Originally published as:

Hrincius, E.R., Wixler, V., Wolff, T., Wagner, R., Ludwig, S., Ehrhardt, C.
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(2010) Cellular Microbiology, 12 (6), pp. 831-843.

DOI: 10.1111/j.1462-5822.2010.01436.x

CRK adaptor protein expression is required for efficient replication of avian influenza A viruses and controls JNK mediated apoptotic responses

Eike R. Hrincius[1], Viktor Wixler[1], Thorsten Wolff[2], Ralf Wagner[3], Stephan Ludwig[1]* and Christina Ehrhardt[1]*

1. Institute of Molecular Virology (IMV), ZMBE, Von Esmarch-Str. 56, D-48149 Muenster, Germany
2. Robert Koch Institute, P15, Nordufer 20, D-13353 Berlin, Germany
3. Paul-Ehrlich-Institute, Paul-Ehrlich Str. 51-59, D-63225 Langen, Germany

* for correspondence:

Prof. Dr. Stephan Ludwig	Dr. Christina Ehrhardt
Institute of Molecular Virology	Institute of Molecular Virology
Westfälische-Wilhelms-University	Westfälische-Wilhelms University
Von Esmarch-Str. 56	Von Esmarch-Str. 56
D-48149 Münster	D-48149 Münster
Germany	Germany
Phone: +49 (0)251-8357791	+49 (0)251-8353010
Fax: +49 (0)251-8357793	+49 (0)251-8357793
e-mail: ludwigs@uni-muenster.de	ehrhardc@uni-muenster.de

This is an Accepted Article that has been peer-reviewed and approved for publication in the *Cellular Microbiology*, but has yet to undergo copy-editing and proof correction. Please cite this article as an "Accepted Article"; doi: 10.1111/j.1462-5822.2010.01436.x

Abstract

The non-structural-protein 1 (A/NS1) of influenza A viruses (IAV) harbors several src-homology domain (SH)-binding motifs that are required for interaction with cellular proteins. The SH3-binding motif at aa212-217 [PPLPPK] of A/NS1 was shown to be essential for binding to the cellular adaptor proteins CRK and CRKL. Both regulate diverse cellular effector-pathways, including activation of the MAP-kinase JNK that in turn mediates antiviral responses to IAV infection. By studying functional consequences of A/NS1-CRK interaction we show here that A/NS1 binding to CRK contributes to suppression of the antiviral-acting JNK-ATF2 pathway. However, only IAV that encode an A/NS1-protein harboring the CRK/CRKL SH3-binding motif PPLPPK were attenuated upon down-regulation of CRKI/II and CRKL, but not of CRKII alone. The PPLPPK site-harboring candidate strains could be discriminated from other strains by a pronounced viral activation of the JNK-ATF2 signaling module that was even further boosted upon knock-down of CRKI/II. Interestingly, this enhanced JNK activation did not alter type-I IFN-expression, but rather resulted in increased levels of virus-induced cell death. Our results imply that binding-capacity of A/NS1 to CRK/CRKL has evolved in virus strains that over-induce the antiviral acting JNK-ATF2 signaling-module and helps to suppress the detrimental apoptosis promoting action of this pathway.

Key Words: A/NS1, CRKI/II and CRKL, JNK-ATF2, JNK-mediated apoptosis

Introduction

Influenza A virus (IAV) infections still pose a major burden to human health. Infections with these pathogens are responsible for thousands of hospitalisations and an enormous economic loss each year.

IAV belong to the family of Orthomyxoviridae, which is characterized by a segmented, single stranded RNA genome with negative orientation. The genome encodes up to eleven viral structural and non-structural-proteins. The non-structural-protein 1 of IAV (A/NS1) is encoded by the smallest of the eight gene segments along with the nuclear export protein NS2 (reviewed by (Hale *et al.*, 2008)). The A/NS1 protein is an important virulence factor of IAV by its interference with the innate immune response of the host cells. Besides suppression of the antiviral acting type I IFN system (reviewed by (Fernandez-Sesma, 2007)) via interplay with RNA-induced signaling and binding to host-cell proteins, A/NS1 was shown to activate signaling factors such as phosphatidylinositol-3 kinase (PI3K) to prevent premature apoptosis (Ehrhardt *et al.*, 2007, Shin *et al.*, 2007a, Zhirnov *et al.*, 2007). Activation of PI3K signaling is induced by direct interaction of A/NS1 with the regulatory subunit p85 of PI3K (Ehrhardt *et al.*, 2007, Hale *et al.*, 2006, Shin *et al.*, 2007b). In addition to these interactions, direct binding of A/NS1 to several other cellular proteins has been described (Hale *et al.*, 2008). This includes the tripartite motif (TRIM) protein TRIM25 that belongs to a new class of antiviral acting molecules (Gack *et al.*, 2009).

A/NS1 is a multifunctional protein consisting of an RNA-binding domain and several protein-protein interaction motifs including three Src-homology (SH) binding motifs (BM), one SH2BM and two SH3BM (reviewed by (Ehrhardt *et al.*, 2009)). Recently, it was shown that A/NS1 proteins of avian virus strains associate with the adaptor proteins CRK and CRK-*like* (CRKL) (Heikkinen *et al.*, 2008). The SH3BM of A/NS1 at aa212-217 was identified as the principal binding site. This PPLPPK SH3BM is, with only a few

exceptions, highly conserved among avian IAV strains (Heikkinen *et al.*, 2008), but is uncommon to IAV of human origin. Major variations in the SH3BM sequence of A/NS1 proteins of different IAV strains are observed at prolines P212 and P215 as well as at the positively charged lysine K217. These amino acid residues seem to be critical for efficient binding to CRK proteins (Heikkinen *et al.*, 2008).

The family of CRK adaptor proteins comprises two splice variants CRKII (40kDa) and CRKI (28kDa) (Matsuda *et al.*, 1992), and the CRKL (39kDa) that is encoded by a different gene (ten Hoeve *et al.*, 1993). CRKII and CRKL possess an N-terminal SH2-domain and two SH3-domains, whereas the truncated CRKI lacks the C-terminal SH3-domain (reviewed by (Feller, 2001)). The members of the CRK family bind to numerous cellular proteins, regulating a variety of cellular signaling processes (Feller, 2001). One prominent signaling cascade that is controlled by CRK adaptor proteins is the Jun-N-terminal kinase (JNK) signaling pathway (Dolfi *et al.*, 1998). Upon stimulation of cells with the epidermal growth factor (EGF), CRK was shown to induce JNK activation via the small GTPase Rac1 (Dolfi *et al.*, 1998), which was probably mediated by Dock180, an SH3-domain binding protein and a nucleotide exchange factor for Rac1 (Feller, 2001). Another study illustrated a direct association of JNK1 with CRKII (Girardin *et al.*, 2001). While EGF-induced JNK activation was strongly dependent on the CRKII-JNK1 interaction, TNFα- or UV-induced JNK activity was shown to be independent of CRKII-JNK1-binding (Girardin *et al.*, 2001), suggesting different modes of JNK activation by different stimuli.

IAV infection is also a potent activator of JNK. Among the JNK effectors the transcription factors c-Jun and ATF2 are most critical for virus replication since these factors co-regulate expression of IFNβ, a very potent antiviral cytokine (reviewed by (Samuel, 2001)). Inhibition of the JNK cascade resulted in impaired IFNβ expression and enhanced IAV replication (Ludwig *et al.*, 2001). Hence, the JNK signaling cascade

appears to be an essential mediator of the antiviral immune response. In addition to modulation of the innate immune response, JNK also has been reported to be involved in regulation of apoptosis, either by promotion of pro-apoptotic gene expression, such as TNFα or Fas-L (reviewed by (Dhanasekaran *et al.*, 2008)) or by interference with anti-apoptotic proteins located in the mitochondria (Kharbanda *et al.*, 2000, Yamamoto *et al.*, 1999). Whether IAV-mediated JNK activation is CRKI/II or CRKL dependent has not been studied yet.

