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Proteome analysis of the HIV-1 Gag interactome

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ABSTRACT

Human immunodeficiency virus Gag drives assembly of virions in infected cells and interacts with host factors which facilitate or restrict viral replication. Although several Gag-binding proteins have been characterized, understanding of virus–host interactions remains incomplete. In a series of six affinity purification screens, we have identified protein candidates for interaction with HIV-1 Gag. Proteins previously found in virions or identified in siRNA screens for host factors influencing HIV-1 replication were recovered. Helicases, translation factors, cytoskeletal and motor proteins, factors involved in RNA degradation and RNA interference were enriched in the interaction data. Cellular networks of cytoskeleton, SR proteins and tRNA synthetases were identified. Most prominently, components of cytoplasmic RNA transport granules were co-purified with Gag. This study provides a survey of known Gag–host interactions and identifies novel Gag binding candidates. These factors are associated with distinct molecular functions and cellular pathways relevant in host–pathogen interactions.

Introduction

Human immunodeficiency virus type 1 (HIV-1) Gag is the key structural protein mediating assembly of virions in infected cells. In the late phase of the HIV-1 replication cycle, the Gag polyprotein is translated on polysomes in the cytoplasm and is targeted to the plasma membrane, the site of Gag multimerization, assembly and budding of virions. During or shortly after budding, the $Pr55^{Gag}$ polyprotein is cleaved by the viral protease, yielding the products matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC) and p6. Specific functions during assembly have been ascribed to distinct domains within Gag, such as the membrane binding domain within MA, the interaction domain in CA and NC which mediates Gag multimerization and the p6 late domain which promotes viral budding. Zinc finger motifs and basic residues in NC are mainly responsible for encapsidation of viral RNA (Sundquist and Kräusslich, 2012 for review). Though Gag is capable of self-assembly *in vitro*, it has become generally accepted that within the context of a living cell, host factors play a decisive role for efficient viral replication. Several host factors that associate with Gag have been identified previously. For instance, HIV-1 Gag p6 interacts with Tsg101 to recruit ESCRT (endosomal sorting complex required for transport) proteins essential for budding (McCullough et al., 2013). The cellular protein Staufen1 has been reported to be involved in Gag multimerization (Châtel-Chaix et al., 2008). Likewise, inhibitory factors such as Trim5alpha (Stremlau et al., 2004, 2006) and APOBEC3G (Cen et al., 2004; Schäfer et al., 2004; Zennou et al., 2004) interact with Gag to restrict viral replication.

Recently, a meta-analysis of host cell genes linked to HIV replication has been published (Bushman et al., 2009). For most of these factors, the mechanism by which they promote or restrict HIV replication remains to be resolved. Interaction with viral proteins is one possibility, and screens for interaction partners of HIV proteins have been performed (Gautier et al., 2009; Bushman et al., 2009; Studamire and Goff, 2008; Jäger et al., 2012). However, despite the progress made in recent years, our understanding of Gag trafficking and assembly remains incomplete. Thus, the search for unknown Gag–host interactions may still provide valuable insights.

Here we report results of a series of affinity purification screens identifying potential novel interaction partners of HIV-1 Gag and

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discuss these in the context of previous studies. After describing the experimental approach and primary data analysis, we relate the screening results to the current literature and finally provide an integrative overview of protein classes and networks discovered in the Gag interaction data. Rather than focusing on functional characterization of individual factors, we consider the HIV-1 Gag interactome as a whole and highlight pathways and networks relevant for HIV-1 replication.

Results and discussion

AP/MS screens for cellular proteins interacting with HIV-1 Gag

A variety of affinity purification (AP) methods have been developed to identify protein–protein interactions. To increase the chance of identifying *bona fide* interaction partners, two approaches are conceivable: (1) performing multiple AP experiments with one method, (2) performing analogous experiments applying several methods. While candidates selected with the first approach are reproducible in a specific experimental setting, candidates identified with different methods may more likely be valid interaction partners. Thus, we chose the second approach and performed a series of six independent screens with three AP methods in 293T cells (Table 1 and Supplementary Table 1). Cells were transfected with plasmids encoding tagged HIV-1 Gag. A tagging approach was used since multimeric Gag assemblies are not well precipitated by many Gag-specific antisera and detecting a heterologous tag avoids masking of binding sites by antibodies against the target protein. Three methods were employed to identify cellular interaction partners of HIV-1 Gag: paramagnetic anti-GFP microbeads (Beads) (Cristea et al., 2005), nanotrapping with GFP Trap_A (Nano) (Rothbauer et al., 2008) and tandem affinity purification (TAP) (Rigaut et al., 1999; Gingras et al., 2005). Two alternative baits were purified with the GFP-based techniques: Gag-EGFP with a C-terminal GFP tag (C) (Hermida-Matsumoto and Resh, 2000) and pCHIV^{EGFP} (Müller et al., 2004), with insertion of the GFP tag between the MA and CA domains of Gag (MA) in the context of an almost complete HIV-1 genome. This was done because differences in conformation or domain mobility due to the position of the tag may affect interactions with host proteins, and because the presence of additional HIV-1 proteins besides Gag may influence the Gag interactome. To obtain quantitative mass spectrometry (MS) data, stable isotope labeling in cell culture (SILAC) (Ong et al., 2002; Vermeulen et al., 2008) was applied in one screen with anti-GFP microbeads for affinity purification after transfection of pCHIV^{EGFP}.

Table 1
AP/MS screens for cellular interaction partners of HIV-1 Gag. Six different AP/MS screens were performed to identify potential cellular interaction partners of HIV-1 Gag. Name of the screen, affinity purification method, bait, negative control bait and total number of hits (potential Gag- interacting proteins) in the respective screen are given in the table.

AP/MS screens				
Screen	AP method	Bait	Control	Hits
BeadsC	Anti-GFP microbeads	pGag-EGFP	pEGFPc1	224
BeadsMA	Anti-GFP microbeads	pCHIV^{EGFP}	pEGFPc1	285
NanoC	GFP Trap_A	pGag-EGFP	pEGFPc1	519
NanoMA	GFP Trap_A	pCHIV^{EGFP}	pEGFPc1	396
CTAP	Tandem affinity purification	pGagCTAP	pGagCTAP	714
SILAC	Anti-GFP microbeads	pCHIV^{EGFP}	pEGFPc1	914

Data extraction and annotation of potential artifacts

Data from all screens including annotations are shown in Supplementary Table 1. In total, 1804 potential Gag interaction partners were identified across all screens. Pseudogenes were not considered as candidates. For the non-quantitative AP/MS screens, proteins identified by at least three peptides and not present in the negative controls were considered significant. For SILAC, proteins for which at least three peptides were detected and which achieved a heavy/light (H/L) ratio greater than 5 (see below) were considered to be significant hits. 895 candidates (46%) met these significance criteria.

