral RNA synthesis. Treatment of Vero cells with either actinomycin D or  $\alpha$ -amanitin, drugs that block cellular mRNA transcription, in the first 8 hours of infection result in a

reduction of the amount of accumulated viral RNA and an absence of detectable viral proteins at 48 hours post-infection. The same effect was not observed for Vero cells infected with stomatitis virus which indicates that inhibition of cellular mRNA synthesis has a specific effect on RV replication and the results observed are not due to generalized cellular deterioration (26).

The 24S subgenomic RNA encodes the 3'ORF only and is translated to produce the p100 polyprotein. P100 has two signal peptides belonging to the E1 and E2 glycoproteins which target it for transport to the ER. Host signalases found in the ER lumen cleave the polyprotein into capsid protein, E1 and E2. A unique feature of the RV capsid protein is its retention of the E2 signal peptide on its carboxy terminus after cleavage which makes its C-terminal end hydrophobic. Similarly, E2 retains the E1 signal peptide (11).



**Figure 2: Overview of rubella gene expression and protein processing.** The 5'ORF of the genome is translated to produce p90 and p150 following autoproteolytic cleavage of the polyprotein p200. P90 is the viral RNA polymerase and transcribes the genome to create the complementary anti-genome from which the genomic RNA and a subgenomic RNA are produced. The subgenomic RNA is translated to produce the viral structural proteins.

### Virion Assembly and Exit

Following proteolytic cleavage of p100 in the ER, E1 and E2 form disulfide-linked heterodimers while capsid protein forms disulfide-linked homodimers. E1 has an ER retention signal which retains E1 subunits and immature E1-E2 dimers in the ER. Folding and heterodimer formation mask the ER retention signal and the E2 transmembrane and cytoplasmic domains target the heterodimer to the Golgi complex which is the main site of virion assembly and budding. The E1 transmembrane and cytoplasmic domains are not required for transport to the Golgi but are necessary for secretion of the virus into the medium and play a critical role in the late stages of viral budding (27).

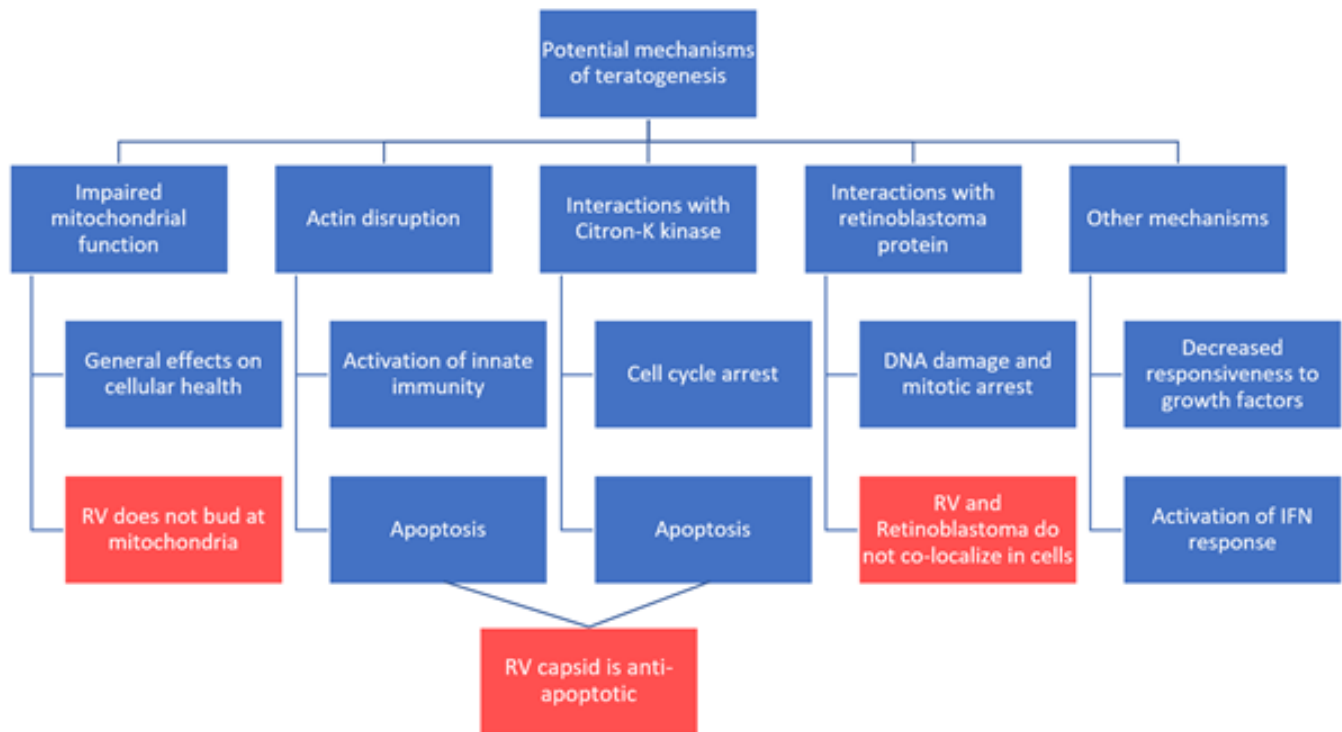
Since capsid protein retains the E2 signal sequence, it has been proposed that this allows the capsid protein to be transported to the Golgi with the viral glycoproteins. Nucleocapsid formation of RV is not fully understood but a 29-nt sequence near the 5' end of the genome interacts with capsid protein. Assembly of the nucleocapsid is regulated by phosphorylation since phosphorylation of capsid protein inhibits nucleocapsid formation (28).