Here, we demonstrate that interaction of the viral A/NS1 with cellular CRK proteins inhibits IAV-mediated JNK activation. Partial depletion of CRK proteins by siRNA mediated knock-down resulted in impaired propagation of IAV due to JNK-mediated increase of premature cell death. The described phenomena were observed only with IAV strains encoding A/NS1 proteins that are able to associate with CRK proteins.

Results

CRK proteins selectively interact with A/NS1 proteins of avian IAV harboring an intact PPLPPK motif

Before studying the functional consequences of the A/NS1-CRK association, we explored this interaction for several IAV strains that were selected based on differences in their amino-acid sequences within their SH3BM. Interaction of CRKI/II and CRKL with A/NS1 proteins was suggested to involve the SH3BM at aa212-217 of A/NS1 (Heikkinen *et al.*, 2008). Hence, we compared the binding properties of A/NS1 proteins from IAV strains, such as the two avian fowl plague viruses (FPV) A/FPV/Bratislava/79 (H7N7) and A/FPV/Rostock/34 (H7N1), the human-origin A/Puerto Rico/8/34 (H1N1) (PR8), and A/Thailand/KAN-1/2004 (H5N1) (KAN-1), which are characterized by different sequences within this region (Fig. 1A). We analysed whether CRKII and CRKL would precipitate with A/NS1 from lysates of infected cells. Interestingly, CRKII and CRKL co-precipitated only

with A/NS1 of the H7N7 and H7N1 strain, but not with A/NS1 proteins of the H1N1 or H5N1 isolates (Fig. 1B, E, F, G). In accordance with these results, immune complexes of either CRKII or CRKL exclusively contained the A/NS1 from the H7N7 but not from the H1N1 strain (Fig. 1C and D). Similar results were obtained with the strain A/Victoria/3/75 (H3N2), that harbors a threonine residue instead of proline 215 in its A/NS1. Accordingly the A/NS1 of this strain failed to bind to CRKII and CRKL (data not shown). These data confirm that proteins of the CRK family are able to interact with A/NS1 proteins and that proline 215, the positively charged lysine 217 and/or proline 212 of the A/NS1 within the SH3BM are elemental for this interaction.

Recently binding of A/NS1 to the p85 regulatory subunit of the phosphatidylinositol-3 kinase (PI3K) was demonstrated (Ehrhardt *et al.*, 2007, Hale *et al.*, 2006, Shin *et al.*, 2007b). P85 and CRKI/II or CRKL were reported to utilize diverse binding sites within the A/NS1 protein (Ehrhardt *et al.*, 2009). We therefore evaluated whether these proteins bind to A/NS1 in a competitive manner. A time course of infection with the avian H7N7 IAV revealed that binding of A/NS1 to CRK proteins correlates very well with the onset of H7N7 A/NS1 expression, similar to the previously described A/NS1-p85 interaction (Ehrhardt *et al.*, 2007, Hale *et al.*, 2006, Shin *et al.*, 2007b). Upon immunoprecipitation of A/NS1 from lysates of H7N7 infected cells not only CRKII and CRKL could be detected in immunocomplexes, but also p85β, confirming binding of both proteins to A/NS1 in a non-competitive manner (Fig. 1G). Analysis of A/NS1 immunocomplexes from cells infected with the human H1N1 IAV strain affirmed the association of A/NS1 with p85β, but not with CRKII or CRKL (Fig. 1G). These data demonstrate strong strain selectivity for binding to CRK and furthermore suggest that A/NS1 can bind to several proteins simultaneously.

Down-regulation of CRKI/II and CRKL results in impaired propagation of the avian H7N7 and H7N1 strains

It is well known that adaptor proteins of the CRK family are able to form multi-protein complexes that convert the signal generated by extracellular stimuli to the activation of tyrosine kinase signaling (reviewed by (Birge *et al.*, 2009)). To elucidate the functional consequences of the association of CRKI/II and CRKL with A/NS1 within infected cells, we knocked-down expression of CRK proteins in A549 cells by siRNA approaches (Fig. 2). Efficient down-regulation of CRKI/II, CRKII and CRKL expression was verified by Western-Blot analysis. Infection of these cells with the avian H7N7 or H7N1 strain revealed significantly reduced progeny virus titers when CRKI/II was knocked-down (Fig. 2A, B panel I, II) with siRNAs against two different target sequences [see Material and Methods: CRKI/II(a), CRKI/II(b)]. Decreased virus titers, albeit less pronounced, were also observed upon down-regulation of CRKL (Fig. 2A, B panel IV). Down-regulation of CRKII expression alone did not reduce virus titers in comparison to untransfected control cells. Moreover, if compared to the scrambled siRNA control, virus titers appeared even slightly increased (Fig 2A, B panel III). In contrast, virus propagation of the H1N1 strain, bearing an A/NS1 protein not able to bind to CRKI/II and CRKL, was not affected by knock-down of the adaptor proteins (Fig. 2C, panel I-IV). Thus, sensitivity of virus strains to CRKI/II or CRKL knock-down directly correlates with the ability of the respective A/NS1 proteins to bind the CRK adaptor proteins. So far, our data suggested that association of CRKI/II and CRKL with the A/NS1 is beneficial for virus replication. The fact that CRKI/II knock-down, but not down-regulation of CRKII alone reduces replication capabilities of the H7N7 and H7N1 strains suggested that CRKI was the most active CRK protein supporting efficient virus replication. Furthermore, the distinct effects upon CRKI/II or CRKL and CRKII down-regulation indicated that the CRK family members exert non-redundant activities in infected cells. These observations, however, also pose the

question why only some but not all A/NS1 proteins have gained the capability to bind CRK adaptor proteins to support virus replication.

CRK knock-down results in elevated JNK and ATF2 activity in cells infected with the avian H7N7 and H7N1 strains

One prominent feature of CRK proteins is to link multiple upstream factors to the JNK signaling pathway (Dolfi *et al.*, 1998). Since JNK was previously shown to be activated upon IAV infection (Ludwig *et al.*, 2001, Ludwig *et al.*, 2002), we focused our attention on the involvement of CRK in IAV induced activation of this pathway.

A549 lung epithelial cells were transfected with specific siRNAs against CRKI/II (Fig. 3A-C), CRKL or CRKII (Fig. 3D) or scrambled siRNA controls and were subsequently infected with the H7N7 (Fig. 3A, B, D), the H1N1 (Fig. 3A, B, D) or the H7N1 strain (Fig. 3C) for different time points. Fig. 3A and B show that in non-transfected control cells JNK phosphorylation was detectable only at late stages of virus replication. Furthermore, within the given time frame and infection doses used, JNK activation was only observed in cells infected with the avian H7N7 isolate but not with the human H1N1 strain. In cells transfected with scrambled siRNA a slight phosphorylation of JNK was already visible 4h post infection, probably due to an additive effect of virus infection and transfection-induced cellular stress. However, cells with down-regulated CRKI/II proteins exhibited an increased phosphorylation of JNK already 4h post infection, which remained elevated until 8h. Phosphorylation of the transcription factor ATF2, a downstream target of JNK, fully reflects the increased activation of JNK upon virus infection. Importantly, the enhanced JNK activation was observed only in cells that where infected with the H7N7 isolate but not with the H1N1 strain, which correlated with the selective ability of the H7N7 A/NS1 protein to bind CRK adaptor proteins. Concomitantly, enhanced JNK activation was also detectable in H7N1 infected cells upon CRKI/II down-regulation on the level of ATF2 phosphorylation (Fig. 3C). In addition to CRKI/II we investigated the

contribution of CRKL and CRKII in IAV induced JNK activation. While CRKL knock-down led to only a marginal increase of ATF2 phosphorylation (Fig. 3D, lane 5), CRKII knock-down did not elevate but rather slightly reduced activation of the JNK pathway (Fig. 3D, lane 6). Fig. 3D further confirmed, that activation of the JNK pathway is not detectable upon H1N1 infection, irrespectively of the CRKL or CRKII expression levels.

Taken together, our data suggested that binding of A/NS1 to proteins of the CRK family exerts an inhibitory effect on virus-induced activation of the stress kinase JNK and identified CRKI as crucial binding factor within this family.