AP screens are prone to false positive results due to non-specific binding of abundant and "sticky" proteins. Likewise, false negatives may result from non-specific binding of a veritable interaction partner to the affinity tag. Potential AP artifacts were annotated using published lists of known common contaminants (e.g. trypsin, keratins) and non-specifically binding proteins (Table 2 and Supplementary Table 2). Overall, 623 (34%) of the 1804 candidate proteins were considered to be potential artifacts. Potential artifact classes common to all screens were ribosomal proteins, DEAD/H-Box proteins, ribonucleoproteins and translation-associated proteins, although there is some variability in the proportions (Supplementary Fig. 1). Recently, Jäger et al. performed a large-scale AP/MS study for human proteins interacting with HIV proteins and developed a scoring system to distinguish specific and non-specific binders. Jäger et al. also reported many RNA helicases, DEAD/H-box proteins, splicing factors and ribosomal proteins which co-purified non-specifically with Gag and Gag processing products (Jäger et al., 2012). However, the DEAD/H box

Table 2
Criteria for artifact filtering. Potential AP/MS artifacts were annotated using the criteria listed in the table. Lists of PANTHER families, GO terms and HGNC gene names can be found in Supplementary Table 2.

Artifact annotation		
Class	Artifacts	Extracted from
Pseudogenes	Database alignment artifact	NCBI gene database
Contaminants	E.g. trypsin, bovine serum albumin, keratins	MaxQuant (Cox and Mann, 2008)
Abundant and “sticky” cellular proteins	Ribosomal proteins	PANTHER families (Thomas et al., 2003)
	Translation factors	GO terms (Ashburner et al., 2000)
	Heatshock proteins	HGNC (Eyre et al., 2006)
	Ribonucleoproteins	
	Histones	
	DEAD/H box helicases	
	Intermediate filaments	
	Cytoskeletal and motility proteins	
Additional common AP artifacts	E.g. proteins binding to affinity matrices	cRAP, (Trinkle-Mulcahy et al., 2008; Max Planck Institute of Biochemistry,)

proteins RNA helicase A (DHX9) (Roy et al., 2006) and DDX49 (Jäger et al., 2012) seem to interact with Gag in a specific manner indicating that this annotation as potential artifacts may not always hold true. Similarly, cytoskeletal proteins are often considered as artifacts, but previous reports have suggested that Gag may specifically interact with actin and actin-binding proteins (Liu et al., 1999; Wilk et al., 1999).

SILAC-based quantitative mass spectrometry has been developed as a tool to discriminate between specific and non-specific protein–protein interactions (Ong et al., 2002; Vermeulen et al., 2008). This method is based on labeling cells expressing the protein of interest and parallel control cells with different amino acid isotopes (heavy (H) and light (L)). Quantifying the H/L ratio of factors co-purifying with the bait allows for relative quantification of recovered proteins, with high H/L ratios indicating a possible specific interaction. For the SILAC experiment, 293T cells labeled with heavy amino acid isotopes were transfected with pCHIV^{EGFP}, whereas control cells maintained in medium with light amino acid isotopes were transfected with pEGFPc1. Non-specific binding should occur roughly equally in the heavy and light fractions leading to a ratio near 1. However, increased non-specific binding to the larger Gag bait compared to controls may occur: for instance, Gag-EGFP, a ~92 kDa protein, provides a far greater contact surface than the EGFP control of 27 kDa. The distribution of H/L ratios for all SILAC hits for non-artifacts, potential artifacts and contaminants is shown in Supplementary Fig. 2. In addition a histogram of the log-transformed H/L ratios is shown in Supplementary Fig. 3. The SILAC data were further analyzed using an empirical Bayes approach. With the Bayesian Information Criterion, a Gaussian mixture model with three components with unequal variance, corresponding to the null model (no regulation), low ratio group (downregulation), and high ratio group (upregulation) was selected as the best model. This model suggested an H/L ratio cutoff of around 4.34 (untransformed) to achieve a 1% significance level using the unregulated class distribution as null model. To reduce the number of false positives, the H/L ratio cutoff was set to 5 in the present analysis. While most contaminants are eliminated using an H/L threshold of 5, some putative artifacts are distributed throughout the range, most of which are ribosomal proteins.

Pr55^{Gag} is considered to be a cytoplasmic and membrane-associated protein. Nuclear and mitochondrial proteins identified in the screens may thus be false positives that could have been brought into proximity of the Gag bait during cell lysis. However, annotation of protein localization may be incomplete. Moreover, a main nuclear protein may be present in the cytoplasm, though at lower abundance. Therefore, non-cytoplasmic and non-membrane proteins were not excluded from the candidate list.

Interaction candidates identified in multiple screens and by H/L ratios exceeding 5

Candidates identified in multiple screens appear most suited for downstream validation experiments. Ninety proteins were significant hits in at least three screens (Supplementary Table 1). The cellular protein Lyric (synonyms MTDH metadherin, AEG-1 astrocyte elevated gene 1) was identified in all six screens, significant in five screens, not annotated as a potential artifact and achieved a high H/L ratio of 50.2. Thus, Lyric was chosen for further characterization (Engeland et al., 2011). Several previously described Gag-interacting proteins were also identified by this method, e.g. Staufen (STAU1) (Mouland, 2000; Châtel-Chaix et al., 2004, 2008). On the other hand, some potential artifacts were retained despite these more stringent criteria.

In total, 371 of the 914 initial SILAC hits were not classified as artifacts and achieved H/L ratios above the threshold of 5.

Table 3
Candidates with high H/L ratios in SILAC. Significant HIV Gag interaction candidates not considered to be potential artifacts which achieved an H/L ratio of >10 in the SILAC screen are classified below. Functional categories include RNP complexes, RNA interference (RNAi), RNA degradation by nucleases, members of the tRNA synthetase complex, cytoskeletal proteins, factors involved in the antiviral response, host restriction factors and proteins with other or unknown biological functions. Several candidates belong to more than one category.

Candidates with H/L ratios >10 in SILAC						
RNP complexes	RNAi	Nucleases	tRNA	Cytoskeleton	Antiviral	Other
STAU1	Lin28B	ZC3HAV1	KARS	MAP4	ZC3HAV1	MTDH (Lyric)
SND1	DICER	EXOSC10	MARS	MYL6	MOV10	ZCCHC3
IGF2BP1	SND1	XRN2	AIMP1	MYH10	HERC5	BAT2L2
IGF2BP2	MOV10		EPRS	MYH9	PRKRA	ATXN2L
IGF2BP3			IARS		NKRF	NAT10
MOV10			DARS		ILF2	MYBBP1A
ILF2			RARS		ILF3	LARP1
ILF3			NSUN2			SRP14
LSM12			LARS			SPATA5L1
ZFR			QARS			CHMP4B
FXR1			TRM1L			
			AIMP2			

The top 51 candidates with H/L ratios of greater than 10 are listed in Table 3 including functional annotation. Proteins involved in RNA transport and processing, the tRNA synthetase complex, several cytoskeletal proteins and also factors involved in the host antiviral response were most prominent among the top SILAC hits.

Host factors reported previously

For an integrative analysis of the available data, interaction candidates identified in multiple screens and/or achieving high H/L ratios in SILAC were related to results from previous studies. These included host factors detected in protein interaction screens, proteins identified as part of the virion proteome or implicated in HIV replication through siRNA screens.

