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The highly conserved orthopoxvirus 68k ankyrin-like protein is part of a cellular SCF ubiquitin ligase complex

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Abstract

The 68k ankyrin-like protein (68k-ank) of unknown function is highly conserved among orthopoxviruses and contains ankyrin repeats and an F-box-like domain. We performed a yeast-two-hybrid screen with 68k-ank to find interacting proteins. From a human and a murine cDNA library, 99% of the interaction partners were S-phase kinase-associated protein 1a (Skp1a), a part of the SCF ubiquitin ligase complex. 68k-ank co-immunoprecipitated with components of the endogenous, mammalian SCF ubiquitin ligase. This interaction was F-box domain dependent and could also be observed in infected cells, indicating that SCF complex formation might be important for the viral life cycle.
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Keywords: Poxvirus; 68k ankyrin-like protein; F-box; SCF ubiquitin ligase complex

Introduction

Orthopoxviruses are large DNA viruses well known to replicate entirely within the cytoplasm of infected cells and to encode over hundred different viral proteins. Many of these proteins are enzymes and co-factors enabling the virus to autonomously replicate and express its genetic information leading to the synthesis of translatable mRNAs with typical eukaryotic features (Moss, 2007). In addition, poxviruses employ numerous proteins to regulate their interaction with the host cell for interference with antiviral defense mechanisms and to create a favorable environment for viral replication. These genes determine the pathogenicity and host range of poxviruses, which can be very diverse. For example, the prototypical orthopoxvirus, vaccinia virus (VACV), has a broad host range, while that of variola virus (VARV), the causative agent of smallpox, is narrow. Previous studies have been able to allocate functions to many proteins encoded by VACV by determining their interactions with cellular factors (Seet et al., 2003). Well described are for example, the extensively studied E3 protein, which binds dsRNA to prevent activation of PKR and OAS (Chang et al., 1992; Ludwig et al., 2006), or proteins that interfere with the induction of apoptosis like N1L, which binds and sequesters proapoptotic Bcl-2 proteins (Cooray et al., 2007). One class of genes frequently present in poxviral genomes and involved in host range function encodes proteins containing ankyrin repeat (ANK) motifs. This motif is a 33-residue sequence that facilitates protein–protein interactions. It can be found in a variety of cellular proteins with functions including, for example, cell-to-cell signaling, cytoskeleton integrity, transcription and cell-cycle regulation (Mosavi et al., 2004). We focused here on the orthopoxviral 68k ankyrin-like protein (68k-ank), which is highly conserved and shows little sequence variation between the highly pathogenic variola virus and the avirulent modified vaccinia virus Ankara (MVA) (Fig. 1). Interestingly, no other genes encoding proteins with ANKs are conserved in the MVA genome (Antoine et al., 1998). The 68k-ank gene also encodes a C-terminal F-box-like domain, which is found in cellular adapter proteins involved in the ubiquitin-dependent proteolytic pathway. To obtain insights into the functional role of 68k-ank, we attempted to identify cellular interaction partners of the protein. The presence of ANKs and an F-box-like domain strongly indicates protein–protein interactions, probably with cellular proteins. Furthermore, the fact that

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A

Protein sequence	identity / similarity to MVA 68k-ank	
VACV [B18R]	98%	99%
CPXV [B16R]	98%	99%
HSPV [194]	98%	99%
RPXV [178]	97%	99%
VARV [B16R]	94%	98%
ECTV [C11R]	93%	97%

B

Fig. 1. Functional domains and conservation of 68k ankyrin-like protein orthopoxvirus orthologs. (A) Comparison of MVA 68k-ank encoded by MVA ORF 186R with 68k-ank sequences from VACV vaccinia virus strain Copenhagen; CPXV cowpox virus strain GRI90; HSPV horsepox virus strain MNR-76; RPXV rabbitpox virus strain Utrecht; VARV variola major virus strain Bangladesh 1975; and ECTV ectromelia virus strain Moscow [respective ORFs according to http://www.poxvirus.org/]. (B) Schematic diagram of 68k-ank. Striped: ankyrin repeat (ANK); checkered: a newly established PRANC (Pox protein Repeats of Ankyrin C-terminal) domain belonging to the F-box motif family (motif analysis and diagram adapted from Pfam database, http://pfam.sanger.ac.uk/).