Down-regulation of CRK does not affect IFNβ or MxA expression

Besides other functions, JNK and ATF2 have been implicated to fulfill antiviral tasks by regulating IFNβ expression in IAV infected cells. Therefore we analysed whether the increased JNK and ATF2 activation upon infection of CRK knock-down cells would correlate with an increased IFN response and thereby might lead to reduced virus replication.

To analyse involvement of CRKI/II in the IAV-induced type I IFN expression we determined mRNA levels of IFNβ and the IFNβ-stimulated gene, MxA (data not shown). Upon IAV infection mRNA synthesis of both genes was induced, whereby the human H1N1 virus strain was more effective than the avian H7N7 isolate. Nevertheless, independent of the virus strain used, CRKI/II down-regulation did not significantly affect mRNA levels of IFNβ and MxA synthesis.

CRK knock-down increases JNK-mediated cell death upon infection with the avian H7N7 strain

The findings that A/NS1-CRKI/II interaction results in suppression of H7N7-induced JNK-ATF2 activation, but did not affect type I IFN expression suggested that other JNK-

mediated antiviral responses may be antagonized by A/NS1-CRK binding. Since JNK is also a regulator of apoptotic responses, we measured the viability of cells with decreased CRKI/II levels versus control cells. One hallmark of cell death is disintegration of cellular membranes, a process that allows nucleic acid staining compounds such as propidium-iodide (PI) to enter the cell. Flow cytometry analysis of PI-positive cells revealed a slight induction of cell death in control or mock-transfected cells upon H7N7 infection (Fig. 4A lane 4 and 5) that was not observed in non-infected (Fig. 4A lane 1 and 2) or H1N1 infected cells (Fig. 4A lane 7 and 8). This IAV induced cell death was further enhanced when CRKI/II was down-regulated, however, selectively in cells infected with the avian H7N7 IAV strain (Fig. 4A lane 6) that possesses CRKI/II binding capability. In cells infected with the human H1N1 IAV strain the number of PI-positive cells was not changed (Fig. 4A lane 9). These results were confirmed with a second CRKI/II specific siRNA (CRKI/II(b)) (Fig. 4B).

To further study whether the increased number of PI-positive cells (Fig. 4A, B lane 6) is due to an enhanced activation of the JNK-ATF2 pathway (Fig. 3), we blocked induction of JNK activity with the specific JNK inhibitor SP600125 (Fig. 4C). While in general enhanced levels of cell death were observed upon infection with the H7N7 IAV strain (Fig. 4C lane 5 and 6) CRKI/II down-regulation led to an even increased number of PI-positive cells (Fig. 4C lane 6) compared to the infected control cells (Fig. 4C lane 5). Inhibition of JNK, however, resulted in a decrease of virus-induced death of cells transfected with either scrambled or CRKI/II-specific siRNA (Fig. 4C lanes 7 and 8). To exclude that decrease of virus-induced cell death is due to reduced virus replication upon SP600125 treatment we investigated viral protein synthesis as an indicator for viral replication (Fig. 4D). H7N7 replication did not significantly differ between solvent or SP600125 treated cells as indicated by similar levels of A/NS1 accumulation six hours post infection (Fig.

4D). These results verified that the increase of H7N7 induced cell death was a result of the enhanced JNK activity upon CRKI/II knock-down.

To further confirm the involvement of CRKI/II in H7N7 induced apoptosis, we performed Nicoletti-assays, which allow to analyse the chromosome-set of cells, due to the level of DNA fragmentation during apoptotic cell death. Flow cytometry analysis of apoptotic cells revealed a slight induction of cell death in scrambled siRNA transfected cells upon infection with the H7N7 strain (Fig. 4E lane 3) in comparison to uninfected cells (Fig. 4E lane 1 and 2). Nevertheless, this enhanced fraction of apoptotic cells was further significantly increased upon knock-down of CRKI/II expression in H7N7 infected cells (Fig. 4E lane 4). Thus, both propidium-iodide (PI)-staining and Nicoletti-assay experiments revealed that CRKI/II knock-down results in increased cell death upon infection with the H7N7 strain, illustrating the involvement of CRK proteins in IAV-induced cell death. In contrast, CRKL or CRKII knock-down in H7N7 infected cells did not result in a significant increase in PI-positive cells (data not shown).

To further assess the role of CRKI/II in regulation of cell survival we investigated the cleavage of poly-(ADP-ribose) polymerase (PARP), a prominent substrate of apoptotic caspases. A cleaved PARP band was detectable in lysates of CRKI/II knock-down cells upon infection with the avian H7N7 IAV strain (Fig. 5A lane 6) but not in cells infected with the H1N1 isolate (Fig. 5A lane 9). The pronounced PARP cleavage shown in this experiment strongly correlated with the enhanced activation of the JNK-ATF2 pathway (Fig. 5A lane 6). Concomitantly, siRNA mediated knock-down of CRKI/II expression in H7N7 infected cells resulted in an activating cleavage of full caspase-9 (Fig. 5B lane 6) in contrast to control cells (Fig. 5B lane 4 and 5).

Disruption of A/NS1-CRK-interaction negatively affects replication ability of IAV in chicken embryonic fibroblasts

To analyse functional consequences of alterations in A/NS1-CRK-interaction, we constructed and examined two recombinant H7N1 IAV viruses expressing either the wt or mutant A/NS1 protein with an amino acid replacement at position 212 within A/NS1 (NS1-wt and NS1-P212S, respectively). To analyse the A/NS1-CRK-binding capacity, we investigated whether CRKII and CRKL were able to co-precipitate with A/NS1 from lysates of infected cells. As expected CRKII and CRKL only co-precipitated with A/NS1 of the H7N1 wild-type (NS1-wt) but not with A/NS1 proteins of the H7N1 mutant (NS1-P212S) virus strain (Fig. 6A). These results confirmed that proline 212 within A/NS1 is essential for CRKII and CRKL binding. Additionally, we analysed the replication-capability of H7N1 wild-type in comparison to H7N1 mutant (NS1-P212S) viruses. Infection of human A549 cells revealed no significant differences in replication of H7N1 wild-type (NS1-wt) or mutant (NS1-P212S) viruses within this cell type (Data not shown). Nevertheless, we observed significant differences in replication of both virus strains in chicken embryonic fibroblasts (CEF`s). Virus titers of H7N1 (NS1-P212S) mutant viruses were significantly reduced in comparison to wild-type viruses (Fig. 6B). Thus, mutation of the predicted CRK-binding sequence within the A/NS1 protein prevents A/NS1-CRK binding and results in reduced virus replication in CEF`s indicating that A/NS1-CRK-interaction is beneficial for efficient virus-growth.

In conclusion, the presented data confirm that CRKI/II and CRKL bind to A/NS1 proteins, which harbor the PPLPPK motif at aa212-217. Further, our results indicate for the first time that the interaction of the CRK and A/NS1 proteins prevents a strong induction of the JNK-ATF2 pathway. Thereby, we showed that predominantly the interaction of A/NS1 with CRKI and to a lesser extent with CRKL decreases IAV induced activation of the

JNK-ATF2-pathway. Consequently, virus induced premature cell death is inhibited enabling efficient virus replication.

Discussion

IAV continue to be a cause of highly contagious respiratory diseases worldwide. These pathogens have co-evolved with their hosts and thereby developed strategies to manipulate the cellular signaling machinery to ensure efficient replication (reviewed by (Wolff *et al.*, 2008)). The best studied viral protein interfering with host cell signaling is A/NS1 that antagonizes the cellular immune response. The A/NS1 protein is able to inhibit activation of antiviral acting signaling mediators, such as JNK, resulting in impaired IFNβ induction (Ludwig *et al.*, 2002). Recently, it became obvious that direct binding of A/NS1 to cellular proteins leads to manipulation of host cell signaling. Besides interaction with the latent protein kinase R (PKR) (Li *et al.*, 2006, Tan *et al.*, 1998), A/NS1 was shown to bind to the p85 regulatory subunit of PI3K (Ehrhardt *et al.*, 2007, Hale *et al.*, 2006, Shin *et al.*, 2007b), and TRIM25 (Gack *et al.*, 2009). Other cellular proteins, which bind to A/NS1 are the cellular adaptor proteins CRKI/II and CRKL. It has been shown that only A/NS1 proteins, which harbor a conserved proline rich sequence (PPLPPK) that represents a SH3BM, are capable to bind CRK proteins (Heikkinen *et al.*, 2008). Aside from a few exceptions this sequence is predominantly present in IAV strains of avian origin but is rarely found in human strains (Heikkinen *et al.*, 2008).