Gag-interacting proteins: A number of cellular proteins interacting with HIV Gag and/or Gag processing products have been reported previously. The NIAID HIV protein interaction database catalogs interactions between HIV-1 and human proteins published in the literature (Fu et al., 2009; Ptak et al., 2008; Pinney et al., 2009). Interactions described in the NIAID dataset are very diverse and range from highly characterized and specific molecular binding data to associations such as colocalization or dependency or regulatory relationships. We reviewed the associated literature to retrieve validated direct protein–protein interactions between HIV-1 Gag or its processing products and host cell factors. Also we searched the literature for additional direct HIV Gag interactors not listed in the NIAID database. 19 out of 29 Pr55^{Gag} interactors (65%) and 30 out of 51 interactors with Gag processing product (59%) found in the literature were recovered in our AP/MS screens (see Supplementary Table 1). We cannot rule out that some interaction partners of processed Gag proteins would not interact with the Gag baits in our experiments, but the similar proportion of previously reported proteins for Gag and its cleavage products suggests that this does not play a major role.

The previously reported Gag interaction partners DHX9 (DEAH box polypeptide 9, synonym RNA helicase A) (Roy et al., 2006), NCL (nucleolin) (Nisole et al., 2002; Ueno et al., 2004), IGF2BP1 (Zhou et al., 2008b) and STAU1 (Staufen1) (Mouland, 2000; Châtel-Chaix et al., 2004, 2008), were significant in multiple screens. NCL, STAU1, KARS (Javanbakht et al., 2003), MOV10 (Abudu et al., 2012), HNRNPD (Lund et al., 2012), DDX49, EEF1E1, EIF2AK2, MRPL11, AIMP1, and AIMP2 (Jäger et al., 2012) achieved high H/L

ratios of over 20 in SILAC. In total, roughly 60% of all cellular proteins which have been reported to directly interact with Gag or Gag processing products were recovered in our screens.

Proteins observed in HIV-1 virions: $Pr160^{GagPol}$ was the only other HIV-1 protein besides Gag detected in our MA EGFP Gag screens. The HIV-1 protein Vpr was previously shown to be incorporated into HIV-1 by interaction with the Gag p6 domain (Selig et al., 1999), but was not detected in our screen. This observation confirms previous results that *bona fide* interaction partners may be missed in unbiased interaction screens (Jäger et al., 2012; Roy et al., 2006). Purified virions derived from T cells, monocytes, macrophages and 293T cells have been characterized previously regarding content of cellular proteins (Chertova et al., 2006; Santos et al., 2012). The results of these studies are documented in the NCI Host proteins in HIV database (NCI Frederick). Host proteins associating with Gag during assembly may differ depending on cell type. It should be noted, however, that host cell proteins may be incorporated into virions specifically through a direct interaction with an HIV protein or non-specifically as bystanders (since virions must contain components of the membrane and cytosol). Supplementary Table 1 shows 158 proteins identified in the six screens, which had been detected previously in the virion proteome. Many of these are considered as possible AP artifacts and it is not unlikely that abundant cellular proteins, such as heat shock or cytoskeletal proteins, are both non-specifically co-purified in AP experiments and incorporated into nascent viral particles. MOV10, SND1, STAU1 and SRP14 were identified in multiple screens, achieved high H/L ratios and are not considered as common AP artifacts, suggesting that an interaction with Gag may lead to incorporation of these host cell proteins into the viral particle.

Host factors identified in siRNA screens: Recently, several siRNA screening results for host factors influencing HIV replication were published (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008a). Another siRNA screen identifying cellular factors involved in the early phase of HIV infection was performed by our group (Börner et al., 2010; Börner and Kräusslich, unpublished data). Identification of candidates in AP/MS and siRNA screens may suggest a mechanism involving Gag–protein interaction. However, apart from the two-part assay carried out by Brass et al. (2008), siRNA screening read-outs mainly addressed the early stages of HIV replication, whereas high levels of Gag are reached at a late stage of the viral replication cycle. Therefore, we did not expect a high overlap between the AP and siRNA screens. Of course, a cellular protein may influence HIV replication at multiple stages. Supplementary Table 1 lists 158 siRNA hits also identified in the AP screens. RBM14, SND1 and SRSF3 were identified in ≥ 3 AP screens. The possible biological relevance indicated by the siRNA data may warrant further investigation of these host factors.

Molecular functions, cellular pathways and interaction networks

We analyzed the complete dataset of 1804 candidates with the DAVID Functional Annotation Tool (Huang et al., 2009a,b) to reveal enrichment of conserved protein domains and factors associated with distinct biological processes, molecular functions and cellular compartments (Tables 4 and 5). To identify validated protein complexes and networks within the screening data, we extracted validated protein–protein interactions from STRING (Franceschini et al., 2013) and subsequently searched for clusters within the interaction data using the MCODE algorithm (Bader and Hogue, 2003). Apart from a cluster of ribosomal proteins, the three major clusters identified were (1) SR family proteins with their interaction partners, (2) a complex of centrosomal and microtubule-associated proteins and (3) the tRNA synthetase complex (Fig. 1).

Table 4
Protein domains enriched in the HIV Gag AP/MS data. Protein domains enriched in the HIV Gag interaction dataset categorized according to their molecular functions are listed in the table.

Protein domains enriched in HIV Gag AP/MS data	
Function	Protein domains
RNA/DNA binding	RNP-1
	Helicase motifs
	Histone core
	dsRNA binding domain
	KH domain
Protein–protein interaction	WD40 repeats
Protein complex assembly	AAA ATPase
Cytoskeletal proteins	Tubulin
Intracellular transport	Intermediate filament motifs
	Myosin head
	Tropomyosin
	Actin conserved sites
Specific molecular functions	Helicase function
	Chaperone motifs
	Exosome proteins (exoribonuclease complex)
	tRNA synthetases

Table 5
GO enrichment analysis of AP/MS hits. Gene ontology terms were used for annotation of AP/MS hits. The table lists the most enriched biological processes, molecular functions and cellular compartments.

GO enrichment analysis of AP/MS hits		
GO biological processes	GO molecular functions	GO cellular compartments
Translation elongation	RNA binding	Ribonucleoprotein complex
RNA processing	Constituent of ribosome	Ribosome
RNP complex biogenesis	Nucleic acid binding	Cytosolic part
Ribosome biogenesis	Helicase activity	Nucleolus
RNA splicing	ATPase activity	Nuclear lumen
ncRNA metabolic process	DNA clamp loader activity	Protein–DNA complex
Nucleosome assembly	Motor activity	Spliceosome
Microtubule-based process	Translation factor activity	Chromosome
RNA stabilization	actin binding	hnRNP complex
tRNA metabolic process	tRNA ligase activity	Cytoskeleton
Actin filament based movement		Centrosome
Microtubule-based process		Myosin complex

In the following we will discuss protein classes, cellular pathways and networks found in the AP/MS dataset and relate our findings to previous studies on HIV-host cell interactions.

