7 Summary

Hepatitis C virus (HCV) is a small RNA virus that causes severe liver pathogenesis in humans. It encodes for 3 structural and at least 7 non-structural proteins. The non-structural protein 5A (NS5A) has been implicated in the deregulation of host cell signal transduction, interfering with signaling pathways that regulate cell growth, cell proliferation and cell survival. Modulation of these signaling pathways is of potential interest with regard to viral replication, immune evasion and virus-associated liver pathogenesis. Therefore, this study was focused on the identification of cellular targets of NS5A that might trigger the NS5A-mediated deregulation of host cell signal transduction.

Using an affinity chromatography approach, c-Raf1 was identified as a novel binding partner of NS5A. Binding was confirmed by immunoprecipitation using baculovirus-infected Sf21 cells. NS5A and c-Raf1 were found to colocalize in transiently-transfected HuH-7 cells and HCV replicon cells. Furthermore, deletion mutants of NS5A that are localized in the nucleus cause the nuclear translocation of endogenous c-Raf1. This demonstrates that NS5A and c-Raf1 interact in a physiologically relevant model system, ie. in human hepatoma cells.

Next, it was analyzed how modulation of c-Raf1 activity affects HCV replication. Inhibition of c-Raf1 by the means of a small-molecule inhibitor (BAY43-9006) lead to a decrease in HCV replication. Reducing c-Raf1 expression by the means of siRNA lead to a similar reduction of HCV replication, arguing that integrity of c-Raf1 is required for viral replication. Disruption of the MAP kinase cascade at the level of MEK (U0126) did not affect viral replication. These data suggest that the c-Raf1-mediated reduction of HCV replication is not due to the disruption of the MAP kinase cascade. Furthermore, these data highlight the exclusive role of c-Raf1 in viral replication.

In the following set of experiments, the impact of NS5A on c-Raf1-mediated signal transduction was investigated. Surprisingly, the level of ERK phosphorylation was not affected by the presence of NS5A. Moreover, NS5A did not affect the level of basal activation of SRF, AP-1, NF-$\varkappa$B or STAT3 – either if expressed as an individual protein or if expressed in the HCV polyprotein context. This argues that NS5A does not modulate c-Raf1-mediated signal transduction.

Since NS5A is a phosphoprotein and c-Raf1 is a kinase, the impact of c-Raf1 on NS5A phosphorylation was analyzed. To that end, both NS5A and GST-Raf were purified by affinity

chromatography. Phosphorylation of NS5A by GST-Raf was reconstituted *in vitro* using highly purified GST-Raf. An inactive mutant of GST-Raf (K375W) did not phosphorylate NS5A, arguing that phosphorylation of NS5A was specific on behalf of c-Raf1. This suggests that c-Raf1 is an NS5A kinase *in vitro*. Moreover, c-Raf1 inhibition by the means of BAY43-9006 lead to a decrease in NS5A hyperphosphorylation in HCV replicon cells, arguing that c-Raf1 contributes to NS5A phosphorylation *in vivo*.

In conclusion, this study describes a novel cellular binding partner of HCV NS5A that is essential for NS5A phosphorylation and HCV replication. Among the multitude of binding partners described for NS5A, there are few binding partners that fit these criteria and provide an impact on understanding this exciting, though enigmatic protein.