Our experiments confirmed that the PPLPPK motif within A/NS1 of IAV is an important part of the principal binding site for CRK and CRKL adaptor proteins. The A/NS1 of the avian H7N7 and H7N1 IAV strains that harbor this moiety in their SH3BM did bind to CRK adaptor proteins, whereas the A/NS1 proteins of the human H1N1 isolate A/PR/8/34, the H3N2 strain A/Victoria/3/75 and the highly pathogenic H5N1 strain A/Thailand/KAN-1/04 that do not exhibit the SH3 binding consensus sequence failed to

do so. Interestingly, concurrent detection of p85 and CRK upon A/NS1 co-immunoprecipitation in H7N7 infected cells suggests the existence of functionally independent SHBMs within the A/NS1 protein. This observation excludes competition of p85 and CRK for the binding to A/NS1. Furthermore, in H1N1 virus infected cells, A/NS1 binds to p85 but does not bind to CRK. These results clearly demonstrate that A/NS1-p85 binding occurs independent from CRK, illustrating that CRK is not the missing cellular bridging factor between A/NS1 and p85, as discussed by Heikkinen and colleagues (Heikkinen *et al.*, 2008). However, it remained enigmatic for which reason A/NS1 proteins differ in their binding capacity to CRK adaptor proteins depending on the virus strain. Here we show an interplay between CRK and A/NS1 that regulates the antiviral acting JNK signaling pathway. Our results reveal an interaction between A/NS1 and CRK adaptor proteins as an additional mechanism of A/NS1 to shape efficient IAV replication. Such a mechanism may only have evolved in virus strains that *per se* provoke a strong activation of JNK. It has been shown by using a A/NS1 deficient virus mutant that IAV strains, which overinduced the JNK signaling pathway, provoke an enormous IFNβ expression (Ludwig *et al.*, 2002). Thus, the A/NS1 may interfere with CRK and suppress JNK activity below a threshold to prevent JNK contribution to IFNβ expression and furthermore keep apoptosis induction to a tolerable limit. Again, this may only be relevant for virus strains that strongly induce these responses. Thus, reduction of CRKI/II and to a lesser extent CRKL protein expression led to decreased virus titers of the H7N7 and H7N1 strains, whereas propagation of the H1N1 strain was not affected. Down-regulation of CRKII did not reduce, but rather enhance H7N7 and H7N1 replication in comparison to scrambled siRNA transfected cells confirming variable functions of the CRK family members. Replication of H1N1 was not affected at all upon down-regulation of protein-expression of any CRK family member.

In addition to reduced replication, reduction of CRKI/II protein expression resulted in higher phosphorylation of JNK and ATF2 in H7N7 and H7N1 infected cells, compared to H1N1 infected cells. Upon CRKL knock-down slightly increased phosphorylation of ATF2 was detectable upon H7N7 infection, whereas down-regulation of CRKII did not elevate but rather slightly reduce activation of the JNK pathway.

Based on these observations we concluded that CRK protein expression is important for IAV, whose A/NS1 possess CRK binding capacity. Further, these results indicate that members of the CRK family do not fulfill equivalent functions during IAV replication. We identified CRKI as most important candidate of the CRK family to support replication of A/NS1-CRK binding-competent IAV, by suppression of virus induced JNK-ATF2 activation.

Interference of the CRK adaptor protein family with the JNK pathway was illustrated by several studies (Birge *et al.*, 2009, Feller, 2001). Upon stimulation with EGF direct binding of CRKII to JNK1 resulted in JNK activation, whereas TNFα or UV induced JNK1 phosphorylation was CRKII-JNK1-binding independent (Girardin *et al.*, 2001). Overexpression of CRK led to JNK activation and c-Jun phosphorylation in HEK293T (Ling *et al.*, 1999) but not in Cos-7 cells (Oehrl *et al.*, 1998) and may therefore occur in a cell type specific manner. In our cell system, the human lung epithelial cell line A549, CRK overexpression *per se* did not affect JNK phosphorylation (data not shown). Nevertheless, in cells infected with the H7N7 strain CRK expression was strongly required to suppress JNK activation and activation of pro-apoptotic factors such as caspase-9. The expression of antiviral acting mediators, such as IFNβ or MxA was not affected, probably due to the predominant impact of IRF-3 in IFNβ expression, upon IAV infection (Talon *et al.*, 2000).

The necessity of A/NS1-CRK-interaction for IAV replication was further confirmed by usage of recombinant IAV; upon disruption of A/NS1-CRK-interaction virus replication was decreased.

Taken together, there appears to be still another strategy, predominantly used by avian IAV, to suppress self-inflicted premature cell death followed from JNK phosphorylation and activation. The presence of CRK proteins was required to efficient virus replication. With regard to the exposure of pandemic outbreaks we revealed a new piece of the puzzle to understand zoonosis descended from avian origin.

Experimental procedures

Cells, viruses and infection conditions

The avian influenza virus A/FPV/Bratislava/79 (H7N7) (FPV) and the human influenza virus A/Puerto-Rico/8/34 (H1N1) (PR8) were taken from the virus strain collection of the Institute of Virology, Giessen. The human influenza virus isolate of a highly pathogenic avian virus strain, A/Thailand/KAN-1/2004 (H5N1) (KAN-1), was a kind gift of P. Puthavathana (Mahidol University, Bangkok, Thailand).

The H7N7 and H5N1 strains were passaged on Madin-Darby canine kidney (MDCK) cells. The H1N1 strain was propagated in 11-days old chicken embryos. For infection, cells were washed with phosphate-buffered saline (PBS) and incubated with IAV at the indicated multiplicities of infection (MOI) diluted in PBS containing 0.2% bovine serum albumin (BSA), 1mM $MgCl_2$, 0.9mM $CaCl_2$, 100U ml^{-1} penicillin, and 0.1mg ml^{-1} streptomycin for 30min at 37°C. The inoculum was aspirated, and cells were incubated with either minimal essential medium (MEM) or Dulbecco modified Eagle medium (DMEM) containing 0.2% BSA and 100U ml^{-1} penicillin, and 0.1mg ml^{-1} streptomycin. MDCK cells were cultured in MEM, while the human lung epithelial cell line A549 was cultivated in DMEM. Chicken embryonic fibroblasts (CEF´s) were isolated by digesting

pieces of 11-day old chicken embryos (head, legs and giblets were removed) with 1x Trypsin/EDTA (0.05%/0.02%) and were cultured in DMEM. Cell-culture media were supplemented with 10% heat-inactivated fetal bovine serum and 100U ml^{-1} penicillin, and 0.1mg ml^{-1} streptomycin.

Generation of recombinant influenza viruses

A set of plasmids allowing the rescue of the recombinant influenza virus strain A/FPV/Rostock/34 (H7N1) (FPV) was used for generating recombinant H7N1 (NS1-wt) and recombinant H7N1 (NS1-P212S) mutant viruses. The reverse genetics system includes eight influenza virus RNA-coding transcription plasmids (pHW2000-PB1, -PB2, -PA, -NP and pHH21, -HA, -NA, -M and -NS) (Wagner *et al.*, 2005). The mutation NS1-P212S in the NS gene segment was introduced by site-directed mutagenesis using the primers NS1-P212S (fwd) (5`gta atg aga atg ggg gaT ctc cac tcc ctc caa ag) and NS1-P212S (rev) (5` ctt tgg agg gag tgg agA tcc ccc att ctc att ac). To generate the recombinant viruses, 1 µg of each of the eight plasmids was transfected into HEK293 with Lipofectamine 2000 (Invitrogen) as described (Basler *et al.*, 2000). Cells were grown in DMEM (100U ml^{-1} penicillin, and 0.1mg ml^{-1} streptomycin, 0,5% heat-inactivated fetal bovine serum and 0.2% bovine serum albumin (BSA)) and 24h upon transfection cell-culture-medium was exchanged. 48h posttransfection the supernatant was removed and used for infection of MDCK cells. After 2-3 days incubation the supernatant was harvested and the virus titer was determined on MDCK cells by plaque assays. For virus propagation recombinant H7N1 wild-type (NS1-wt) and mutant (NS1- P212S) viruses were passaged on Madin-Darby canine kidney (MDCK) cells. The presence and propriety of the desired mutation was confirmed by sequencing.