Helicases: Helicases were abundant among the screening candidates. Though often considered as AP artifacts, several helicases were significant in the AP/MS screens which have been associated with HIV previously: DHX9 has been reported to bind Gag (Roy et al., 2006) and may induce conformational changes in viral RNA (Xing et al., 2011). It has been suggested that DDX24 plays a role in packaging of viral RNA (Ma et al., 2008). After depletion of DDX6, infectious progeny particles were reduced (Reed et al., 2012). The helicase MOV10 has been shown to interact with Gag NC in an RNA-dependent manner (Abudu et al., 2012). MOV10 was also identified in our siRNA screen (Börner and Kräusslich, unpublished data). MOV10 has been characterized as a viral restriction factor which inhibits reverse transcription, reduces Gag protein levels and processing as well as virion production (Wang et al., 2010; Burdick et al., 2010; Furtak et al., 2010).

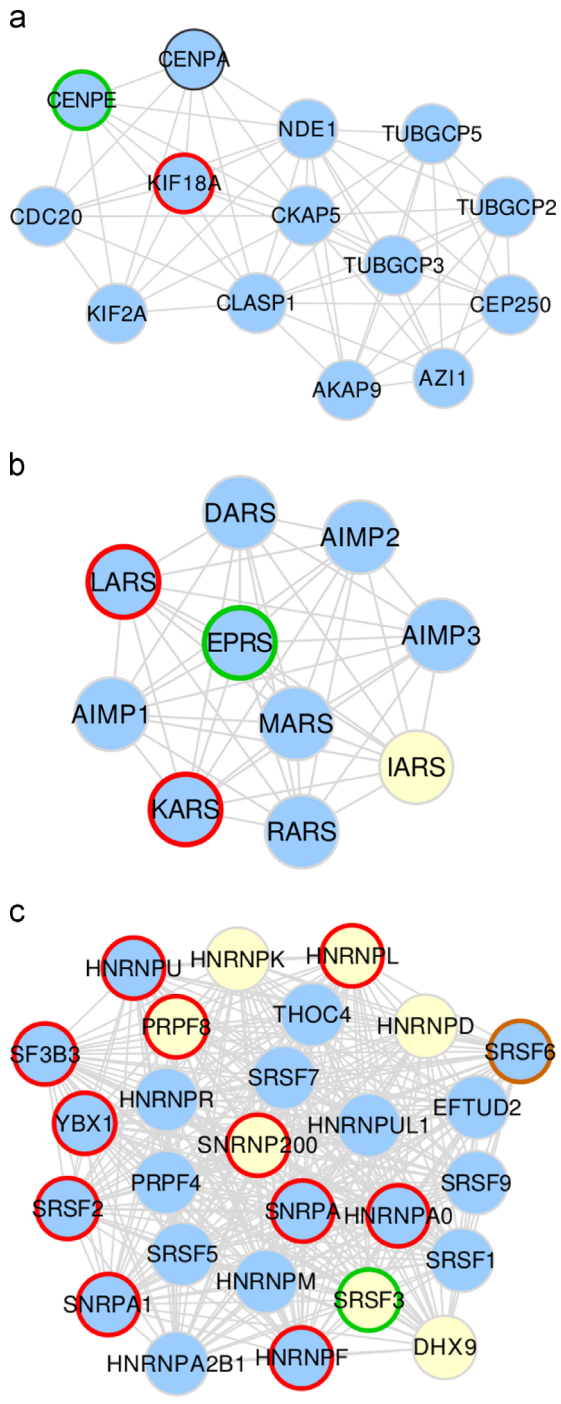

Fig. 1. Molecular clusters in the HIV Gag AP/MS data. Clusters identified by protein–protein interaction and subsequent MCODE analysis. Blue nodes: AP/MS hits; yellow nodes: proteins identified in HIV particles; red border: impaired viral replication after siRNA knockdown; green border: siRNA knockdown enhanced HIV replication in siRNA screens. (a) A cellular complex of centrosomal proteins, gamma tubulins and kinesin; (b) the tRNA synthetase complex; (c) the SR protein family.

Cytoskeletal and motor proteins: A number of cytoskeletal proteins were identified in the AP/MS screens. Actin and actin-binding proteins were abundant among the AP/MS hits. However, most of these were not significant, with the exception of myosins in the TAP screen and tropomyosins in SILAC. Tubulins were present in negative controls and achieved low H/L ratios (<1). Centrosomal proteins, microtubule-associated proteins MAP1B and MAP4, dynein, kinesin and the kinesin-binding protein kinectin scored significantly in the TAP and SILAC screens. Cluster analysis identified a complex of centrosomal proteins, gamma tubulins and kinesin among the significant Gag interaction candidates (Fig. 1a).

Interactions of Gag with cytoskeletal proteins and motor proteins in the late stages of the HIV replication cycle have been reported previously (reviewed in Fackler and Kräusslich, 2006; Naghavi and Goff, 2007; Stolp and Fackler, 2011). Poole et al. reported that Gag interacts with centrioles and captures viral RNA near the MTOC (Poole et al., 2005). Previous studies have suggested association of Gag with actin (Liu et al., 1999; Wilk et al., 1999) and the microtubule motor protein KIF4A (Tang et al., 1999; Martinez et al., 2008). An interaction of filamin with Gag has been reported which may be involved in particle release (Cooper et al., 2011). Jolly et al. reported that actin and tubulin were required for assembly of HIV in T cells (Jolly et al., 2007). Gladnikoff et al. observed actin remodeling during assembly and budding (Gladnikoff et al., 2009). Altogether, how Gag interacts with the cytoskeleton during trafficking and assembly remains to be resolved in detail.

Chaperones and protein folding: Several molecular chaperones were found in the AP screens. The chaperonin-containing T complex TriC consisting of TCP1 and CCT2-8 as well as the heat shock proteins Hsp40, 60, 70 and 90 were identified. However, chaperones often co-purify non-specifically in AP experiments and were thus present in negative controls and achieved low H/L ratios in SILAC.

On the other hand, some proteins that are *bona fide* Gag interactors were not identified. This was the case for Cyclophilin A (PPIA), which was not significant in our screens and also did not achieve a significant score in the analysis by Jäger et al. (Jäger et al., 2012). Interaction of Gag with Cyclophilin A has previously been characterized in detail (Luban et al., 1993; Franke et al., 1994; Thali et al., 1994; Colgan et al., 1996).

Interaction of HIV Gag p6 and the Gag protein of the Mason-Pfizer monkey retrovirus with TCP-1gamma has been reported and this interaction may contribute to retroviral assembly (Hong et al., 2001). Gurer et al. reported binding of Gag to Hsp60 and Hsp70 (Gurer et al., 2002). HP68 has also been implicated in HIV assembly (Lingappa et al., 2006). Joshi et al. reported that HSP90AB1 can rescue infectivity of HIV CA mutants and suggested that this chaperone may stabilize the HIV viral core (Joshi and Stoddart, 2011; Joshi et al., 2013).

Proteins containing WD40 repeats and AAA ATPase domains were overrepresented in our interaction data. These domains are known to assist protein folding and protein complex assembly. The distinct role of many of these factors remains poorly defined. Further studies are needed to address whether these proteins specifically assist HIV assembly.

