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Interaction of the replication proteins and the capsid protein of porcine circovirus type 1 and 2 with host proteins

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ABSTRACT

Porcine circovirus type 2 (PCV2) is an important pathogen in swine, whereas porcine circovirus type 1 (PCV1) is apathogenic. To analyze the interactions between PCV and its host, we have used a yeast two-hybrid assay to identify cellular proteins interacting with Cap and Rep proteins of both PCV genotypes. Six cellular proteins were found to interact with Cap (MKRN1, gC1qR, Par-4, NAP1, NPM1 and Hsp40) and three with Rep (ZNF265, TDG and VG5Q). These interactions were confirmed by co-immunoprecipitation. Investigation of the localisation of the proteins by immunofluorescence revealed in some cases only limited spatial overlapping with Cap, while in others a clear co-localisation and prominent protein redistribution was observed. The nine cellular proteins are associated with distinct aspects of viral lifecycle and our data is likely to support future research in the field of PCV2 pathogenesis.

Introduction

Porcine circoviruses (PCV) belong to the family *Circoviridae*, which comprises small icosahedral non-enveloped viruses containing a small circular single-stranded DNA genome. Two genotypes of PCV have been described, which differ with respect to their pathogenicity: Experimental infection with PCV type 1 (PCV1, 1759 nt) does not result in any disease, while PCV type 2 (PCV2, 1768 nt) is the etiological agent of the post-weaning multisystemic wasting syndrome (PMWS). PMWS was first reported in Canada in 1991 (Clark, 1997, Harding, 1996) and has a severe impact on swine production worldwide (Segales et al., 2005). Prominent PMWS symptoms are wasting, fever, lymphadenopathy and lymphocyte depletion of lymphoid tissues. It has also been suggested that PCV2 infections trigger other porcine diseases, e.g. the porcine dermatitis and nephropathy syndrome (PDNS) and the porcine respiratory disease complex (PRDC) (Allan and Ellis, 2000, Segales et al., 2005). The molecular basis of PCV pathogenicity is not well understood, since infection with PCV2 does not necessarily result in PMWS and PMWS is regarded as a multifactorial disease. Therefore, intrinsic factors as pig genetics and virus strains, as well as extrinsic criteria like nutrition or vaccination schedules are thought to influence the course of the disease.

The genomes of PCV1 and PCV2 have similar genome organisation and display a nucleotide homology of approximately 70% (Hamel et al., 1998). PCV encode only two major open reading frames, which are divergently transcribed. The *rep* gene is located on the viral plus strand and encodes the two replicases Rep and Rep′, both indispensable for the initiation of viral replication (Mankertz and Hillenbrand, 2001, Mankertz et al., 2003), which occurs via a rolling-circle mechanism (Cheung, 2006, Steinfeldt et al., 2006, 2007). The *cap* gene is located on the viral minus strand and encodes the capsid protein Cap, the only structural component of the virion and the dominant immunogenic agent of PCV (Nawagitgul et al., 2002).

Due to the small genome size and the limited coding capacity, it is generally acknowledged that the viral life cycle of PCV must depend extensively on host factors. Since expression of multifunctional proteins will be beneficial for the virus, Rep and Cap may not only serve as replicase and packaging units but might perform an important function in the virus–host interaction. With a bacterial two-hybrid approach Timmusk et al. have identified the intermediate filament protein and the transcriptional regulator c-Myc as cellular proteins interacting with Rep of PCV2 and the complement component C1qB was shown to bind the Cap protein of PCV2 (Timmusk et al., 2006). In order to gain a more detailed knowledge of the virus–host interaction, we have used a yeast two-hybrid approach. With respect to the distinct pathogenic potential of the two PCV, Rep and Cap proteins from both genotypes were used to identify cellular

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interacting proteins. Six porcine proteins interacted with the Cap proteins, and three bound to the Rep proteins. The physical interaction between Cap, Rep and Rep′ and the respective porcine proteins was verified by co-immunoprecipitation in cell culture, and the subcellular localisation of viral and cellular interacting partners was analyzed after co-transfection in cell culture.

Results

Cap of PCV1 and PCV2 interacts with MKRN1, gC1qR, Par-4, NAP1, NPM1 and Hsp40

To identify cellular proteins that interact with the Cap protein of both PCV genotypes, a cDNA library derived from the spleen of a healthy pig was screened in a yeast two-hybrid approach. Cap was used as bait to screen approximately 5×10^6 cDNA library clones. A total number of 40 positive clones, 20 for each of the two Cap proteins, were rescued, sequenced and analyzed by a BLAST search for homology to human and porcine proteins. To verify the interaction, prey plasmids encoding putative interacting partners were co-transformed with the bait constructs into yeast strain AH109. Transformed colonies were cultivated on double drop out (DDO) and quadruple drop out plus X-α-Gal (QDO/X) plates. Growth on DDO plates indicated co-transformation, while growth of blue colonies on QDO/X plates demonstrated the physical interaction of the two encoded proteins. Table 1 lists the cellular interacting partners and indicates the frequency, with which the clones were observed. Six cellular proteins interacted with the Cap proteins of PCV: (I) the makorin-1 RING zinc finger protein (MKRN1); (II) the receptor protein for the globular heads of complement component C1q (gC1qR), also termed protein p32, p33, or hyaluronan binding protein 1 (HABP1), (III) the nucleosome assembly protein-1 (NAP1); (IV) the prostate apoptosis response-4 (Par-4) protein, also referred to as PAWR (PRKC, apoptosis, WT1, regulator protein); (V) nucleophosmin-1 (NPM1); and (VI) the heat shock protein 40 (Hsp40). The interaction was reflected by growth of blue colonies on QDO/X plates (Fig. 1). Since not all six Cap-interacting proteins were identified in both independently performed screens, a specific retesting was done by co-transformation of the putative interaction partner and Cap (PCV1) and Cap (PCV2), respectively. All six proteins bound not only to Cap of PCV1 (Fig. 1, line 4) but also to Cap of PCV2 (line 5). To exclude autoactivation of the ADH1 promoter by the Hsp40 protein (Fig. 1, panel G6), QDO/X plates were supplemented with 4 mM 3-AT to repress residual transcription, resulting in the suppression of growth (panel H6). Since blue colonies were observed under these more stringent conditions (panels H4 and H5), autoactivation of the ADH1 promoter by Hsp40 was excluded.