68k-ank is highly conserved among orthopoxviruses implies an important role in the viral life cycle. We performed an extensive yeast-two-hybrid (Y2H) screen to find cellular interaction partners of 68k-ank using a human and a mouse cDNA library.

Results

The MVA-encoded 68k ankyrin-like protein is highly conserved among orthopoxviruses

The highly attenuated and chicken cell adapted MVA lacks about 30 kb coding sequence of its genome being most likely responsible for the replication-defectiveness of MVA in most cells of mammalian origin (Meyer et al., 1991; Sutter and Moss, 1992). This phenotype indicates the probable importance of genes that are conserved in the MVA genome as essential factors in the viral life cycle. With particular attention to proteins likely involved in the regulation of VACV host interaction, we compared different orthopoxvirus genomes (see http://www.poxvirus.org/viruses.asp) and looked for highly conserved proteins being encoded by MVA. Interestingly, we found that the only ANK motif-containing gene in the MVA genome, encoding the 68k ankyrin-like protein (68k-ank), is extremely well preserved among orthopoxviruses. It shares 98% sequence identity and 99% similarity with orthologs from VACV, horsepox virus (HSPV) and cowpox virus (CPXV) (Fig. 1A). It is expressed during early time points after infection of chicken embryo fibroblasts with MVA, as well as with ectromelia virus (ECTV) and VACV strain Copenhagen (data not shown). Conservation with the rabbitpox virus (RPXV) and the VARV orthologs is only slightly reduced, with 97% and 94% identity, respectively. There is hardly any variation in the variola orthologs of MVA186R among the approximately 50 sequenced isolates of VARV. The ECTV ortholog has an extended N-terminus and a calculated molecular weight of about 70 kDa, but still 93% sequence identity and 97% similarity (Fig. 1A). According to the Pfam database (http://pfam.sanger.ac.uk/), all 68k-ank proteins have four ankyrin repeats (ANK; entry PF00023) and a so-called PRANC domain (Pox protein Repeats of Ankyrin C-terminal; entry PF09372 assigned after Mercer et al. (2005); Fig. 1B). The PRANC domain is closely related to the F-box domain, which mediates protein–protein interactions and is found in proteins that target other proteins to E3 ubiquitin ligase complexes for polyubiquitinylation and subsequent degradation via the proteasomal pathway.

Yeast-two-hybrid (Y2H) screen reveals Skp1a as a cellular interaction partner of the 68k-ank protein

The presence of protein–protein interaction motifs in the highly conserved 68k-ank protein encouraged us to perform a Y2H screen to identify cellular interaction partners. We selected the 68k-ank of MVA (ORF 186R) as the representative ortholog. Its yeast codon-optimized sequence was cloned into the pGBT-9 bait vector and analyzed for interactions with a prey library. The screen was performed with two different mammalian cDNA libraries, either generated from human kidney tissue or murine tissue of 11-day-old embryos, to verify the relevance of a potential interaction. Amazingly, the results from the Y2H screen were very clear. A total of 39 interacting fragments from the human and 120 from the mouse cDNA library was analyzed by PCR sequencing, of which 38 and 119, respectively, yielded the sequence of the S-phase kinase-associated protein 1a (Skp1a). Additionally, two other genes were identified only once. The RIKEN cDNA 0610009D07 gene, also named splicing factor 3B was isolated from the mouse library and the TCTA (T-cell leukemia translocation altered) gene from the human library. TCTA is ubiquitously expressed with highest levels in kidney, which might explain its appearance in the human kidney tissue cDNA library screen. In summary, 99% of the analyzed positive interactions could be attributed to Skp1a, indicating a high significance and conservation of this interaction.