Cellular HIV restriction factors: TRIM2, 6, 21, 25, 28, 34 and 68 were found in our AP/MS screens. TRIM5alpha is an HIV restriction factor which binds the incoming viral capsid (Stremlau et al., 2004, 2006). Aside from TRIM5alpha, binding of TRIM6, TRIM21 and TRIM34 to Gag has been reported (Li et al., 2006). These three TRIM proteins were identified in our screens, but achieved low scores. Jäger et al. found TRIM25 to be associated with Gag and TRIM26 with NC, though both did not achieve significant scores (Jäger et al., 2012).

The cytidine deaminase APOBEC3G is known to bind Gag and – in the absence of Vif – restrict HIV replication by inhibiting reverse transcription and inducing G-to-A mutations (reviewed in Goila-Gaur and Strebel, 2008). A recent study reports that not only APOBEC3G, but also APOBEC3D, -F and -H restrict HIV replication (Hultquist et al., 2011). APOBEC3G was not identified in any of our screens, independent of the presence or absence of Vif. However, APOBEC3D was identified in one screen (NanoC). In all screens

using the plasmid pCHIV^{EGFP} as a bait, expression of *Vif* probably led to degradation of APOBEC.

Vesicular trafficking, membrane proteins: The clathrin adaptor–protein complex subunits AP2A1, AP2A2, AP2B1, AP2M1, AP3B1, AP3D1, and AP3M1 were identified in SILAC, but did not achieve significant H/L ratios. AP1mu has been implicated in HIV-1 budding previously (Camus et al., 2007). Dong et al. reported an interaction between Gag and the adaptor protein complex AP-3delta, suggesting a requirement of AP-3 for Gag targeting to late endosomes and MVBs early in HIV assembly (Dong et al., 2005).

Tsg101, which recruits the ESCRT complex necessary for HIV budding (McCullough et al., 2013), was not identified in any of the AP/MS screens. CHMP4B was the only ESCRT component identified in the AP/MS screens and achieved a high H/L ratio of 10.9. CHMP4B interacts with Alix and is necessary for Alix-mediated rescue of HIV late domain mutants (Usami et al., 2007). Conversely, overexpression of CHMP4B inhibits HIV-1 release (Carlton et al., 2008). Jäger et al. also did not detect Tsg101 and Alix and presumed that these interactions may be too weak for detection in their AP/MS approach (Jäger et al., 2012). Moreover, membrane-associated proteins tend to be undersampled in AP experiments with cell lysates.

The Gag matrix domain interacts with phosphatidylinositol-4,5-bisphosphate, thus targeting Gag to the plasma membrane (reviewed in Chukkapalli and Ono, 2011). More specifically, association of Gag with lipid rafts and tetraspanin-rich domains has been studied (for review, see Ono, 2010). However, raft proteins or tetraspanins were not recovered in our screening data. Lipid-mediated interactions may not be detected by AP of tagged proteins. Thus, proteins which associate with Gag at membranes or proteins involved in vesicular trafficking may not be identified with our experimental approach.

Ubiquitin, proteasome: HIV Gag ubiquitinylation has been studied in detail (reviewed in Martin-Serrano, 2007). In our Gag interaction screens, ubiquitin was identified in SILAC with an H/L ratio of 3.9. Several ubiquitin ligases (UBE2E1, UBE2G1 and UBE20) were significant in the TAP screen. However, other members of the ubiquitin/proteasome pathway previously implicated in HIV replication (meta-analysis in Bushman et al., 2009) were not identified.

Autophagy: Kyei et al. described colocalization and co-immunoprecipitation of the autophagy factor LC3 with Gag (Kyei et al., 2009). It has been suggested that early phases of autophagy assist virion formation in host cells (reviewed in Dinkins et al., 2010). LC3 (MAP1LC3A) was not recovered in our screens, only the autophagy factors ATG3 and ATG16L2 were significant in one screen.

Translation factors: Cellular factors involved in RNA translation were overrepresented in the AP/MS data and complexes of translation factors such as the EIF3 complex were identified. Though many translation factors were not considered as significant hits, EIF2S1-3 were significant in multiple screens and achieved high H/L ratios in SILAC. Interaction of Gag with translation factors EEF1A1 (Cimarelli and Luban, 1999), EEF1E1 and EIF2AK2 (Jäger et al., 2012) has been reported previously. The interaction between EEF1A1 and Gag MA inhibits translation *in vitro*, and a model in which accumulating Gag inhibits translation to favor packaging of viral RNA has been suggested (Cimarelli and Luban, 1999).

tRNA synthetase complex: The tRNA synthetase complex which was identified as one of the main clusters in the AP/MS data is shown in Fig. 1b. Aspartyl tRNA synthetase (DARS) was significant in four screens. Members of the tRNA synthetase complex were the largest group with high H/L ratios in SILAC. The recent HIV protein interaction study by Jäger et al. also identified tRNA synthetases as binding partners of Gag and specifically its matrix domain (Jäger et al., 2012).

HIV uses tRNA^{Lys} as a primer for reverse transcription and Gag mediates incorporation of tRNA^{Lys} into virions via an interaction with lysyl tRNA synthetase (KARS) (reviewed in Kleiman et al., 2010). In a recent study, tRNA^{Ile} and other tRNAs were detected in virions, leading to the hypothesis that HIV selectively packages tRNAs specific for rare human codons which are frequently used by HIV, e.g. AUA for isoleucine (Pavon-Eternod et al., 2010). Thus, interactions of Gag and tRNA synthetases aside from KARS may be required for recruitment of additional tRNAs, e.g. IARS for incorporation of tRNA^{Ile}.

SR proteins: Three members of the SR protein family (SRSF3, SRSF1 and SRSF7) were significant in multiple screens. The SR proteins SRSF2-6, 9, 10 and 12 were also identified. The SR protein-specific kinase SRPK1 and TOP1 were significant in multiple screens (Supplementary Table 1). SRPK2 was significant in SILAC. The SRSF1-interacting protein PRPF4 was identified in 5 AP/MS screens (significant in 2 screens). Fig. 1c depicts the SR protein network as identified by MCODE analysis of the AP/MS data.

Though first characterized as splicing factors, the serine–arginine-rich (SR) family of proteins has multiple functions in RNA metabolism from mRNA processing over RNA transport to translational regulation (reviewed in Shepard and Hertel, 2009; Zhong et al., 2009; Twyffels et al., 2011). SR proteins have been implicated in HIV replication previously: Bennett et al. reported interaction of HIV-2 Gag with PRPF4 which leads to inhibition of PRPF4-mediated phosphorylation of SRSF1 (Bennett et al., 2004). An increase of p24 CA release after overexpression of SRSF4, SRSF5 and SRF6 and after phosphorylation of SRSF4 by SRPK2 has also been reported (Fukuhara et al., 2006). When overexpressing SRSF1, SRSF2 and SRSF7, Jacquenet et al. observed a reduction of Gag expression and accumulation of Gag at the plasma membrane and in intracellular compartments (Jacquenet et al., 2005). After overexpression of SRSF1, SRSF2 and SRSF5 a decrease in virion production and viral infectivity was observed (Jablonski and Caputi, 2009). Swanson et al. reported that SRSF5 and SRSF6 enhance Gag translation (Swanson et al., 2010). SRSF3 was also significant in our siRNA screen (Börner and Kräusslich, unpublished data). Taken together, interaction of HIV with SR proteins may merit further exploration.

ncRNA/RNAi effector complex: Factors involved in RNA interference were enriched in the AP/MS dataset (Table 5). The RISC components DICER, Ago2 (EIF2C2) and TARBP (Chendrimada et al., 2005) were identified in the AP/MS screens and achieved high H/L ratios of 51.4, 8.6 and 10.0, respectively.