Table 1
Identified cDNA clones coding for Rep and Cap interacting proteins

Number of cDNA clones found		Description of cDNA clones and best hits of homolog proteins identified in databases
Rep PCV1	Rep PCV2	
12	7	Complete cDNA encoding aa 1–328 of porcine ZNF265 (*Sus scrofa* ABF72033, E value=0; *Homo sapiens* AAD09746, *E* value=7e−89)
1	–	Complete cDNA encoding aa 1–410 of TDG similar to human TDG (*H. sapiens* AAC50540, *E* value=0)
2	3	Partial cDNA encoding aa 143–410 of VG5Q with similarity to VG5Q of cattle and human (*Bos taurus* AAI19838, *E* value=1e−119; *H. sapiens* AAH32844, *E* value=2e−108)
Cap PCV1	Cap PCV2	
5	3	Complete cDNA encoding aa 1–282 of gC1qR (*B. taurus* NP001029699, *E* value=2e−139; *H. sapiens* NP001203 *E* value=1e−128)
6	9	Partial cDNA encoding aa 246–429 of MKRN1 (*Equus caballus* XP001496388, *E* value=7e−93; *H. sapiens* EAW83951, *E* value=1e−91)
2	1	Complete cDNA encoding aa 1–340 of Par-4 (*H. sapiens* CAG38786, *E* value=2e−71)
2	–	Complete cDNA encoding aa 1–235 of NAP1 similar to human NAP1-like 4 isoform CRA_a (*H. sapiens* EAX02532, *E* value=3e−110)
1	–	Complete cDNA encoding aa 1–244 of Hsp40 (*B. taurus* AAI09904, *E* value=2e−137; *H. sapiens* AAH11837, *E* value=3e−138)
1	3	Complete cDNA encoding aa 1–121 similar to NPM1 of *Canis familiaris* (*C. familiaris* XP866739, *E* value=3e−68; *H. sapiens* ABP97432, *E* value=1e−67)

Rep of PCV1 and PCV2 interacts with ZNF265, TDG and VG5Q

A similar approach used for Rep of both PCV genotypes (PCV1 and PCV2) resulted in identification of three cellular interaction partners (Fig. 2, columns B to D): the zinc finger protein 265 (ZNF265), the thymine DNA glycosylase (TDG) and the angiogenic factor VG5Q (Table 1). Verification by co-transformation revealed for TDG light blue colonies in the negative control (panel D8), which were not observed upon addition of 4 mM 3-AT (panel E8), indicating that growth was not triggered by autoactivation. Since the *rep* gene encodes also the spliced Rep′ protein, ZNF265, TDG and VG5Q were tested for their ability to bind also to the Rep′ of PCV1 (lane 6). ZNF265 bound to the full length Rep protein of both PCV strains, but it did not interact with Rep′ (panel B5 to B7). Interaction of TDG and Rep′ (panel E6) seemed to be stronger than interaction with Rep (PCV1) and Rep (PCV2) (panels E5 and E7). VG5Q interacted with Rep of both PCV genotypes and also with Rep′ of PCV1 (panel C5 to C7).

Co-immunoprecipitation of cellular proteins with Cap, Rep or Rep′ of PCV1

Co-immunoprecipitation (co-IP) was applied to confirm the physical interaction between HA-tagged Cap, Rep or Rep′ protein of PCV1 and the cellular target proteins. HEK 293 Graham cells were co-transfected with pHA-CapPCV1 in combination with pMyc-MKRN1, pMyc-gC1qR, pMyc-Par-4, pMyc-NAP1, pMyc-NPM1 or pMyc-Hsp40 expressing the respective Myc-tagged interacting partners. pHA-EGFP co-transfected with the pMyc-constructs served as negative controls. Expression of Myc-tagged proteins was demonstrated by immunoblotting with a mouse anti-Myc antibody (Fig. 3b, upper panel), expression of HA-tagged Cap and EGFP with a mouse anti-HA antibody (Fig. 3b, lower panel). Complex formation between HA-Cap and the Myc-tagged interaction partners was detected by precipitation of cell lysates with a rabbit anti-HA antiserum in conjunction with protein G sepharose. The immunoblot corroborated the results of the yeast two-hybrid approach by demonstrating that all Myc-tagged interaction partners were co-precipitated with the HA-Cap protein (Fig. 3a), while no signal was observed with HA-EGFP instead of HA-Cap. A parallel co-IP experiment investigated binding of ZNF265, TDG and VG5Q to Rep and Rep′ of PCV1. HEK 293 Graham cells were co-transfected with either pHA–RepPCV1 (expressing HA–Rep and HA–Rep′), pHA–Rep*PCV1 (only HA–Rep), pHA–Rep′PCV1 (only HA–Rep′) or pHA-EGFP plus either pMyc-ZNF265, pMyc-TDG or pMyc-VG5Q. A mouse anti-HA antibody was used to detect expression of the HA-tagged Rep, Rep′ and EGFP proteins (Fig. 4b, lower panel), expression of the Myc-tagged ZNF265, TDG and VG5Q proteins was shown with a mouse anti-Myc antibody (Fig. 4b, upper panel). Immunoblotting revealed up to four ZNF265-specific protein bands of approximately 30 kDa, while a double band was observed for TDG and VG5Q. Precipitation of HA–Rep protein complexes was carried out with a mouse anti-HA antibody and protein G sepharose. In agreement with the results of the yeast two-hybrid assay, the ZNF265 protein was precipitated by Rep or Rep/Rep′, but not by Rep′ alone (Fig. 4a). Myc-tagged TDG and VG5Q bound to Rep as well as to Rep′ (Fig. 4a). Indicative of a Rep-specific precipitation, HA-EGFP did not precipitate ZNF265, TDG and VG5Q. β-actin was used in both experiments as a loading control (Fig. 3b and 4b, lower panel). When the co-IP was