Skp1a is part of the cellular SCF (Skp1a-Cullin-1-F-box protein) ubiquitin ligase complex and usually serves as an adaptor protein between an F-box protein (FBP) and the E3 ubiquitin ligase Cullin-1 (Cul-1). FBPs are the substrate-recognition components of E3 ubiquitin ligases and confer substrate specificity to these complexes (Fig. 2B). They perform this task because, on the one hand, they are connected to the E3 complex via their F-box, and on the other hand, they have additional protein–protein interaction motifs, WD or leucine-rich repeats (LRR), through which they bind proteins that are ubiquitinylated by the E3 ligase and subsequently degraded via the proteasomal pathway (Cardozo and Pagano, 2004). A very well characterized Skp1a-interacting FBP is the S-phase kinase-associated protein 2 (Skp2), which promotes the polyubiquitinylation of the cell-cycle regulator protein $p27^{KIP}$ by the SCF complex (Carrano et al., 1999). By comparing the F-box domain from Skp2 with the F-box-like domains of the different 68k-ank orthologs (regions were assigned according to

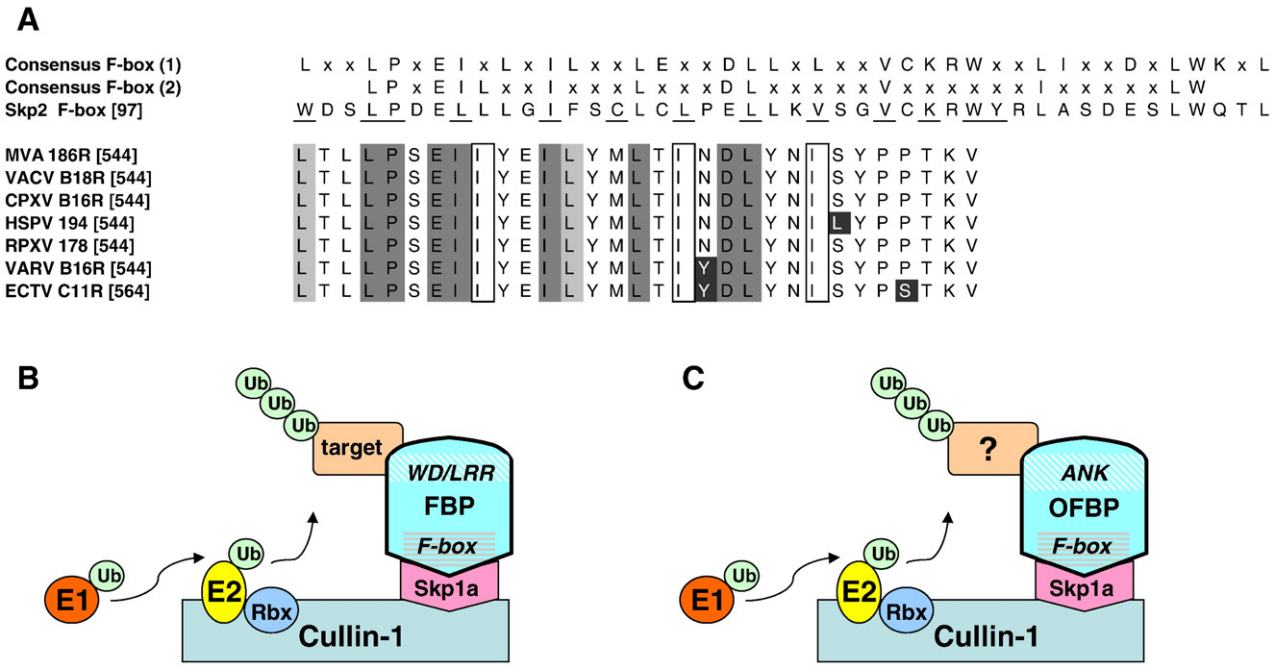

Fig. 2. The 68k ankyrin-like protein is part of a SCF ubiquitin ligase complex. (A) Comparison of the F-box-like domains of the 68k-ank orthologs with the F-box domain from Skp2 and consensus F-box sequences [first amino acid]. The F-box consensus sequences (1) and (2) were adapted from Schulman et al. (2000) and Kim et al. (2002), respectively. Underlined residues indicate Skp1a contact points in Skp2 as reported by Schulman et al. (2000). Light gray = identical to one consensus sequence; dark gray = identical to both consensus sequences; framed = similar to one consensus sequence or Skp2; black = variation within 68k-ank sequences. (B) Schematic overview of the cellular SCF complex with a cellular F-box protein (FBP) as target-recognition component. (C) Model for the 68k-ank orthopoxvirus F-box protein (OFBP). OFBP associates with the E3 ubiquitin ligase complex in the same manner as the cellular FBP. Target proteins are unknown, as indicated by the question mark.

the ExPASy PROSITE database, http://expasy.org/prosite/), we noted that the sequences share a common motif previously described as F-box consensus sequences (Kim et al., 2002; Schulman et al., 2000) Here most residues are identical to one or both consensus sequences or are similar, such as isoleucine replacing leucine (Fig. 2A). In addition, eight of the thirteen Skp2 residues that have been identified as contact points with Skp1a (underlined in Fig. 2A) are also present in the consensus sequence of 68k-ank, which further support the Y2H data.