The RNAi pathway has been implicated in HIV replication previously (reviewed in Klase et al., 2012). HIV mRNA has been reported to associate with RISC components (Chable-Bessia et al., 2009; Nathans et al., 2009). The RISC component TARBP binds the TAR element of HIV RNA. Knockdown of TARBP impairs processing of microRNA, but also seems to decrease HIV replication (Christensen et al., 2007). Knockdown of the RNAi effectors Dicer and Drosha apparently leads to an increase in HIV particle production, while Dicer appears to inhibit HIV replication (Chable-Bessia et al., 2009; Triboulet et al., 2007). Bouttier et al. reported that Gag recruits Ago2 to viral RNA. This interaction did not appear to affect RNA stability but supported HIV replication (Bouttier et al., 2012). Previous studies as well as the AP/MS data suggest a possible interaction of Gag with several RISC components. The precise role of Gag in RNA interference during HIV replication remains to be defined.

RNA degradation by nucleases: Aside from factors involved in RNA interference, factors mediating RNA degradation by nucleases were among the top AP/MS hits. ZC3HAV1 was among the candidates with the highest H/L ratios in the SILAC screen. The exosomal RNAse components EXOSC1-10 were identified in the AP/MS screens, with EXOSC5-8 and 10 achieving high H/L

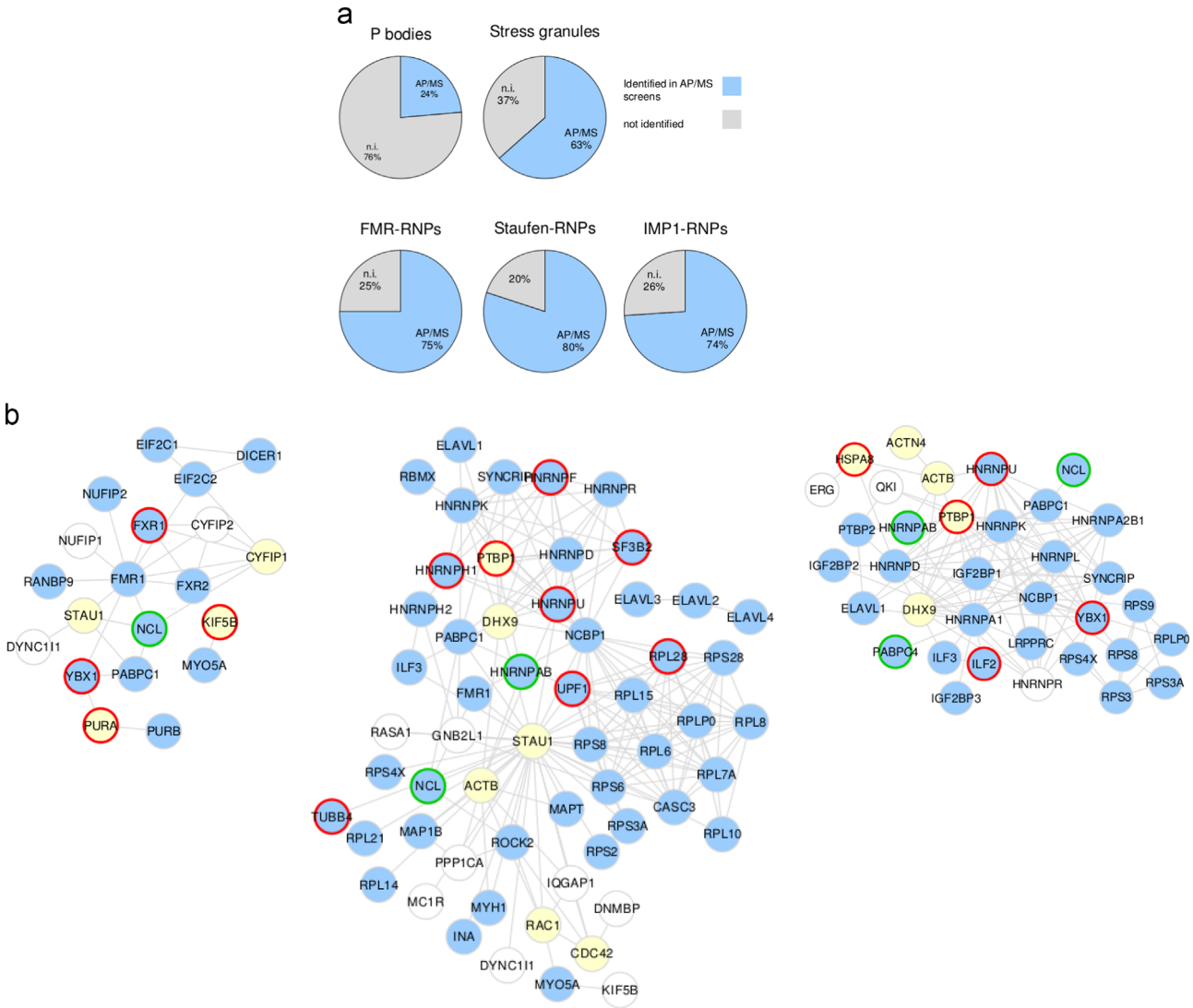

Fig. 2. Cellular ribonucleoprotein complexes. (a) Overlap of Gag interaction screening hits with defined cellular protein-RNA complexes. AP/MS gives the percentage of RNP components recovered in the interaction screens; n.i.: not identified in the Gag AP/MS screens. (b) FMR1, IMP1 (IGF2BP1), and Staufen (STAU1)-containing ribonucleoprotein complexes with annotation of proteins identified in virions, HIV Gag AP/MS and siRNA screening hits. Blue nodes: AP/MS hits; yellow nodes: proteins identified in HIV particles; red border: impaired viral replication after siRNA knockdown; green border: siRNA knockdown enhanced HIV replication in siRNA screens; brown border: both enhanced and impaired viral replication after knockdown were observed.

ratios, though only EXSOC9 and 10 were considered significant. Also the RNA exonuclease XRN2 was identified in two screens and significant in SILAC.

In an shRNA experiment, knockdown of EXOSC5 affected Gag trafficking (Yeung et al., 2009). Zhu et al. reported that overexpression of ZC3HAV1 leads to degradation of HIV mRNAs by recruiting exonucleases and decapping enzymes (Zhu et al., 2011). Factors involved in RNA degradation may non-specifically co-purify with Gag and viral RNA. However, Gag may also shield viral RNA from degrading enzymes during trafficking.