### Potential Mechanisms of Viral Teratogenesis

The direct pathway by which rubella virus causes teratogenesis has yet to be elucidated but several studies have shown various effects of RV infection on normal host cell function which may contribute to teratogenesis. These include mitochondrial changes, cytoskeletal abnormalities, interactions with cellular proteins, and inhibition of cytokinesis which will be discussed in further detail.

#### Mitochondrial Changes

In 1976, cardiolipin was reported to be present in rubella virions. Since this phospholipid is specific to the inner mitochondrial membrane, this finding suggested that RV budding may occur at mitochondria. Furthermore, in the first hour of infection, cellular ATP levels of BHK-21 cells decrease while alanine synthesis, glycolysis, and respiration increase which suggests mitochondria are important during RV infection. Electron microscopy studies in RV-infected Vero cells revealed electron-dense zones associated with



**Figure 3: Overview of potential mechanisms of RV-mediated teratogenesis.** Blue boxes describe a potential mechanism described in the literature, red boxes indicate contradicting data or areas that require further investigation.

mitochondria along with a change in the morphology of mitochondria to a club shape; similar changes were not observed in mock-infected cells. In addition, mitochondria cluster around RV replication complexes since they are sites of high-energy requirement and may therefore induce mitochondria to migrate. Finally, RV-infected cells have a fourfold increase in the levels of mitochondrial stress proteins and mitochondrial chaperones (29). All those observed changes suggest mitochondrial function may be impaired during RV infection which may have a significant effect on overall cellular function. This is especially critical if it occurs in progenitor cells involved in fetal organogenesis and may be a way by which RV causes birth defects.

Since it is now more firmly established that rubella virion budding occurs at the Golgi and not the mitochondria (14), more research needs to be conducted to explain the molecular basis of the observed mitochondrial changes and to confirm the presence of those same changes occur *in vivo* in cells of infected human fetuses.

### Cytoskeletal Factors

Actin is a major cytoskeletal component. Immunofluorescence studies using antibodies to actin have shown that 16 hours post-infection with RV, the arrangement of actin filaments is altered. Actin depolymerizes and is detected as amorphous clumps instead of the filamentous cables normally observed in uninfected cells. These changes were not observed in microtubules, another component of the cellular cytoskeleton (30).

Disruption of the actin cytoskeleton in human epithelial cells has been associated with activation of nuclear factor-kappaB which activates transcription of many genes involved in the inflammatory response (31, 32). Thus, inflammation and the activation of innate immunity may be causing the fetal damage observed in CRS (33). Actin disruption has also been shown to activate p53 which can induce apoptosis (34). In a developing embryo, the infection and subsequent apoptosis of progenitor stem cells could have devastating effects on organ development and may explain the teratogenic effects of rubella.

However, recent studies have suggested that RV capsid protein may be an anti-apoptotic protein which is beneficial to maintain a persistent infection of the embryo (35). Those conflicting results suggest more research is needed to better understand the true effect of rubella infection on cellular apoptosis and its role in teratogenesis.

### Interactions with Retinoblastoma Protein

Retinoblastoma protein (RB) is a tumor suppressor responsible for a major G<sub>1</sub> checkpoint. It represses transcription of genes required for the transition from G<sub>1</sub> to S phase thus blocking cell cycle progression (36). Sequence

of analysis of viral protein p90 revealed the presence of an RB-binding motif, furthermore it was demonstrated that p90 binds RB *in vitro*. In addition, deletion of the RB-binding motif in p90 was found to be lethal and RB null mouse cells show decreased virus production following infection (37). All these observations suggest that RB likely serves as an obligatory host factor to support RV replication.