The 68k ankyrin-like protein is part of a SCF ubiquitin ligase complex

Although the Y2H screen was very clear, it cannot be assumed that an interaction found in the yeast system can also be detected in a mammalian cell-based assay. To address this question, we cloned the yeast codon-optimized sequence of the MVA 68k-ank open reading frame into the mammalian expression vector pFLAG-CMV-2, thereby introducing an N-terminal Flag tag. The resulting plasmid, pFLAG-CMV-2-68k, was transfected into 293T cells. Two days after transfection, immunoprecipitations were performed from cell lysates with an antibody directed against either Skp1a or the Flag tag, and were analyzed by western blotting. Using the anti-Flag tag antibody, we were able to immunoprecipitate the 68k-ank protein (Fig. 3A, lane 6a) and detected the co-immunoprecipitation of endogenous Skp1a and Cul-1 (Fig. 3A, lanes 6b, c). Precipitating Skp1a allowed the co-immunoprecipitation of 68k-ank (Fig. 3A, lane 9a) and again Cul-1 (Fig. 3A, lane 9c). A weak interaction of Skp1a and Cul-1 could be detected in untransfected cells (Fig. 3A, lane 7c); however, the presence of 68k-ank enhanced this complex formation (Fig. 3A, lane 9c). Both immunoprecipitations confirmed the interaction of 68k-ank with components of the SCF ubiquitin ligase complex in mammalian cells.

Additionally, we constructed a truncated version of the 68k-ank gene deleting the F-box-like domain, encoding an N-terminally Flag-tagged version of 68k-ank containing amino acids 1–397. We used this construct to elucidate if 68k-ank binds directly to Skp1a, which would be expected from the Y2H screens or indirectly via Cul-1. Again co-immunoprecipitation analysis was performed after transfection of the construct into 293T cells. Immunoprecipitation with an antibody directed against the Flag tag allowed the immunoprecipitation of the truncated version (Fig. 3A, lane 5a). Although the amount of protein precipitated was comparable with the full-length 68k-ank version (Fig. 3A, lanes 5a and 6a) the data clearly show that deletion of the F-box-like domain also eliminated Skp1a binding (Fig. 3A, lane 5b). In addition, Cul-1 did not bind to the truncated 68k-ank protein (Fig. 3A, lane 5c), indicating that the F-box-like domain is the site of interaction with Skp1a and that Cul-1 interacts with the complex via Skp1a.