Plaque titration

Supernatants of infected cells, were collected at the indicated times p.i. and used to assess the number of infectious particles (plaque titers) in the samples. Briefly, MDCK-cells grown to a monolayer in 6-well dishes were washed with PBS and infected with serial dilutions of the collected supernatants in PBS/BA for 30min at 37°C. The inoculum was aspirated and cells were supplemented with 2ml MEM/BA (medium containing 0.2% BSA, 1mM $MgCl_2$, 0.9mM $CaCl_2$ and 100U ml^{-1} penicillin, and 0.1mg ml^{-1} streptomycin) containing 0.6% Agar (Oxoid), 0.3% DEAE-Dextran (Pharmacia Biotech) and 1.5% $NaHCO_3$ and incubated at 37°C with 5% CO_2 for 2-3 days. Virus plaques were visualized by staining cells with neutral red and virus titers were depicted as PFU/ml.

siRNA-transfection, Western-Blots and immunoprecipitation

For silencing CRKI/II or CRKL protein expression, siRNAs for human CRKI/II (Qiagen) (a) Hs_CRK_1 HP siRNA (SI00073780), (b) Hs_CRK 5 HP Validated siRNA (SI00299929), CRKL (Santa Cruz Technologies) or CRKII (Santa Cruz Technologies) were used. Scrambled siRNA served as a control. In brief, A549 cells seeded in 12 well-dishes were transfected with 50pmol siRNA, using Lipofectamine 2000 (Invitrogen) as described (Basler *et al.*, 2000). Transfected cells were incubated at 37°C with 5%CO_2 for 48h. After infection for the indicated times, cells were lysed on ice with RIPA lysis buffer (25mM Tris-HCl pH 8.0, 137mM NaCl, 10% glycerol, 0.1% SDS, 0.5% DOC, 1% NP40, 2mM EDTA pH 8.0, 5µg ml^{-1} leupeptin, 5µg ml^{-1} aprotinin, 0.2mM pefablock, 1mM sodium vanadate and 5mM benzamidine) for 30min. Cell lysates were cleared by centrifugation and protein concentration was determined by the Bradford method. Cell lysates were used for analysis of protein expression by SDS-PAGE and Western-Blot.

For immunoprecipitation (IP) cells were lysed on ice with Triton lysis buffer (TLB; 20mM Tris-HCl pH 7.4, 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA, 50mM

sodium glycerophosphate, 20mM sodium pyrophosphate, 5µg ml^{-1} leupeptin, 5µg ml^{-1} aprotinin, 0.2mM pefablock, 1mM sodium vanadate and 5mM benzamidine) for 30min. Cell lysates were processed as described above. For IPs the following antibodies or antisera were used; rabbit anti-A/NS1 polyclonal antiserum (RKI, Berlin, Germany), mouse monoclonal antibody A/NS1 (clone NS1-69-1; developed at the IMV Münster, Germany), rabbit CRKII polyclonal antiserum (H-53) (Santa Cruz Technologies), and rabbit CRKL polyclonal antibody (C-20) (Santa Cruz Technologies) coupled to protein A or G agarose (Roche). Sera of non-immunized mice or rabbits were used for control purposes. For IP-input controls, lysates were directly subjected to SDS-PAGE and Western-Blot. Phosphorylated JNK or ATF2 were detected in crude cell lysates by a phosphospecific JNK (pT183/pY185) mouse antibody (BD Transduction Laboratories) or a phosphospecific ATF2 (pT71) rabbit antibody (Cell Signaling Technologies), respectively. Phosphorylated c-jun was detected by a phosphospecific c-jun (pS63) rabbit antibody (Cell Signaling Technologies). The A/NS1 protein was visualized by the A/NS1 rabbit antiserum, mentioned above or the A/NS1 mouse antibody (clone NS1-23-1; developed at the IMV Münster, Germany). CRKI/II and CRKL were detected by a CRKI/II mouse antibody (BD Transduction Laboratories) and a CRKL rabbit antibody (C-20) (Santa Cruz Biotechnologies) or a CRKL mouse antibody (clone 5-6) (Millipore). Detection of p85β was executed by a p85β mouse antibody (AbD Serotec). Apoptosis specific markers were visualized with a caspase-9 (Asp330)-specific rabbit antibody or a Poly-(ADP-ribose) polymerase (PARP)-specific mouse antibody (BD Transduction Laboratories). Pro-caspase-9 was detected by a mouse anti-caspase-9 (C9) antibody (Cell Signaling Technologies). For loading controls a pan-ERK2- (Santa Cruz Biotechnologies), a pan-JNK1- (Santa Cruz Biotechnologies) or a pan-ATF2-antibody (Cell Signaling Technologies) were used. Protein bands were visualized by a standard enhanced chemiluminescence reaction. The specific JNK inhibitor SP600125 (ENZO

lifescience) was dissolved in DMSO and was added into the medium directly after infection at a final concentration of 10-40µM.

Cell death analysis

Quantification of propidium-iodid (PI) positive cells was achieved by flow cytometry (FACS) analysis and used to quantify the amount of dead cells. siRNA transfected A549 cells were infected with the avian H7N7 strain (MOI=10) or the human H1N1 strain (MOI=10) for 6h. Cells present in supernatants and still adherent cells were combined, washed twice with PBS and subjected to PI-staining. Briefly, cells were incubated with PI (50µg ml^{-1} in PBS) for 10 min at room temperature. The fraction of PI-positive cells was detected by flow-cytometry using the FL2-H channel of the FACS Calibur cytometer (Becton Dickinson). For detection of apoptotic cells, the Nicoletti-Assay (Nicoletti *et al.*, 1991) was performed. The Nicoletti-Assay can be used for determination of apoptotic cell death, thereby apoptotic hypodiploid cells will be detected by fluorescence-activated cell sorter analysis. For Nicoletti-Assay analysis siRNA transfected A549 cells were infected with the avian H7N7 strain (MOI=0.1) for 24h. Adherent cells and detached cells were combined, washed twice with PBS and incubated in Nicoletti-buffer (50µg ml^{-1} propidium-iodid, 0.1% Triton X-100, 0.1% sodium citrate) at 4°C for 4h. The fraction of apoptotic cells was analysed by flow-cytometry using the FL2-H channel of FACS Calibur cytometer (Becton Dickinson).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, grant DFG EH 235 1/1, the fund “Innovative Medical Research” of the University of Muenster Medical School and the Fonds der Chemischen Industrie (FdChI).