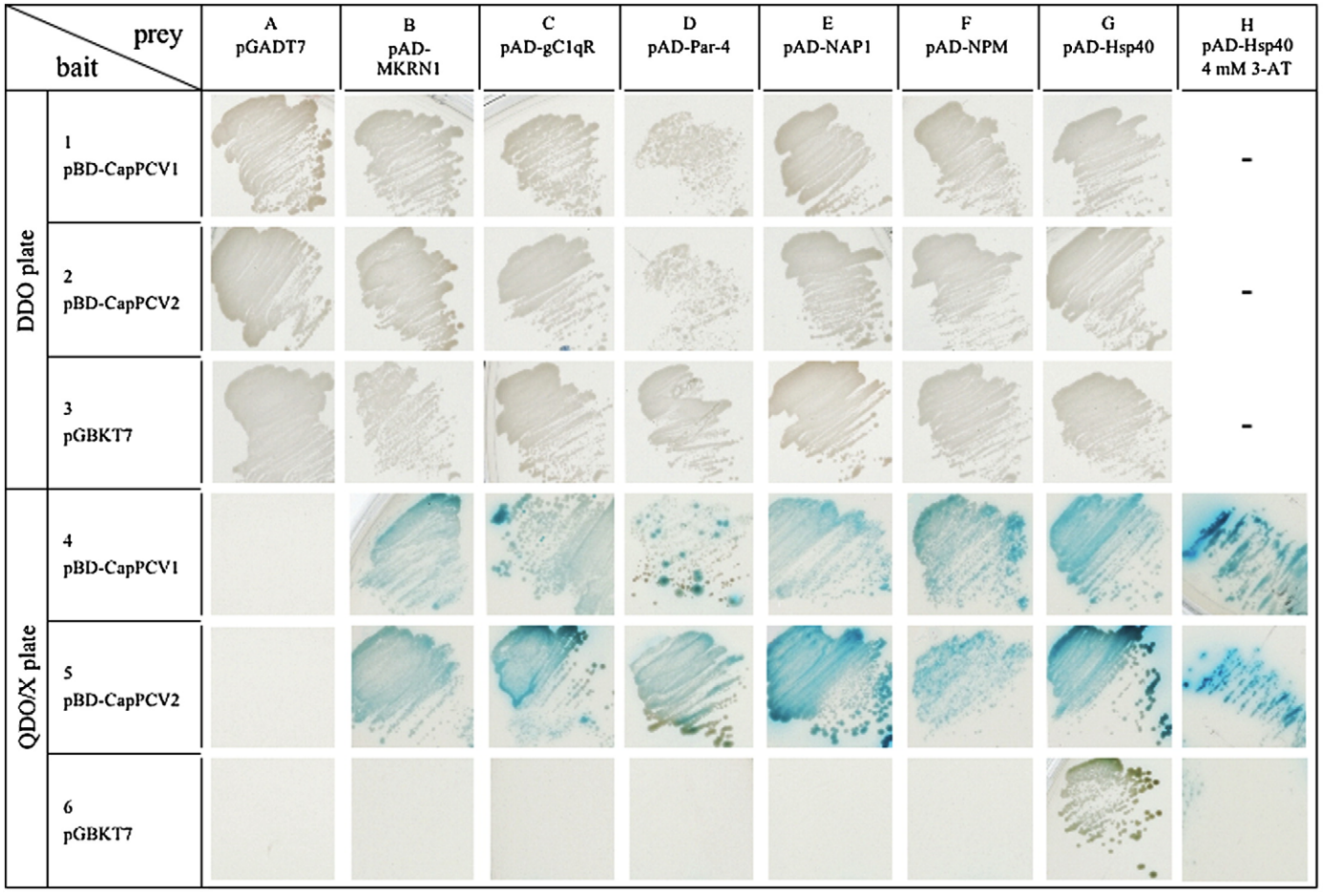

Fig. 1. Yeast two-hybrid assay with PCV Cap proteins and host proteins. Yeast strain AH109 was co-transformed with pGBKT7-based bait and pGADT7-based prey plasmids and selected for the presence of both plasmids on DDO plates lacking tryptophane and leucine. Interaction of the Cap proteins with the six cellular proteins MKRN1, gC1qR, Par-4, NAP1, NPM1 and Hsp40 is indicated by growth of blue colonies on QDO/X plates. In case of Hsp40, autoactivation was excluded by addition of 4 mM 3-AT.

performed with PCV2-encoded Cap and Rep proteins, results were not deviating (data not shown).

Subcellular localisation of PCV-encoded proteins and the cellular interacting partners

Since the subcellular localisation of a protein will usually reflect its function and co-localisation of proteins may support a functional interaction, we have examined the localisation of Cap and Rep/Rep′ of PCV1 in conjunction with their interaction partners by confocal microscopy. To compare the localisation with and without the interacting proteins, HEK 293 Graham cells were first transfected solely with the constructs pHA-CapPCV1 and pMyc-MKRN1, pMyc-gC1qR, pMyc-Par-4, pMyc-NAP1, pMyc-NPM1 or pMyc-Hsp40. Co-localisation and reallocation of the proteins was investigated after co-transfection of the plasmids. When Cap was expressed in HEK 293 Graham cells from pHA-CapPCV1, it was seen in the nucleus, accumulated either in the nucleoplasm or in the nucleoli (Fig. 5a). MKRN1 and Hsp40 proteins formed both speckle-like structures within the nucleoplasm. Co-expressed with the Cap protein, the two proteins co-localised clearly with Cap. The original localisation patterns were altered, and Cap, MKRN1 and Hsp40 proteins were observed in a loose pattern within the nucleoplasm (Figs. 5b and g). The gC1qR protein has been described as a multi-compartment protein, which is localised to the mitochondria, the cytoplasm, the nucleoplasm, but is also attached to the outer cell membrane (van Leeuwen and O'Hare, 2001). In accordance, gC1qR-specific fluorescence signals were obtained in the nucleus, the cytoplasm and in small droplet-like structures in the cytoplasm (Fig. 5c). Co-expression of gC1qR and Cap led to a reallocation of both proteins. The two proteins co-localised mainly in globular structures in the nucleoplasm, surrounding circular and omitted areas, probably the nucleoli. Translocation of gC1qR upon binding to viral or bacterial antigens has been described (Bryant et al., 2000, Hall et al., 2002). Par-4 was seen attached to a fibrillar structure in the cytoplasm (Fig. 5d) corroborating its described association with the cytoskeleton (Vetterkind et al., 2005). Upon co-expression with Cap, the fibrillar structure seemed to be less prominent and Par-4 was partially translocated to the nucleoplasm. Translocation of Par-4 to the nucleus is described for activated or apoptotic cells (Lee et al., 2007). NAP1 functions as a histone chaperone protein facilitating nucleosome assembly, which implies a nuclear localisation. In contrast to its cellular function, distribution of NAP1 to the cytoplasm has been reported (Dong et al., 2003, Park et al., 2008) concordant with our results (Fig. 5e). It was proposed that NAP1 acts as a shuttling factor that transports H2A/H2B dimers from the cytoplasm to the nucleus (Mosammaparast et al., 2002). After co-transfection the localisation of NAP1 did not change, while the Cap protein was found more prominently in the cytoplasm (Fig. 5e). NPM1 accumulated in the nucleoli of HEK 293 Graham cells (Fig. 5f) as described (Mamrack et al., 1979). After co-expression of Cap and NPM1, both proteins localised predominantly in the nucleoli but co-localisation was also seen in the nucleoplasm. To test localisation of Rep and its interaction partners, HEK 293 Graham cells were transfected with pHA–RepPCV1 (expressing Rep and Rep′), pMyc-