RNA binding proteins, RNP complexes: By far the most prominent group in our AP/MS screening data were RNA binding proteins, which is similar to previous Gag interaction studies (Roy et al., 2006; Jäger et al., 2012). Interactions detected in AP experiments may be either direct protein–protein interactions, or may be mediated by another factor. RNA-binding proteins such as HIV-1 Gag are expected to pull down other RNA-binding proteins through interaction with the RNA in the complex. If specifically searching for direct interactions, AP experiments could be performed with Gag variants that do not bind RNA (Poon et al., 1996; Cimarelli et al., 2000) or following RNAse digestion. The different baits in our experiments are expected to interact with different RNAs: RNA expressed from pCHIV^{EGFP} contains the HIV-1 specific packaging signal and is thus expected to interact with Gag. This is not the case for RNA expressed from the GagEGFP plasmid, and Gag in this case will bind non-specifically to cellular RNAs, which are usually shorter than the viral genome and do not contain potential cis-acting elements. Comparing the list of RNA-binding proteins obtained for these two different baits, we observed similar enrichment of RNA-associated proteins and RNA recognition motifs (Supplementary Table 3).

Previous studies have suggested that HIV-1 Gag may capture viral RNA near the centriole (Poole et al., 2005) with intracellular localization of HIV RNA depending on Gag (Lehmann et al., 2009). Furthermore, the formation of an RNP transport complex consisting of Gag, RNA and host cell proteins has been suggested (Cochrane et al., 2006). Different classes of cellular ribonucleoproteins (RNPs) have been described, including P bodies, stress granules (SGs), U bodies, neuronal transport RNP granules, RNP-rich granules in germline cells, sponge bodies, TAM bodies and cytoplasmic PrP-induced RNP granules (for review, see Moser and Fritzler, 2010). A recent study suggested that HIV replication is

independent of P body components and that Gag does not co-localize with P bodies (Phalora et al., 2012), while previous reports observed localization of HIV mRNAs within P bodies and enhanced HIV replication after P body disruption (Chable-Bessia et al., 2009; Nathans et al., 2009). The P body component MOV10 has been shown to influence HIV replication (Burdick et al., 2010; Furtak et al., 2010). Recruitment of DDX6 and additional P body components by Gag to facilitate assembly has been hypothesized recently (Reed et al., 2012). Blocking of SG formation by HIV has been reported (Abrahamyan et al., 2010) and sequestration of HIV RNA in SG's mediated by APOBEC3G has been suggested (Kozak et al., 2006). The RNP component Staufen has been implicated in viral genomic RNA encapsidation previously (Mouland, 2000; Châtel-Chaix et al., 2004). Live-cell imaging of Staufen in HIV-infected cells has been performed (Milev et al., 2010) and Staufen-HIV RNP granules which neither represent stress granules nor P bodies have been characterized (Abrahamyan et al., 2010; Milev et al., 2012).

We investigated whether constituents of distinct RNP complexes were present in the Gag interaction dataset (Fig. 2a). 13 of 55 GO-listed P body components were identified. The requisite P body component GW182 was not identified, while EIF2C2 (Ago2) and RCK/p54 (DDX6) were detected. Proteins unique to P bodies (DCP1/2, LSM1-7, EDC1-4, CCR4, POP2, PAN2 and MEX3A) were not identified. of 30 GO-listed stress granule components were identified. The SG-characteristic proteins PABPC1, 40S ribosomal subunits, ELAVL1 (HuR), G3BP, EIF4A and G, Staufen, FMRP, FXR1 and 2, RACK1 (GNB2L1) and TRAF2 were identified, while TIA-1/R, ZBP1, RSK2, Mex67 were not detected. The Gag interaction data include a large proportion of proteins found in cytoplasmic RNA granules containing FMR1 (Ohashi et al., 2002; Angenstein et al., 2002), IGF2BP1 (Jonson et al., 2007; Weidensdorfer et al., 2009) and Staufen (STAU1) (Brendel et al., 2004; Villace et al., 2004): of 20 FMR1-RNP components were identified, 52 of 65 STAU1-RNP proteins and 34 of 46 IGF2BP-1 components were represented in the AP data. 43 of the 77 Staufen RNP-associated proteins reported by Milev et al. (2012) were recovered in our screens. We extracted binary interactions between proteins in FMR1-, IGF2BP1- and Staufen-RNA granules from the STRING database (Franceschini et al., 2013) and created networks depicting these complexes with annotation of AP/MS hits and factors implicated in HIV replication (Fig. 2b). Taken together, our AP/MS results favor association of Gag with RNP complexes which are distinct from P bodies and stress granules.

Conclusions

In this report we describe results of six independent affinity purification screens to identify potential interaction partners of the HIV-1 structural protein Gag. 1804 candidate proteins were identified, 90 of these were significant hits in ≥ 3 screens. Filtering of primary data and quantitative MS data from a SILAC screen was used to distinguish specifically co-purifying proteins from potential AP artifacts. Recovery of previously characterized interactors shows that the AP/MS approach is in principle suitable for the identification of cellular proteins which interact with HIV Gag. Proteins previously found in HIV particles were also detected, indicating that an interaction with Gag may lead to incorporation of these proteins. Some hits from siRNA screens were identified as potential Gag interaction partners, although these screens primarily addressed early steps of HIV replication.

Analysis of the proteins identified showed an enrichment of distinct protein motifs and molecular pathways. A large number of helicases, translation factors and cytoskeletal proteins co-purified with HIV Gag. Three cellular complexes were identified in the AP/MS experiments: the tRNA synthetase complex, a network of SR proteins and their interaction partners as well as a complex consisting of centrosomal proteins, gamma tubulins and kinesins. The major proportion of screening hits were RNA binding proteins. Factors involved in RNA degradation and RNA interference were significant hits. Most prominently, RNP complex components were enriched in the dataset. RNP components in the interaction data overlapped less with P bodies and stress granules than with Staufen-, FMR1- and IGF2BP1-containing cytoplasmic RNA transport granules, suggesting that HIV may hijack RNA transport compartments during trafficking of viral RNA. In summary, this study provides a rich source of potential novel cellular interaction partners of HIV Gag. Further investigations addressing the biological function of these host cell factors may contribute to our understanding of HIV replication.

Methods

AP/MS screens

Affinity purification using anti-GFP microbeads (Cristea et al., 2005), GFP Trap_A (Rothbauer et al., 2008) and tandem affinity purification (Rigaut et al., 1999; Gingras et al., 2005) with pGagC-TAP, pGag-EGFP (Hermida-Matsumoto and Resh, 2000) and pCHI-V^{EGFP} (Müller et al., 2004) as baits and mass spectrometry were performed as described in Engeland et al. (2011). For details, see Supplementary methods.