After showing the 68k-ank protein-SCF ubiquitin ligase complex formation in mammalian cells, we were interested to confirm this interaction with virus-expressed 68k-ank. We therefore performed the immunoprecipitations in 293T cells infected with MVA for 4 h at an MOI of 20. Since our polyclonal antiserum directed against the 68k-ank protein did not work in immunoprecipitations, we precipitated Skp1a, which allowed the co-immunoprecipitation of 68k-ank (Fig. 3B, lane 4a). Also during

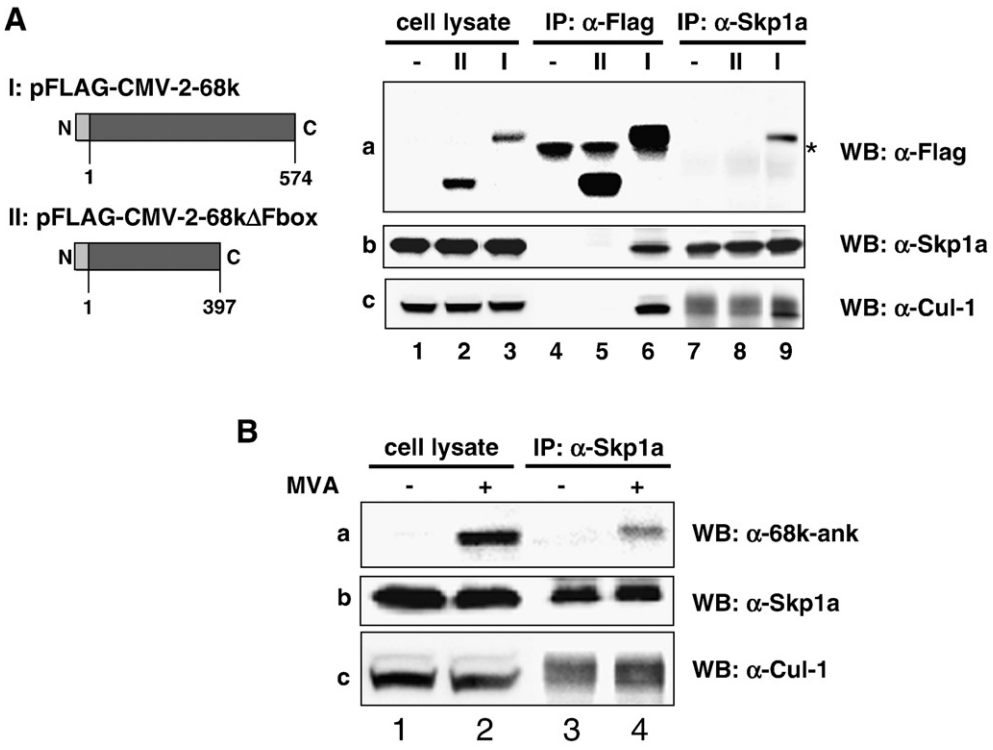

Fig. 3. (A) The 68k ankyrin-like protein interacts with endogenous Skp1a and Cul-1 via its F-box-like domain. Human 293T cells were transfected with pFLAG-CMV-2-68k (I) or pFLAG-CMV-2-68kΔFbox (II). (Schematic overview of proteins encoded by the transfected plasmids is shown on the left with the light area indicating the N-terminal FLAG peptide). Precipitations were carried out with an antibody directed against either Skp1a or the Flag tag (IP). Co-immunoprecipitation of the SCF ubiquitin ligase complexes was analyzed by western blot using the specific antibodies indicated in panels a, b and c. Specificity of the interactions was controlled by immunoprecipitation with untransfected cells (lanes 4 and 7). Asterisk indicates binding of the secondary anti-mouse antibody to heavy chain molecules of anti-Flag antibodies used for immunoprecipitations. (B) The 68k ankyrin-like protein interacts with endogenous Skp1a in MVA infected cells. Human 293T cells were infected with MVA at an MOI of 20 for 4 h. Precipitations were carried out with an antibody directed against Skp1a (IP). Co-immunoprecipitation of the SCF ubiquitin ligase components with viral 68k-ank was analyzed by western blot using the specific antibodies indicated in panels a, b and c. Specificity of the interaction was controlled by immunoprecipitation with uninfected cells (lane 3).

infection, the viral 68k-ank protein interacts with Skp1a and endogenous Cul-1 is also present in the co-immunoprecipitation (Fig. 3B, lane 4c). These interactions indicate that this SCF complex formation is likely to have a functional role during orthopoxviral infections.