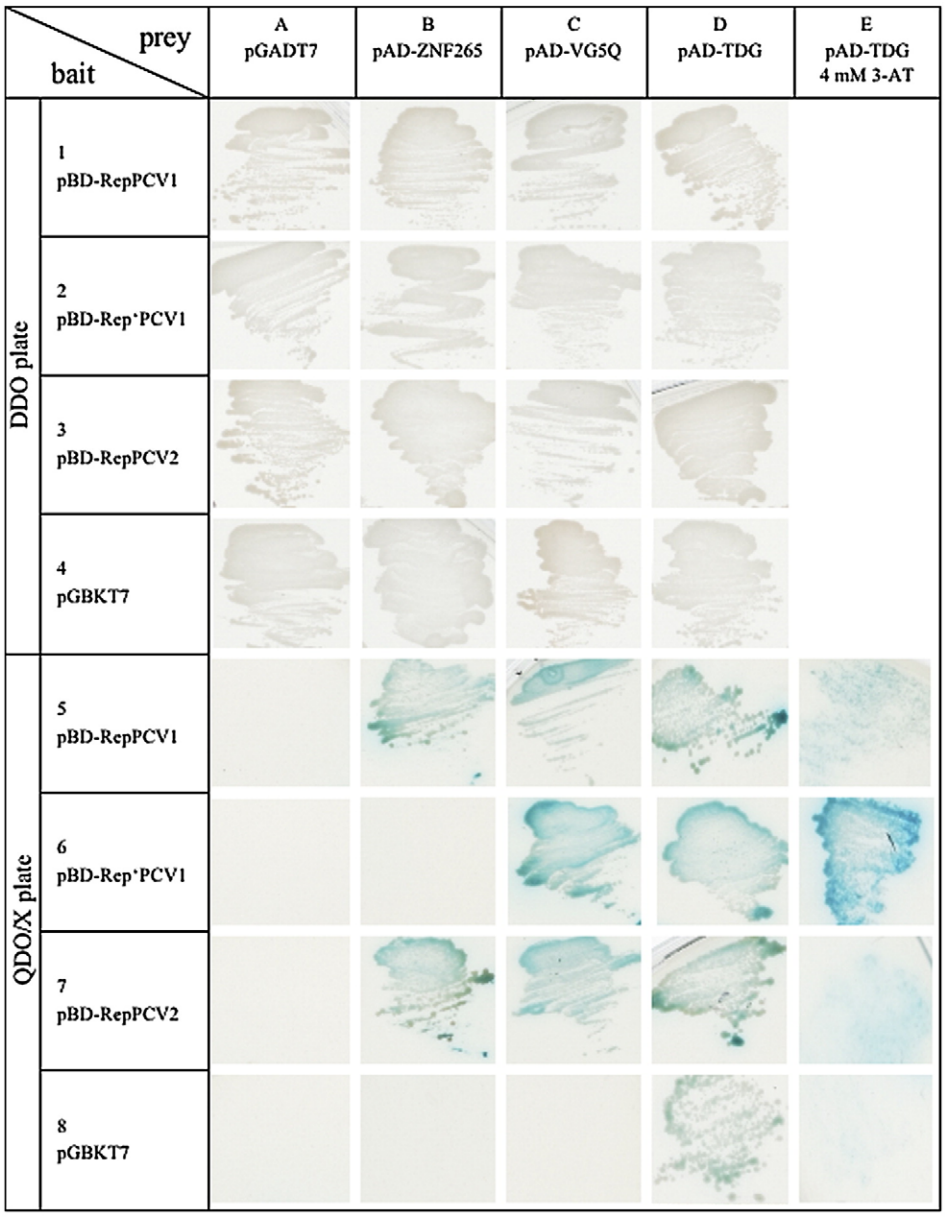

Fig. 2. Yeast two-hybrid assay with *rep* gene products. Specific interaction of the two replication proteins Rep (PCV 1 and PCV2) and Rep′ (only PCV1 shown) with host proteins ZNF265, TDG and VG5Q in yeast cells. After transformation of yeast cells with bait and prey plasmids, colonies on DDO plates verified presence of both plasmids. Interaction of replication proteins with cellular proteins was indicated by growth of blue colonies on QDO/X plates. Autoactivation of TDG was no longer seen after addition of 4 mM 3-AT.

TDG, pMyc-VG5Q or pMyc-ZNF265. After transfection of pHA–RepPCV1, Rep/Rep′ were seen in the nucleus but not in the nucleoli (Fig. 6a), corroborating earlier results (Finsterbusch et al., 2005). TDG accumulated in the nucleoli and to a lesser extent to the nucleoplasm, where co-localisation with Rep/Rep′ was observed (Fig. 6b). In cells with a strong Rep/Rep′ signal TDG was reallocated from the nucleoli to the nucleoplasm, possibly due to interaction with Rep/Rep′. VG5Q was seen in the nucleoplasm identical to Rep/Rep′ (Fig. 6c). After co-expression, VG5Q and Rep/Rep′ were evenly spread in the nucleoplasm. Additionally, spots of high intensity were seen with a speckle-like morphology. ZNF265 was seen in multiple compartments (Fig. 6d). The strongest signal was observed in the nucleoplasm, the nucleoli were stained less intensive, but ZNF265 was also observed to a lesser extent in the cytoplasm. Co-expression of Rep/Rep′ and ZNF265 led to a distinct alteration of the localisation pattern, since both proteins were augmented in condensed punctiform structures within the nucleoplasm. This experiment was also performed with Cap and Rep/Rep′ of PCV2 and gave similar results (data not shown).

Discussion

The molecular mechanism of PCV2 pathogenicity, as well as the dependency of viral growth on host cell factors is still poorly understood. To gain knowledge about the virus host interplay we have used a yeast two-hybrid screen to identify porcine host proteins interacting with Cap and Rep proteins of both PCV genotypes. Six porcine protein fragments or full-length proteins were identified to bind to the Cap protein and three more interacted with the Rep protein of PCV. Almost all interaction partners are annotated in the literature as proteins with multiple functions but there is no indication of a common ontology. Therefore functions of the described interacting proteins regarding PCV infection will be discussed.

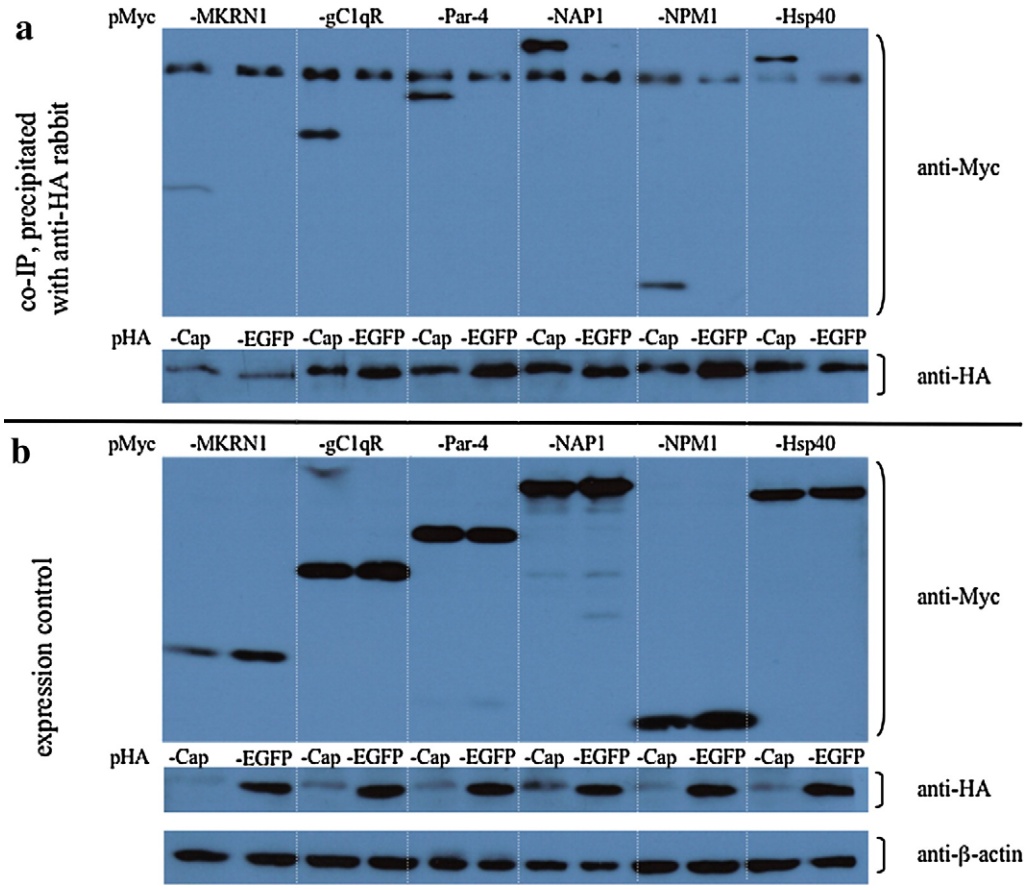

Fig. 3. Co-immunoprecipitation of Cap interacting proteins. HEK 293 Graham cells were co-transfected with plasmids expressing Myc-tagged proteins MKRN1, gC1qR, Par-4, NAP1, NPM1 or Hsp40 and plasmids expressing HA-tagged Cap (PCV1). EGFP was used as a negative control. (a) Extracts were precipitated with rabbit anti-HA antiserum and protein G Sepharose, separated by SDS-PAGE, blotted and detected with mouse anti-Myc and anti-HA antibodies, respectively. (b) Expression control of cell extracts was performed by immunoblotting using mouse anti-Myc and anti-HA antibodies. Detection of β-actin by a mouse anti-β-actin antibody served as internal loading control.