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Figure legends

Figure 1: A/NS1 proteins harboring an SH3BM with the amino-acid sequence PPLPPK bind to CRKII and CRKL

(A) The amino-acid (aa) sequence of the SH3 binding-motif (BM) at aa212-217 of the A/NS1 protein of A/FPV/Bratislava/79 (H7N7) (FPV), A/FPV/Rostock/34 (H7N1) (FPV), A/Puerto Rico/8/34 (H1N1) (PR8), and A/Thailand/KAN-1/2004 (H5N1) (KAN-1) and the class II SH3 binding consensus are depicted. X indicates any aa, ϕ denotes a hydrophobic residue, and + indicates a positively charged aa. (B-F) A549 cells were infected with the H7N7, the recombinant H7N1, the H1N1, or the H5N1 strain (MOI=5) for 6h (B, E) or 8h (C, D, F) and subsequently harvested. Cell lysates were subjected to immunoprecipitation (IP) with an anti-A/NS1- (B, E, F), an anti-CRKL- (C), an anti-CRKII-antibody (D). As control a mouse or rabbit serum was used for IP. Co-immunoprecipitated CRKL (B, E, F), CRKII (B, F), or A/NS1 (C, D) were detected by Western-Blot (WB) analysis. Amounts of equal protein precipitation of A/NS1 (B, E, F), CRKL (C), and CRKII (D) in the immunoprecipitates were verified using specific

antibodies. The viral A/NS1 protein and endogenous CRKII or CRKL of crude cell lysates served as a control. (G) A549 cells were infected with the H7N7 or the H1N1 (MOI=5) for the indicated times and subsequently lysed. Cells were subjected to IP with an anti-A/NS1-antibody. As control a rabbit serum was used for IP. Co-immunoprecipitated p85β, CRKII, and CRKL were detected by WB. Amounts of equal protein precipitation of A/NS1 was verified using a specific A/NS1 antiserum. The viral A/NS1 protein and endogenous p85β, CRKII or CRKL of crude cell lysates served as a control.

Figure 2: IAV able to bind to CRKII/CRKL are attenuated upon CRKI/II or CRKL down-regulation

A549 cells were transfected with scrambled siRNA, siRNA directed against CRKI/II(a) (A, B, C panel I), CRKI/II(b) (A, B, C panel II), CRKII (A, B, C panel III), or CRKL (A, B, C panel IV), or were left untreated. 48h after transfection cells were infected with the H7N7 (MOI=0.01) (A), the recombinant H7N1 (MOI=0.05) (B) or the H1N1 (MOI=0.5) (C) strain. Supernatants were assayed for progeny virus yields 20h p.i. in standard plaque titrations. Virus yields are depicted in PFU/ml. Down-regulation of CRKI/II, CRKII or CRKL was analysed by WB. Equal protein load was verified by ERK2 and viral protein expression was shown by A/NS1 detection. Statistical significance of the differences between scrambled and specific siRNA transfected cells was assessed of at least three independent experiments by student´s t-test: [A (I) p=0.022; A (II) p=0.003; A (III) p=0.014; A (IV) p=0.027; B (I) p=0.0005; B (II) p=0.04; B (III) p=0.012; B (IV) p=0.048].

Figure 3: Down-regulation of CRKI/II expression results in increased induction of the JNK signaling pathway in cells infected with the avian H7N7 and H7N1 strains.

A549 cells were transfected with siRNA directed against CRKI/II(a) (A, B), CRKI/II(b) (C), CRKL (D), or CRKII (D), scrambled siRNA (A-D), or were left untreated (A, B). 48h after

transfection, cells were infected with the H7N7 or H1N1 strain (MOI=5) (A, B, D), or the recombinant H7N1 strain (MOI=5) (C) for the times indicated or were left uninfected (A-D). Phosphorylated JNK (pT183/pY185) (A-B) and phosphorylated ATF2 (pT71) (A-D) were detected by WB. Down-regulation of CRKI/II (A-C), or CRKL and CRKII (D) was analysed by WB. Equal protein loads were verified using CRKL (A-C), CRKI (D), ATF2 (A-D), JNK1 (A, B) and ERK2 (A-D) antibodies. Viral protein synthesis was visualized via A/NS1 WB (A-D).

Figure 4: Down-regulation of CRKI/II protein expression results in enhanced cell death upon infection with IAV strains harboring CRKI/II binding capacity

A549 cells were transfected with scrambled siRNA (A-C, E), siRNA directed against CRKI/II(a) (A, C, E), CRKI/II(b) (B), or were left untreated (A, B). 48h after transfection, cells were infected with the H7N7 (A-C) or the H1N1 (A, B) strain (MOI=10) for 6h (A-C) or the H7N7 strain (MOI=0.1) for 24h (E) or were left uninfected (A-C, E). (C) Upon infection cells were incubated with the JNK inhibitor SP600125 (40µM) or solvent control (DMSO). (A-C) For cell death analysis adherent and attached cells were subjected to PI (50µg ml^{-1}) for 10 min. PI-positive cells were detected in the FL2-H channel by FACS analysis. (D) A549 cells were infected with the H7N7 strain (MOI=5) for 6h in presence of the JNK inhibitor SP600125 (10µM or 40µM) or solvent control (DMSO). Inhibition of JNK activity was analysed by phosphorylation of c-jun (pS63) by WB. Viral protein synthesis was visualized via A/NS1 WB. Equal protein load was verified using ERK2 antibody (E). For Nicoletti-assay adherent and detached cells were collected and incubated in Nicoletti-buffer at 4°C for 4h. The fraction of apoptotic cells was analysed in the FL2-H channel by FACS analysis. The averages of PI-positive cells of two independent samples are depicted (A, C) or mean value of at least three independent experiments (B). (E) The averages of apoptotic cells of two independent samples are depicted. Statistical

significance of the differences obtained in the assays between scrambled and specific siRNA transfected and H7N7 infected cells was assessed of at least three independent experiments by student´s t-test: [A (lane 5-6) p= 0.004; B (lane 5-6) p= 0.015; C (lane 5-6) p=0.047; E (lane 3-4) p= 0.02].

Figure 5: Reduction of CRKI/II protein expression leads to induction of PARP and Pro-caspase-9 cleavage upon H7N7 infection

(A, B) A549 cells were transfected with siRNA directed against CRKI/II(a), scrambled siRNA, or were left untreated. 48h after transfection, cells were infected with the H7N7 or H1N1 strain (MOI=5) (A) or the H7N7 strain (MOI=10) (B) for the times indicated. Cleavage of PARP (A), phosphorylation of ATF2 (pT71) (A) or cleavage of caspase-9 (B) were detected by WB. Analysis of uncleaved PARP (A) and pro-caspase-9 (B) was also investigated by WB. (A, B) Down-regulation of CRKI/II was analysed by WB. Equal protein loads were verified using ATF2 and ERK2 (A) or ERK2 (B) antibodies. Viral protein synthesis was visualized via A/NS1 WB.

Figure 6: Disruption of A/NS1-CRK-interaction negatively affects replication ability of IAV in chicken embryonic fibroblasts

(A) A549 cells were infected with recombinant H7N1 wild-type (NS1-wt) or H7N1 mutant (NS1-P212S) virus (MOI=5) for 8h and subsequently harvested. Cell lysates were subjected to immunoprecipitation (IP), using an anti-A/NS1-antibody. As control a rabbit serum was used for IP. Co-immunoprecipitated CRKL and CRKII were detected by Western-Blot (WB) analysis. Amounts of equal protein precipitation of A/NS1 in the immunoprecipitates were verified using specific antibodies. The viral A/NS1 protein and endogenous CRKII or CRKL of crude cell lysates served as a control. (B) Chicken embryonic fibroblasts were infected with the recombinant H7N1 wild-type (NS1-wt) or

mutant (NS1-P212S) virus (MOI=0.5). Supernatants were assayed for progeny virus yields 8h p.i. in standard plaque titrations. Virus yields are depicted in PFU/ml. Statistical significance of the differences obtained in the assays was assessed of at least three independent experiments by student´s t-test; [B, $p=0.01$].