Discussion

We have shown here that the highly conserved orthopoxviral 68k-ank protein is part of a SCF ubiquitin ligase complex. A previously published comparative sequence analysis of poxviral proteins that comprise ANKs and F-box-like motifs already indicated an interaction with cellular ubiquitin ligases (Mercer et al., 2005), which is now confirmed by experimental data. Within the SCF complex, the core component Cul-1 functions as a molecular scaffold that simultaneously interacts at the N-terminus with the adaptor protein Skp1a and with a RING-finger protein, Rbx-1, at the C-terminus (Fig. 2B). In addition, a respective E2 or ubiquitin-conjugating enzyme (UBC), which delivers ubiquitin to the E3 ligase, associates with the C-terminus. The specificity of ubiquitinylation is defined by the FBP, and selective substrate binding by FBPs is achieved by protein–protein interaction motifs, mostly WD repeats or LRR (Fig. 2B). The orthopoxviral FBP (OFBP) 68k-ank, however, does not contain WD repeats or LRRs, but rather ANKs that facilitate protein–protein interactions. We therefore propose that the 68k-ank OFBP functions in the same manner as cellular FBPs and guides the cellular degradation machinery to its target (Fig. 2C). The major challenge now is to identify the OFBP target. Although both cellular and viral proteins might be targeted for ubiquitinylation, we initially favored a viral protein. This is because, although the presence of ANKs strongly indicated additional protein–protein interactions, only Skp1a was found to interact with 68k-ank in the Y2H screen, which might suggest that 68k-ank has a viral target not present in the screen. In a previously described genome-wide analysis of vaccinia virus protein–protein interactions, 68k-ank has not been identified as interaction partner with viral proteins (McCraith et al., 2000). However, for our screen we used a codon-usage optimized 68k-ank gene, which gives rise to high level yeast protein expression and only this modification allowed protein expression in eukaryotic cells (data not shown). This strategy was different from the one followed by McCraith et al. (2000) and might explain why they did not find the 68k-ank protein in their global screen. Yet, failure to identify interaction partners of 68k-ank might also reflect a low abundance of these proteins in yeast or the requirement of posttranslational modifications to allow interaction, which might be caused in response to vaccinia virus infection.

Many viruses have been reported to have evolved different strategies to utilize the ubiquitin-proteasome pathway for their own benefit by targeting cellular factors for degradation.

Herpesviruses, in particular, have been reported to encode viral E3 ligases that primarily target mediators of interferon (IFN) signaling for degradation. The RTA protein of Kaposi's sarcoma-associated herpesvirus (KSHV), for example, is able to circumvent IFN-α signaling by directing ubiquitinylation of IRF-7, and herpes simplex ICP0 protein has also been shown to target various factors associated with IFN signaling. Furthermore, ICP0 leads to degradation of UbcH3, an E2 ubiquitin-conjugating enzyme, and thereby prevents its association with respective SCF complexes, which is required to regulate cellular protein turnover. Human herpesvirus-8 encodes two E3 ligases, K3 and K5, that target MHC-I molecules for degradation. For review see Chen and Gerlier (2006) and Gao and Luo (2006).