Like rubella virus, human cytomegalovirus (HCMV) commonly causes birth defects when it infects fetuses. HCMV protein IE2 86 is known to interact with RB, causing premature DNA synthesis causing chromosomal damage and mitotic cell arrest. These effects have been implicated in HCMV-induced teratogenesis (38). It is therefore possible that p90 interaction with RB may be similarly involved in disrupting fetal growth.

It is important to note, however that RB is primarily located in the nucleus while the entire RV lifecycle occurs in the cytoplasm, so it remains to be shown that either RV undergoes a nuclear phase or some RB is present in the cytoplasm in order to prove that an interaction between RV and RB could have relevant effects *in vivo* (37).

### Interaction with Cytokinesis Regulator Citron-K Kinase

Citron-K kinase (CK) is a cytokinesis regulatory protein located in the cytoplasm which was found to interact with p90 (37). During cellular expression of p90, a subpopulation of cells containing tetraploid nuclei were identified, such tetraploid status is indicative of cell cycle arrest after S phase suggesting that p90 perturbs cytokinesis. RV infection induces cytopathic effects and apoptosis in Vero cells attributed to caspase-3-dependent programmed cell death (39) (Pugachev and Frey, 1998). In independent studies, it was shown that CK deficiencies in cell culture lead to tetraploidy followed by apoptosis (40) (DiCunto *et al.*, 2000). This observation further confirms that p90 may be interacting with CK which perturbs cytokinesis leading to tetraploidy which causes caspase-3-dependent induction of apoptosis. This is clinically significant because one of the manifestations of CRS is a reduced number of cells in the affected organs. RV infection of progenitor stem cells early in embryogenesis causing reduced growth and apoptosis could easily lead to a reduced number of cells in the organ that eventually develops (11). Those observations suggest there could be a direct link between a specific protein-protein interaction and the documented CRS pathology in fetuses.

### Other Potential Mechanisms of Teratogenesis

Studies in human embryonic mesenchymal cells have shown that persistent RV infection causes a significant decrease in responsiveness to epidermal growth factor

which could be a mechanism by which RV stunts organ development (41).

Studies of vaccinia virus showed that the presence of dsRNA activates dsRNA-dependent protein kinase which is involved in the interferon-mediated host response and apoptosis (42). This has not been shown in RV but the presence of the double-stranded replicative forms and intermediates of rubella along with the observation that 90% of RV-infected cells express alpha-interferon suggest that RV dsRNA may elicit the same response as vaccinia virus (43).

### Future Research Directions

Although rubella virus only encodes 5 proteins, it has been shown to have a variety of cellular effects ranging from mitochondrial changes to actin depolymerisation and cell cycle alterations. As discussed above, protein-protein interactions between RV and host proteins are essential for viral replication and likely play an important role in RV-induced teratogenesis, as suggested by studies of p90 and CK, yet very few interactions between RV and host proteins have been characterized. In order to further investigate potential mechanisms of RV-induced teratogenesis, host-virus protein-protein interactions should be further characterized and studied. A better understanding of these interactions could lead to the development of therapeutic agents that specifically block them. This could serve to either cure the mother of rubella at early stages of infection prior to viremia or directly inhibit pathways of teratogenesis in the fetus.

An animal model for congenital rubella syndrome has recently been developed using pregnant ferrets. This model showed that infection in pregnant ferrets disseminates to the placenta and fetus where RV causes a persistent systemic infection and viral-induced teratogenesis observed in ferrets is dependent upon the stage of gestation at which infection occurs (44). Both these observations suggest that ferrets are a promising model for CRS which could prove useful for future *in vivo* studies of RV-induced teratogenesis and to test potential therapeutic agents.

### Conclusion

The development of a highly effective vaccine and wide vaccination policies have greatly reduced the incidence of rubella and congenital rubella syndrome which led to reduced research efforts. Yet it is still estimated that around 100 000 infants are born with CRS globally every year. More research is needed to expand our understanding of the mechanisms underlying rubella-induced teratogenesis. The development of therapeutics that target those mechanisms could be used as an antiviral intervention if an unvaccinated mother acquires rubella

during pregnancy to prevent development of CRS in the fetus. A better understanding of the pathways of RV teratogenesis may also lead to elucidation of the paradigm of teratogenesis by other viral agents such as Zika virus which is particularly relevant today given the current epidemic.

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