MKRN1 belongs to the E3 ubiquitin ligase family. It has been reported to modulate telomere length homeostasis by ubiquitination and proteasome-mediated degradation of the telomerase subunit hTERT (Kim et al., 2005). We found that solely expressed MKRN1 accumulates in speckle-like structures within the nucleoplasm. These structures were dissolved after co-expression with Cap and both proteins were found in a loose dotty pattern in the nucleoplasm. Additionally, expression of Cap was reduced in the presence of MKRN1 (data not shown) implying that MKRN1 may promote degradation of Cap. Hsp40, like other heat shock proteins, is a chaperone known to prevent aggregation and misfolding of proteins (Hartl, 1996). In parallel to MKRN1, Hsp40 is involved in proteasome-mediated protein degradation, e.g. Hdj1, a human Hsp40 protein, promotes the ubiquitination and degradation of α-synuclein (Fan et al., 2006). Moreover, Hsp40 affects the replication of diverse viruses, it suppresses hepatitis B virus replication through destabilization of viral core and X proteins (Sohn et al., 2006), whereas human immunodeficiency virus type 1 (HIV-1) gene expression and replication is enhanced by interaction of Hsp40 with the HIV-1 Nef protein (Kumar and Mitra, 2005). gC1qR, the complement receptor for C1q and thereby part of the cellular host defence, has been described as a protein which binds to a large number of cellular, viral and bacterial proteins (Ghebrehiwet et al., 2001). Interaction of several viral proteins including the HIV-1 Tat and Rev (Berro et al., 2006, Tange et al., 1996), the rubella capsid protein (Beatch et al., 2005) and the core proteins of adenovirus (Matthews and Russell, 1998) with gC1qR has been reported, promoting viral transcription, replication, capsid assembly and protein transport. Binding of gC1qR on human monocyte-derived dendritic cells with hepatitis C virus (HCV) core protein inhibited toll-like receptor-induced interleukin (IL)-12 production (Waggoner et al., 2007). In a recent report, repression of IL-12 in PCV2-infected blood peripheral mononuclear cells was also observed but attributed to PCV2-induced IL-10 secretion (Kekarainen et al., 2008). Binding of viral proteins to gC1qR often occurs via arginine-clusters of the interacting protein (Bryant et al., 2000, Hall et al., 2002, Wang et al., 1997), a mechanism that might also apply for Cap, since it displays a highly basic N-terminus rich in arginine. Par-4 was first identified as a pro-apoptotic gene product upregulated in prostate cancer cells undergoing apoptosis (Sells et al., 1994) and interacting with the Wilms' tumor suppressor protein WT1 (Johnstone et al., 1996). The pro-apoptotic function of Par-4 is linked to its ability to associate with actin filaments and to recruit pro-apoptotic proteins as DAP-like kinase and Amida to the actin cytoskeleton (Boosen et al., 2005, Vetterkind et al., 2005). Recently, it was described that Par-4 targets the zipper interacting protein kinase to the actin cytoskeleton of differentiated vascular smooth muscle cells and thereby regulates the contractility of these cells (Vetterkind and Morgan, 2008). Since a characteristic symptom of PMWS is the lymphocyte depletion, induction of apoptosis by PCV has been addressed repeatedly. Resendes et al. showed that lymphoid depletion was not related to apoptosis since PMWS-affected pigs had significantly lower rates of apoptosis than the controls (Resendes et al., 2004), while another group concluded that apoptosis was to be attributed to pyrexia rather than effects of PCV on target cells (Krakowka et al., 2004). Therefore, the association of Cap and Par-4 might rather play a role for the transport of viral proteins or particles than in triggering apoptosis. Internalization of PCV2 viral like particles (VLPs) is accomplished by clathrin-mediated endocytosis after

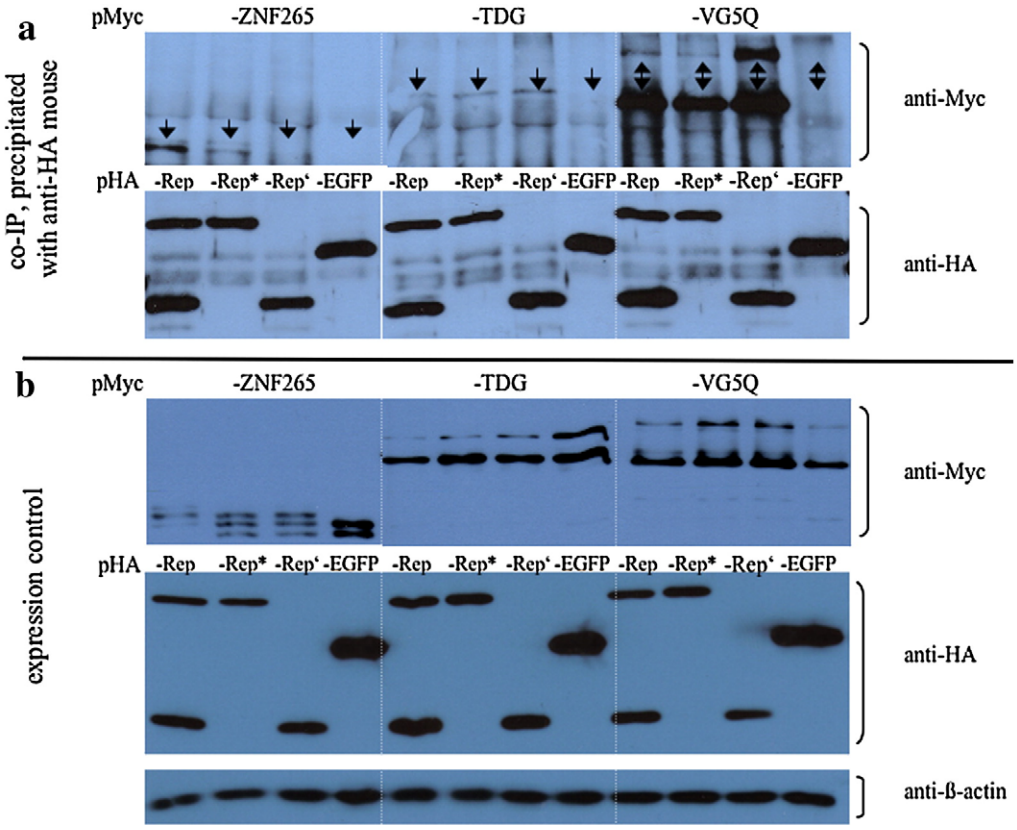

Fig. 4. Co-immunoprecipitation of Rep interacting proteins. HEK 293 Graham cells were co-transfected with pHA–RepPCV1 (expressing HA–Rep and HA–Rep′), pHA–Rep*PCV1 (only HA–Rep), pHA–Rep′PCV1 (only HA–Rep′) or pHA-EGFP in combination with pMyc-ZNF265, pMyc-TDG or pMyc-VG5Q. (a) Extracts were precipitated with rabbit anti-HA antiserum and protein G Sepharose and analyzed by immunoblotting with mouse anti-Myc and anti-HA antibodies. (b) Protein expression in cell lysates was examined by immunoblotting using Myc- and HA-specific mouse antibodies. Arrowheads indicate position of interacting proteins. Detection of β-actin by a mouse anti-β-actin antibody served as internal loading control.