No interactions with the SCF complex have been previously described for orthopoxviruses. Nevertheless, some of these viruses encode viral E3 ligases to control the abundance of cellular proteins. For example, p28 of ECTV is a single subunit RING finger-type E3 ligase (Huang et al., 2004; Nerenberg et al., 2005). It is also highly conserved among orthopoxviruses, however the p28 gene products are either truncated or interrupted in culture-adapted VACV strains like WR, COP or MVA. In contrast to this single subunit E3 ligase, which recognizes the substrate directly, the 68k-ank protein has an F-box which facilitates its binding to the multiprotein complex SCF, which contains small RING-finger proteins as ubiquitin ligases (Fig. 2B). Myxoma virus, which belongs to a different genus of poxviruses, encodes the virulence factor M-T5, which has been shown to interact with Cullin-1 and direct $p27^{KIP}$ polyubiquitinylation. This arrests the cell cycle, providing a favorable environment for viral replication (Johnston et al., 2005). However, this study did not demonstrate an interaction with Skp1a nor discuss that M-T5 is the target-recognition subunit of the E3 ubiquitin ligase complex.

We suggest that, due to the high level of conservation, the formation of a Skp1a-Cul-1-68k-ank ubiquitin ligase applies to all highly conserved 68k-ank orthologs and that this high conservation indicates relevance for orthopoxviruses. Further studies should aim at identifying the potential target protein that interacts with the 68k-ank protein and becomes degraded. Deleting the 68k-ank orthologs from the respective orthopoxvirus genomes will allow investigation of the functional relevance of this new interaction of an orthopoxviral protein with cellular components for the viral life cycle and/or virulence.

Materials and methods

Plasmids and antibodies

The full-length nucleotide sequence of 68k-ank, ORF 186R of MVA, was synthesized with yeast-based codon-optimization (Geneart, Regensburg, Germany) and cloned into the Y2H bait vector pGBT9 (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). The resulting plasmid pGBT9-186R encodes the full-length protein N-terminally fused to a GAL4-binding domain.

An N-terminal Flag tag was introduced into the 68k-ank gene by cloning the yeast codon-optimized sequence mentioned above into pFLAG-CMV-2 (Sigma-Aldrich, Taufkirchen, Germany) to yield pFLAG-CMV-2-68k. The truncated version of 68k-ank lacking the F-box-like domain was generated by inserting the yeast codon-optimized sequence encoding amino acids 1–397 of 68k-ank into pFLAG-CMV-2 yielding the plasmid pFLAG-CMV-2-68kΔFbox.

Rabbit polyclonal antibodies against Skp1a were purchased from Dunn Labortechnik, (Asbach, Germany), anti-Cullin-1 from Abcam, (Cambridge, England) and anti-Flag M2 antibody was obtained from Sigma-Aldrich (Taufkirchen, Germany). Secondary antibodies were peroxidase-conjugated anti-mouse or anti-rabbit (Dianova, Hamburg, Germany). A polyclonal antibody directed against the 68k-ank protein was raised by immunization of rabbits with two peptides derived from the MVA 68k-ank protein (Eurogentec, Köln, Germany).

Yeast-two-hybrid (Y2H) screen

pGBT9-186R expressing the full-length ORF 186R of MVA N-terminally fused to a GAL4-binding domain served as the bait vector. Two automated Y2H screens were performed at the German Resource Center for Genome Research (RZPD now imaGenes, Berlin, Germany) as described before (Albers et al., 2005). Matchmaker GAL4™ libraries from Clontech (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) were used as cDNA prey libraries; either human kidney (pooled from 8 male/female Caucasians, ages 24–55) or mouse 11-day embryo (pooled from Swiss Webster/NIH mice).

Cell culture and co-immunoprecipitation (Co-IP)

293T cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and antibiotics at 37 °C in 5% CO_2.

For Co-IP experiments, 293T cells were transfected with the respective plasmids for 48 h or infected with MVA for 4 h at an MOI of 20. IPs were performed in NET-N buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol) supplemented with the protease-inhibitor cocktail Roche complete (Roche, Mannheim, Germany) as described before (Harlow and Lane, 1999), and analyzed by western blotting. About 5% of the cell lysates were loaded on the gel (cell lysate) to verify protein expression of the transfected plasmids and the endogenous proteins of interest.

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