Figure 1

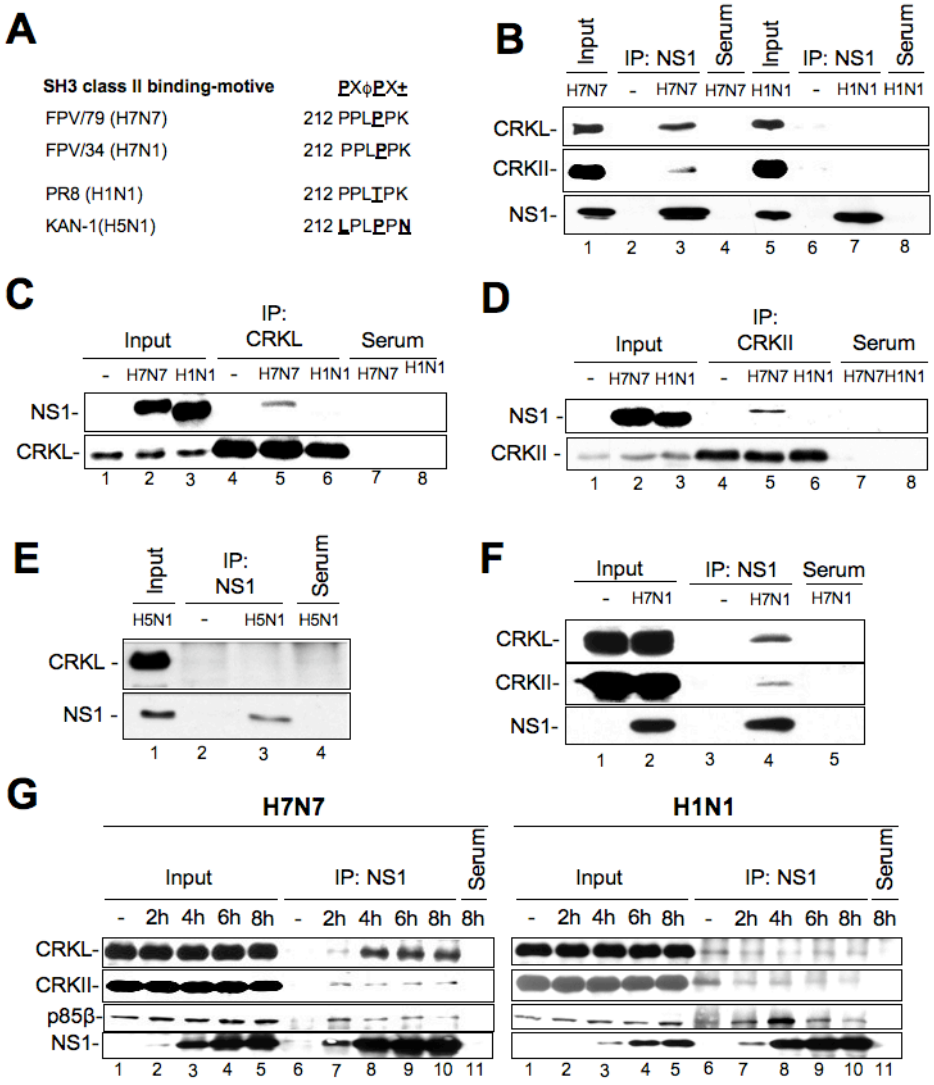

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Figure 2

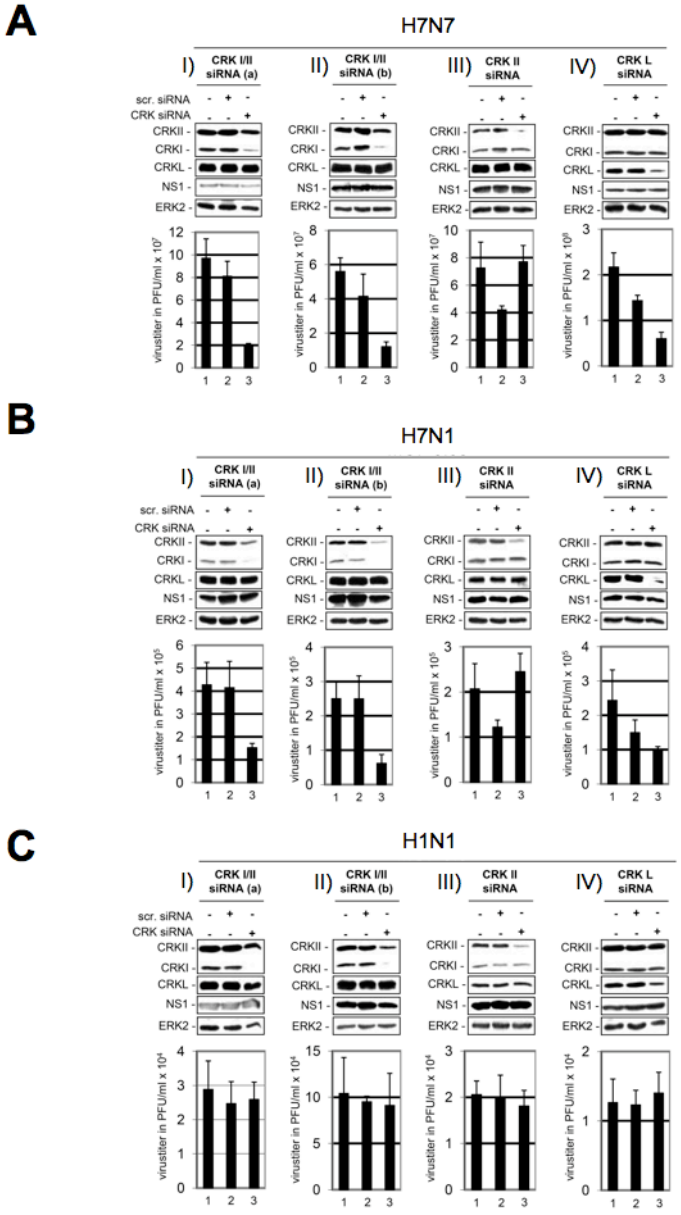

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Figure 3

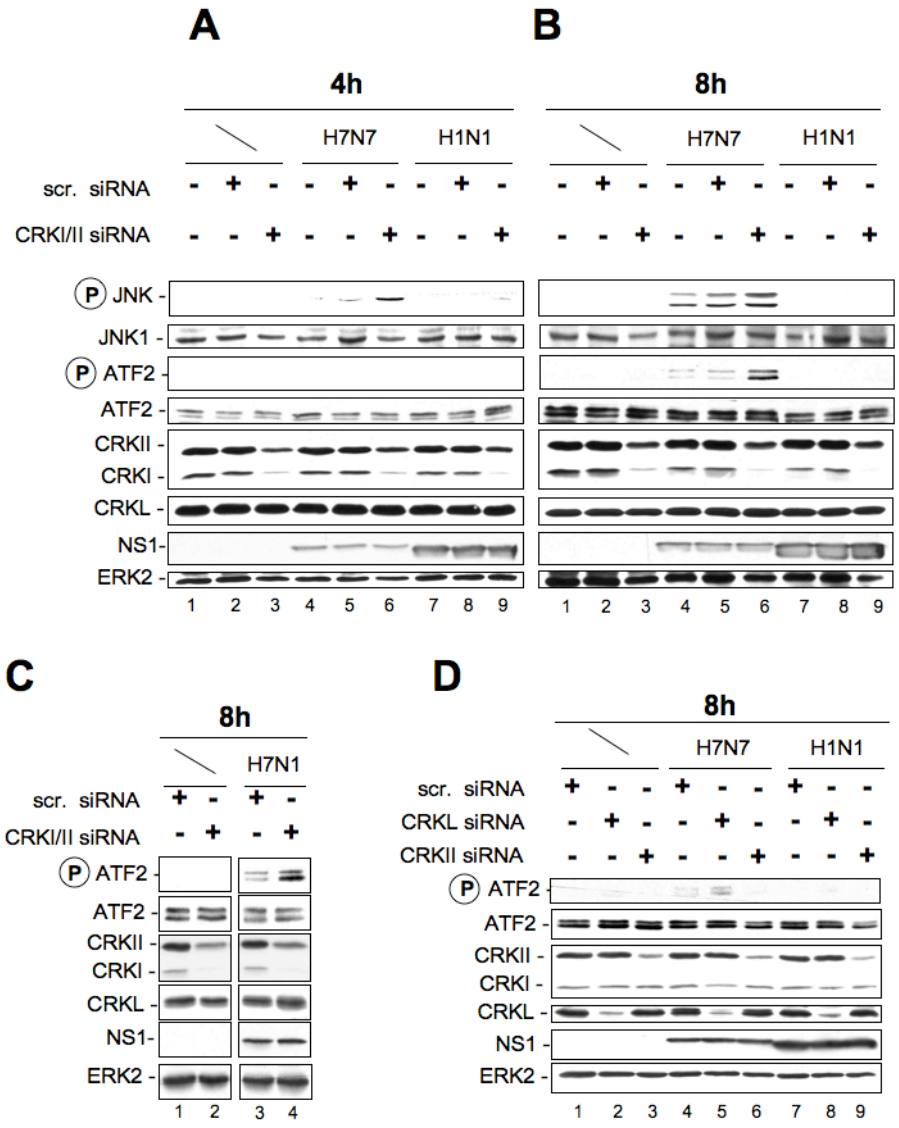

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Figure 4