attachment of PCV2 to glycosaminoglycans (Misinzo et al., 2005). Later on, VLPs were observed in the early endosomes and lysosomes (Misinzo et al., 2008). Endosome trafficking is a highly complex cellular process, whose regulation involves many different proteins, which serve in the selection of cargo or act like Par-4 as scaffolds for effector proteins linking them to the actin cytoskeleton (Murray and Wolkoff, 2003). It may be speculated that PCV particles are recruited by Par-4 to actin microfilaments and that the complex may subsequently be transported into the nucleus of infected cells. The Cap interacting proteins NAP1 and NPM1 are also involved in intracellular transport processes, since both serve as chaperones and bind to histones and other basic proteins. NAP1 was initially identified as a chromatin-assembly factor (Kawase et al., 1996) moreover, it shuttles histones H2A/H2B from the cytoplasm to the nucleus (Mosammaparast et al., 2002), controls mitotic events by interaction with cyclins (Altman and Kellogg, 1997) and regulates transcription by direct interaction with the transcriptional coactivators p300/CBP (Asahara et al., 2002). Binding of NAP1 to viral basic proteins as HIV-1 Tat and papillomavirus E2 protein has been described (Vardabasso et al., 2008; Rehtanz et al., 2004), whereby it promotes viral transcription. Previous reports have suggested both nuclear and cytoplasmic pools of NAP1, a GFP-fused NAP1 was predominantly cytoplasmic and appeared to be excluded from the nucleus (Mosammaparast et al., 2002), as was corroborated by our results. Interaction of Cap and NAP1 could be beneficial for several aspects of PCV infection, (i) the transport of Cap or PCV particles, (ii) the assembly of viral particles and the encapsidation of viral DNA or (iii) the regulation of transcription, as described for other viral proteins. NPM1 is a nucleolar phosphoprotein involved in ribosome biogenesis (Savkur and Olson, 1998), protein transport (Szebeni et al., 1995), centrosome duplication (Okuda et al., 2000) and has chaperone protein characteristics (Szebeni and Olson, 1999). NPM1 interaction is often mediated by nuclear localisation signals of the interacting partner (Szebeni et al., 1995, 1997). In an earlier study we have demonstrated that transiently expressed Cap is restricted to the nucleoli, while during infection it was localised first in the nucleoli of porcine kidney cells and later on seen in the nucleoplasm as well as in the cytoplasm (Finsterbusch et al., 2005). Co-expression of Cap with NPM1 in HEK 293 Graham cells resulted in a pronounced nucleolar accumulation of Cap. Nucleolar localisation of viral proteins induced by NPM1 has been described for Adeno-associated virus (AAV) Rep68 and Cap proteins, Rev and Tat proteins of HIV, the core proteins of HCV and japanese encephalitis virus (Bevington et al., 2007, Fankhauser et al., 1991, Mai et al., 2006, Marasco et al., 1994, Tsuda et al., 2006). In the case of AAV it is speculated that NPM1 may promote the assembly of viral capsids in subnucleolar areas (Bevington et al., 2007), supporting the hypothesis that interaction with NPM1 might serve for a similar purpose in case of PCV.

Three porcine proteins homologous to ZNF265, TDG and VG5Q interacted with the Rep protein of PCV. By western blotting, we observed four bands for ZNF265 and two for TDG and VG5Q. This suggests that several isoforms differing in their post-translational modification, e.g. phosphorylation, may bind to the *rep* gene products and thereby refining the regulation of Rep-mediated processes. While VG5Q and TDG interacted also with the Rep′ proteins of both PCV, ZNF265 bound only the full-length Rep protein, indicating that the interaction is putatively mediated by the C-terminal sequence differing between Rep and Rep′. ZNF265 is localised within active transcriptional compartments as an alternative component of the spliceosome, which can replace the essential constituent SF2/ASF

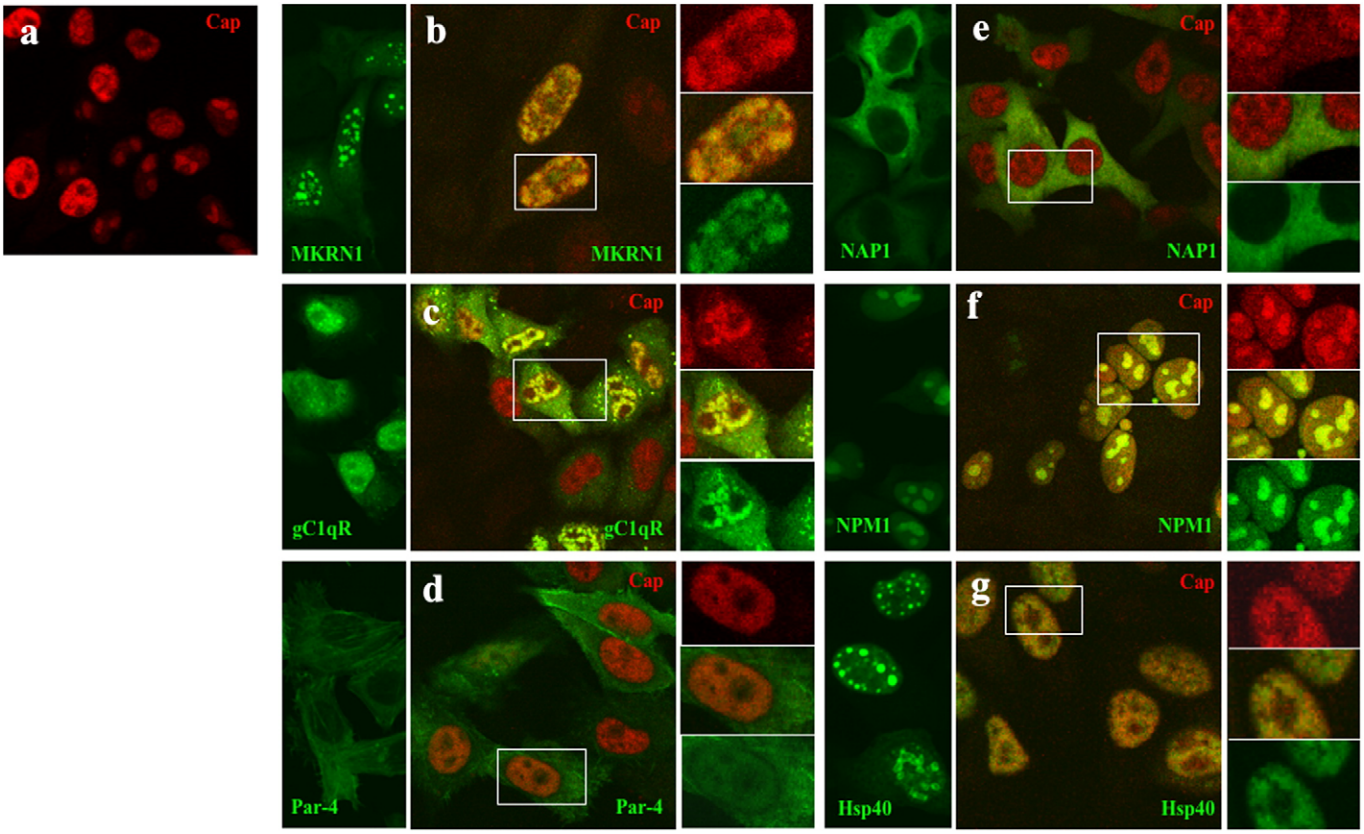

Fig. 5. Intracellular distribution of Cap and its interacting proteins. HEK 293 Graham cells grown on coverslips were co-transfected with pHA-CapPCV1 in combination with pMyc-MKRN1, pMyc-gC1qR, pMyc-Par-4, pMyc-NAP1, pMyc-NPM1 or pMyc-Hsp40. As a control, cells were transfected with only one plasmid. 48 h post transfection cells were fixed and subjected to IFA. Solely expressed HA-Cap was detected with Alexa Fluor 594-labelled anti-HA antibody (a, red), whereas solely expressed Myc-tagged interacting proteins were labelled with monoclonal anti-Myc FITC conjugate (b–g left image, green). After co-transfection cells were double labelled and each fluorochrome was recorded in independent scans. Overlay images are shown in b–g (middle) and areas marked by a rectangle are given enlarged on the right side as insets with red signal indicative of Cap (upper), green signal for the interacting protein (bottom) and overlay signal (middle). Pictures were taken by confocal laserscanning microscopy.

(Adams et al., 2001). Thus, interaction of Rep and ZNF265 may influence transcription and alternative splicing, since co-expression of ZNF265 with Rep/Rep′ in HEK 293 cells led to their accumulation in condensed nucleoplasmatic punctiform structures, resembling transcriptional compartments. VG5Q and TDG could also be linked to transcriptional regulation. TDG was initially described as a DNA repair protein (Neddermann et al., 1996), but several studies have implied a role of TDG in the regulation of gene expression, since TDG interacts with transcriptional activators and coactivators as CBP/p300 (Tini et al., 2002), estrogen receptor alpha (Chen et al., 2003) and the thyroid transcription factor-1 TTF1 (Missero et al., 2001). VG5Q is an angiogenic factor expressed in bloodvessels. A genetic defect of VG5Q in patients leads to the vascular disease Klippel–Trenaunay syndrome characterised by malformations of capillary, venous and lymphatic vessels (Tian et al., 2004).

In this study, we have identified nine porcine proteins interacting with the two major gene products of PCV, which can be associated with several aspects of viral replication such as transcriptional regulation and intracellular transport processes. Future studies will map the domains responsible for the interaction and study the impact of these interactions upon virus infection.

Materials and methods

Plasmid construction

The *rep* and *cap* genes of both PCV genotypes were cloned into vector pGBKT7 (Clontech). The resultant plasmids express the viral proteins fused to the Gal4 DNA binding domain, which were used as bait proteins in yeast two-hybrid screens. DNA fragments were amplified via standard PCR techniques using the High Fidelity PCR System (Merck Biosciences) and restriction site-tagged primers (Table 2). Cycling parameters were 94 °C, 2 min, 30×(94 °C, 15 s; 60 °C, 30 s; 72 °C, 45 s), 72 °C, 7 min. PCR products were inserted via the newly introduced restriction sites into pGBKT7 and sequenced with the pGBKT7-specific primer 891 and the reverse PCR primer (Table 2). All cloning steps were performed according to standard techniques (Sambrook and Russell, 2001). The *rep* gene of PCV1 (strain GER1, GenBank Y09921) was amplified from pORF4A (Mankertz et al., 1998) with primer pair 621/167. The PCR fragment was cloned into EcoRI and BamHI restricted plasmid pGBKT7 and plasmid pBD-RepPCV1 was obtained. Similarly, the following templates and primer pairs were used for plasmid construction: plasmid pBD-CapPCV1 (strain GER1, Y09921), primer pair 141/622, template pSK144 (Finsterbusch et al., 2005); pBD-Rep′PCV1 (GER1), 621/167, pAM4 (Mankertz and Hillenbrand, 2001); pBD-Rep*PCV1 (GER1), 621/167, pAM9 (Mankertz and Hillenbrand, 2001); pBD-RepPCV2 (FRA3, AF201311), 814/815, pSVL-RepPCV2 (Mankertz et al., 2003) and pBD-CapPCV2 (GER3, AF201307), 816/817, pSVL-CapPCV2 (Mankertz et al., 2003). The *rep* and *cap* genes were subcloned via SfiI and NotI restriction sites into pCMV-HA (Clontech) for co-immunoprecipitation (co-IP) and immunofluorescence analysis (IFA). The resultant constructs were designated pHA–RepPCV1, pHA–Rep′PCV1, pHA–Rep*PCV1, pHA-CapPCV1, pHA–RepPCV2 and pHA-CapPCV2. As negative control in co-IP assays construct pHA-EGFP was used. The coding sequence of the enhanced green fluorescent protein (EGFP) was subcloned from pEGFP-N3 (Clontech) into pCMV-HA via XhoI and NotI restriction sites. Cellular interaction partners of

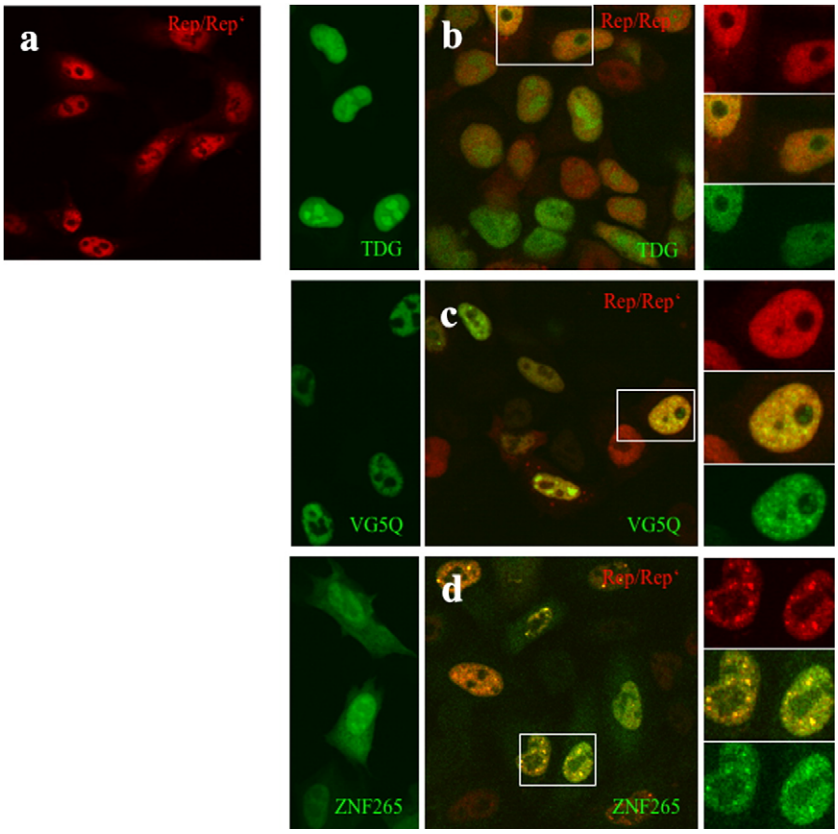

Fig. 6. Intracellular distribution of Rep and Rep interacting proteins. Subcellular localisation of HA–RepPCV1, Myc-ZNF265, Myc-TDG and Myc-VG5Q was analyzed in HEK 293 Graham cells 48 h post transfection with the respective plasmid(s). Cells were fixed and subjected to immunofluorescence analysis. Solely expressed HA–Rep was detected with Alexa Fluor 594-labelled anti-HA antibody (a, red), whereas solely expressed Myc-tagged interacting proteins were marked with monoclonal anti-Myc FITC conjugate (b–d left image, green). Double staining of co-transfected cells is shown in the overlay of both signals (b–d middle image). Rectangle-marked areas are shown enlarged on the right side as insets with red signal for Rep (upper), green signal indicating the interacting protein (bottom) and overlay signal (middle). Pictures were taken by confocal laserscanning microscopy.