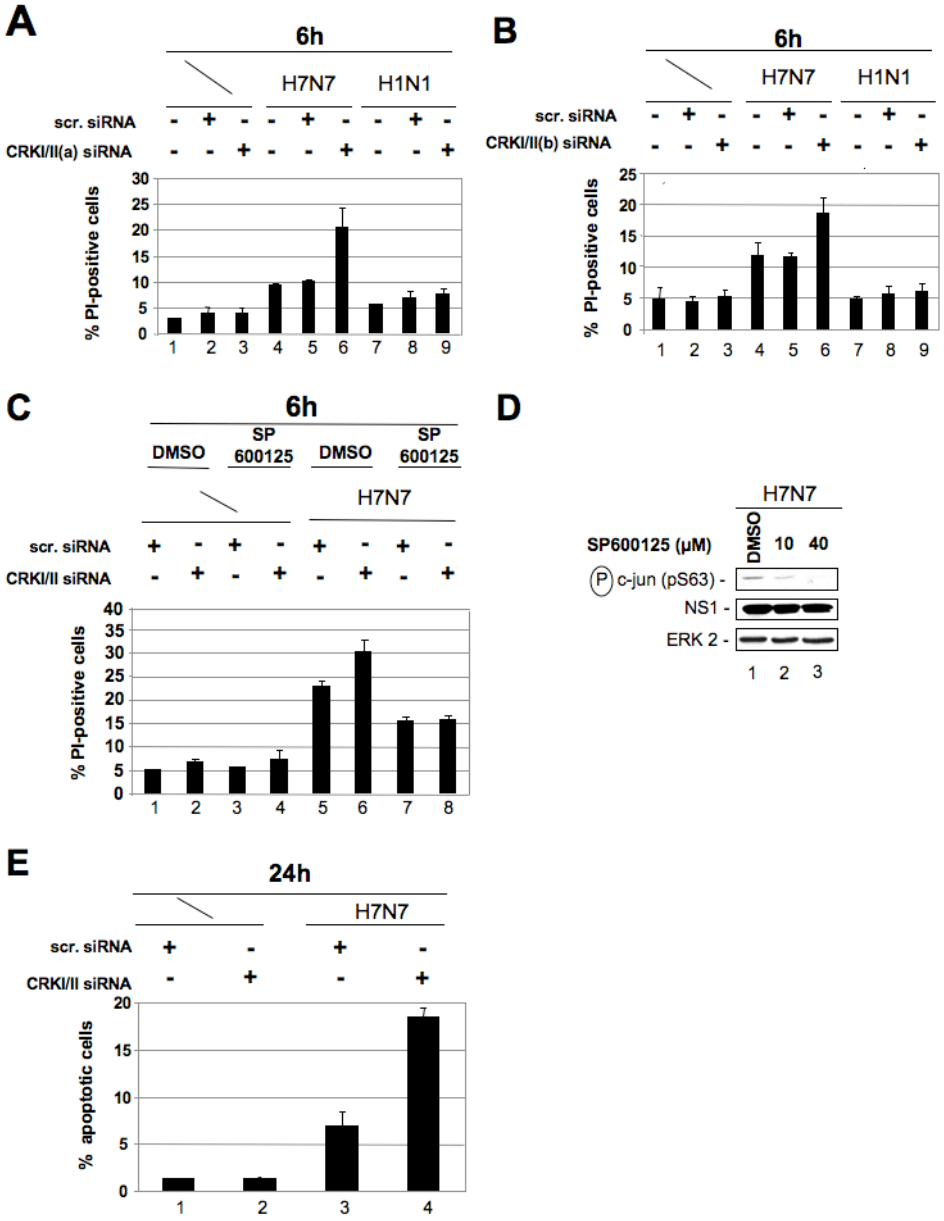

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Figure 5

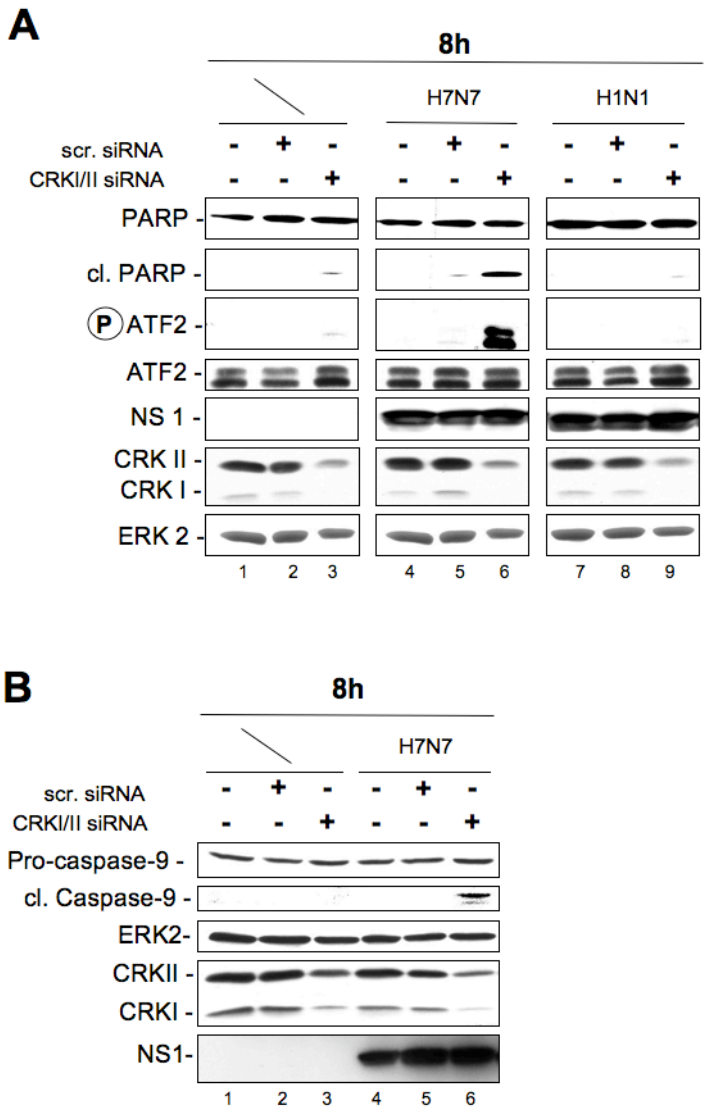

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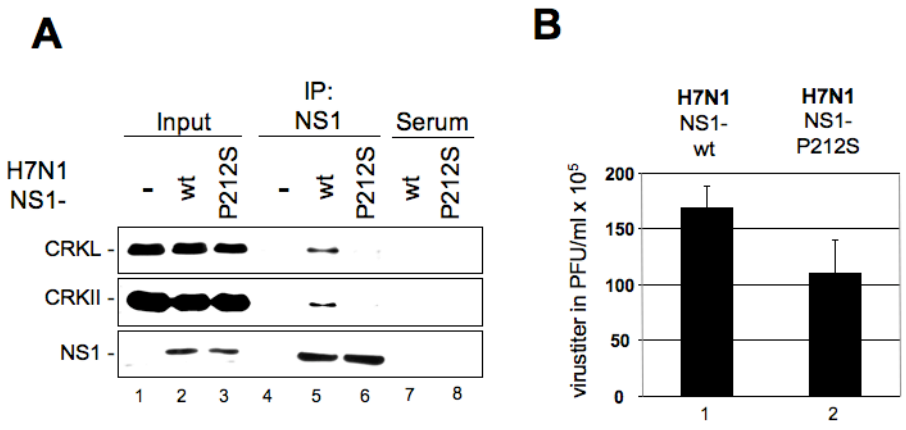
Figure 6



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