Rep and Cap were subcloned into plasmid pCMV-Myc (Clontech) via SalI and NotI restriction sites for further analysis by co-IP and IFA. All pCMV constructs were sequenced with the plasmid specific forward and reverse primers 891 and 892 (Table 2).

Yeast two-hybrid screen

The Matchmaker Gal4 Two-Hybrid system 3 (Clontech) was used according to the manufacturer's instructions. The complete coding sequences of *rep* and *cap* of both genotypes were cloned into pGBKT7 (Clontech) and used as bait to screen a cDNA library prepared from the spleen of a healthy, non-PMWS affected pig in pGADT7 (RZPD, Heidelberg). Yeast strain AH109 (Clontech) was transformed simultaneously with bait and prey (pGADT7 cDNA library) plasmids and selected on quadruple dropout (QDO) plates lacking adenine, histidine, tryptophan and leucine for one week. Yeast colonies were restreaked on QDO/X plates containing 5-bromo-4-chloro-3-indoyl-α-D-galactopyranoside (X-α-Gal, Clontech) to test α-galactosidase expression. Blue colonies were screened five times for growth on QDO/X plates to rescue additional library plasmids and to eliminate false positives. For plasmid isolation, colonies were grown in 1.5 ml double dropout (DDO) medium lacking leucine and tryptophan for 48 h at 30 °C and 200 rpm. Yeast cultures were precipitated and resuspended in 10 µl Lyticase (Sigma-Aldrich) and incubated for 1 h at 37 °C and 250 rpm. 10 µl of 20% SDS was added to samples and plasmid DNA was isolated after freeze-thawing using the Qiagen DNA mini kit (Qiagen). *Escherichia coli* DH5α cells were transformed with DNA samples and grown on LB agar plates with 70 µg/ml ampicillin. Prey plasmids were recovered for DNA sequencing and a BLAST analysis was performed using the NCBI BLASTP program. To confirm results, the respective bait and prey plasmids were co-transformed into yeast strain AH109 and selected on DDO and QDO/X plates. Since the encoded putative interaction partner may possess an intrinsic DNA binding activity, autoactivation was tested. Therefore, the plasmids encoding the interaction partners were co-transformed with the vector pGBKT7 into yeast strain AH109 and tested for absence of growth on QDO/X plates. Optional 4 mM 3-amino-1,2,4-triazole (3-AT) was added to QDO/X plates to repress autoactivation.

Table 2
Primers used in this study

Primer	Sequence[a]	Restriction site
141	5′ CGGGATCCATTTTTATTTATTTAGAGGGTCTTTTAGGA 3′	BamH1
167	5′ CGGGATCTTACGATGTGATAACAAAAAAGACTCAGT 3′	BamHI
621	5′ GGAATTCCCAAGCAAGAAAAGCGGC 3′	EcoRI
622	5′ GGAATTCACGTGGCCAAGGAGGCGTTA 3′	EcoRI
814	5′ CGGAATTCCCCAGCAAGAAGAATGGAAG 3′	EcoRI
815	5′ CGGGATCCAACCATTACGAAGTGATAAAAAAGA 3′	BamHI
816	5′ AACTGCAGTTTAGGGTTTAAGTGGGGGGTCTTTAA 3′	PstI
817	5′ CGGGATCCTGACGTATCCAAGGAGGCGTT 3′	BamHI
891	5′ TAATACGACTCACTATAGGGC 3′	–
892	5′ AGATGGTGCACGATGCACAG 3′	–
1219	5′ GGATGTTGCCTTTACTTCTAG 3′	–
1042	5′ ATCTTATCATGTCTGGATCC 3′	–

[a] Restriction sites are underlined.

Immunoprecipitation and immunoblot analysis

HEK 293 Graham cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum,

100 U/ml penicillin and 100 μg/ml streptomycin. Transfection was performed with Effectene transfection reagent (Qiagen). Subconfluent cells in 6-well plates were simultaneously transfected with 200 ng of pCMV-Myc based constructs expressing Myc-tagged cellular proteins and 200 ng of the respective pCMV-HA constructs expressing HA-tagged viral proteins or EGFP as control. 48 h post transfection cells were washed with PBS and incubated on ice for 1 h in lysis buffer (20 mM Tris/HCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1% NP40) containing one tablet complete mini protease-inhibitor (Roche Diagnostics) and 100 μl phosphatase inhibitor cocktail I/II (Sigma) per 10 ml. Soluble proteins were subjected to immunoprecipitation with rabbit anti-HA antiserum or mouse monoclonal anti-HA antibody (Covance) at 4 °C for 2 h. After addition of 20 μl protein G Sepharose for 2 h at 4 °C the adsorbates were washed four times with ice-cold lysis buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots of total cell lysates (50 μl) were included as input controls. After SDS-PAGE, proteins were blotted onto Immobilon-P transfer membranes (Millipore). The membranes were blocked with 5% milk powder in PBS and 0.05% Tween 20 (PBS-T) for 1.5 h at room temperature and probed with primary antibody in PBS-T containing 0.5% milk powder for 2 h at room temperature. Membranes were washed three times with PBS-T and treated with secondary antibody diluted in PBS-T containing 0.5% milk powder for 1 h at room temperature. The membranes were washed again three times and antigen–antibody complexes were visualised by chemiluminescence using the ECL–Western blotting detection system (Amersham). Immunoblot assays were performed with the following antibodies: monoclonal anti-HA (1 μg/ml, Covance), monoclonal anti-Myc (1 μg/ml, Covance) or monoclonal anti-β-actin (0.2 μg/ml, Sigma) antibody in conjunction with horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum (0.1 μg/ml, Dianova) and rabbit anti-HA (1 μg/ml, Covance) or rabbit anti-Myc (1 μg/ml, Covance) antisera in conjunction with HRP-conjugated goat anti-rabbit antiserum (0.1 μg/ml, Dianova).

Immunofluorescence analysis

HEK 293 Graham cells were grown on coverslips in 24-well plates. For each transfection, a total of 200 ng plasmid DNA was used. Cells were simultaneously transfected with the respective combination of pCMV-HA based (PCV-specific) and pCMV-Myc based plasmid (host protein specific) or separately with only one plasmid. 48 h post transfection cells were washed with PBS and fixed with 4% formaldehyde in PBS for 10 min at room temperature, afterwards cells were permeabilised with 0.1% Triton X-100 in PBS for 10 min. Cells were blocked for 1 h with blocking solution (PBS, 0.05% Tween 20, 1% BSA) and incubated with the respective antibody for 1 h at room temperature. HA-tagged proteins were stained with 1 μg/ml monoclonal Alexa Fluor 594 labelled anti-HA antibody (Covance) and Myc-tagged proteins were stained with 5 μg/ml monoclonal anti-Myc FITC conjugate (Sigma-Aldrich). Coverslips were washed three times with PBS-T (0.05% v/v), mounted in Mowiol (Merck) and examined with a confocal laserscanning microscope (cLSM 510, Zeiss).

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