Data extraction and filtering

Protein identifiers for all hits were extracted from Mascot (Beads, Nano and CTAP screens) (Matrix Science Ltd., 2008) and MaxQuant (SILAC screen) (Cox and Mann, 2008) and stored in a relational database (PostgreSQL). Common AP artifacts as listed by the Global Proteome Machine Organization (cRAP), the Max Planck Institute of Biochemistry, Martinsried and by Trinkle-Mulcahy et al. (2008) were used to annotate the screening data with filters based on PANTHER protein family and subfamily terms (Thomas et al., 2003), NCBI filtered GO terms (Ashburner et al., 2000) and official protein names (Eyre et al., 2006). For a more detailed description, see Supplementary methods.

Data analysis, annotation and protein interaction networks

Previously characterized HIV/host protein interactions from the NIAID HIV interaction dataset (Ptak et al., 2008) were obtained from the NCBI (data as of 2010-08-20). The dataset includes a wide range of types of interaction data for the Gag (Pr55) polyprotein and its processing products matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and p6. We reviewed the associated literature for these to identify validated direct Gag–protein interactions. Proteins incorporated in virions were extracted from NCI Frederick and Santos et al. (2012). Host factors identified in siRNA screens were extracted from Konig et al. (2008), Brass et al. (2008), Zhou et al. (2008a). Gene ontology annotation (Ashburner et al., 2000) of candidates and enrichment analysis of protein domains (Hunter et al., 2012) within the dataset was performed with the DAVID Functional Annotation Tool (Huang et al., 2009a, 2009b). Protein–protein interaction data were obtained from the STRING database (Franceschini et al., 2013). Protein–protein interaction networks were visualized using Cytoscape (Shannon et al., 2003).

Supplementary methods

Tissue culture and DNA transfection

293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM). For stable isotope labeling by amino acids in cell culture (SILAC), DMEM deficient in Arg and Lys was used (Pierce SILAC quantification kit, Thermo Fisher Scientific, Bonn, Germany) as described in Ong et al. (2002). 293T cells were seeded at a density of 3×10^6 cells per 10 cm dish 24 h prior to transfection with Fugene (Roche Diagnostics) according to the manufacturer's instructions. Expression of Gag-EGFP, MA-EGFP Gag and EGFP was monitored by fluorescence microscopy.

Affinity purifications

In this study, three different affinity purification methods were employed: purification with magnetic microbeads, GFP nanotrap and tandem affinity purification (TAP). For the Beads screens, purification with magnetic anti-GFP microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed using standard methods as described previously (Engeland et al., 2011). For the Nano screens, affinity purification with GFP Trap_A (ChromoTek, Martinsried, Germany) was carried out as described by Rothbauer et al. (2008). pEGFPc1 (Invitrogen, Carlsbad, CA) was used as negative control for the microbead and nanotrap screens. The CTAP tandem affinity purification screen was performed as described by Gingras et al. (2005) using a Gag expression plasmid with a C-terminal TAP tag (pGagCTAP) and the TAP tag only (pCTAP) as negative control. For the SILAC screen, cells grown in heavy media were transfected with pCHIV^{EGFP} (Müller et al., 2004), encoding MA-EGFP Gag, while cells grown in light media were transfected with pEGFPc1 (Invitrogen). Affinity purification with anti-GFP microbeads was performed with lysates from both cell populations. Affinity purification samples were resolved on 12.5% low cross-linking polyacrylamide gels. After separation, gels were stained with Coomassie (Imperial Protein Stain, Pierce) and 2 mm slices were excised for analysis by mass spectrometry.

Mass spectrometry

Mass spectrometry for microbeads and nanotrap screens: Gel pieces were reduced with DTT, alkylated with iodoacetamide and digested with trypsin (Catrein et al., 2005) using a Digest pro MS liquid handling system (Intavis AG, Cologne, Germany). Following digestion tryptic peptides were extracted from the gel pieces with 50% acetonitrile/0.1% TFA, concentrated nearly to dryness in a speedVac vacuum centrifuge and diluted to a total volume of 0 µl with 0.1% TFA. 25 µl of the sample was analyzed by a nanoHPLC system (Dionex, Amsterdam, Netherlands) coupled to a ESI LTQ Orbitrap mass spectrometer (Thermo Fisher). Sample was loaded on a C18 trapping column (Inertsil, LC Packings, Amsterdam, Netherlands) with a flow rate of 10 µl/min 0.1% TFA. Peptides were eluted and separated on an analytical column (75 µm × 150 mm) packed with Inertsil 3 µm C18 material (LC Packings) with a flow rate of 200 nl/min in a gradient of buffer A (0.1% formic acid) and buffer B (0.1% formic acid, acetonitrile): 0–6 min: 3% B; 6–60 min: 3–40% B; 60–65 min: 60–90% B. The column was connected with a nano ESI emitter (New Objectives, Woburn, MA). 1500 V were applied via liquid junction. One survey scan (res: 60,000) was followed by 5 information dependent product ion scans in the LTQ. Only doubly and triply charged ions were selected for fragmentation. Tandem mass spectra were extracted by Mascot Daemon without grouping or smoothing and analyzed using Mascot (version 2.2.04, Matrix Science Ltd., 2008). Mascot was set up to search the International Protein Index (IPI, version 3.48) and NCBI nr (version 2008-08-20) databases, using trypsin as protease, a fragment ion mass tolerance of 0.20 Da and a parent ion tolerance of 4.0 ppm. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine, oxidation of methionine and phosphorylation of serine and threonine were specified in Mascot as variable modifications. Only protein hits with a probability of $p < 0.05$ for a random match were listed. *Mass spectrometry for TAP screen*: Tryptic peptides from Coomassie-stained proteins were prepared for mass spectrometry as described in Shevchenko et al. (1996). Samples were analyzed using a Bruker Reflex III MALDI TOF instrument, and proteins were identified using Mascot/MOWSE (version 2.1) and the NCBI nr database (version 2007-03-02), with mass tolerance set to 65 ppm.

Mass spectrometry for SILAC: Tryptic digestion, protein identification and quantification was performed as recently described (Lange et al., 2010). In brief, after tryptic in-gel digestion, the extracted peptide solution was taken to dryness under vacuum and samples were reconstituted in 6 µL of 0.1% (v/v) TFA, 5% (v/v) acetonitrile in water. LC MS/MS analyses were performed on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) equipped with an Eksigent 2D nanoflow LC system (Axel Semrau GmbH, Sprockhovel, Germany). Separations were performed on a capillary column (Atlantis dC18, 3 µm, 100 Å, 150 mm × 75 µm i.d., Waters, Milford, MA) at an eluent flow rate of 250 nL/min using a linear gradient of 040% B in 50 min. Mobile phase A was 0.1% formic acid (v/v) in water; mobile phase B was 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan (with a resolution of 60,000) in the Orbitrap and MS/MS scans of the five most intense precursor ions in the LTQ. The MS survey range was m/z 3501500. The dynamic exclusion time (for precursor ions) was set to 120 sec and automatic gain control was set to 3×10^6 and 20,000 for Orbitrap MS and LTQ MS/MS scans, respectively. Identification and quantification of proteins were carried out with version 1.0.12.31 of the MaxQuant software package (Cox and Mann, 2008). Generated peak lists (msm files) were submitted to a Mascot search engine (version 2.2, Matrix Science Ltd., 2008) and searched against an IPI human protein database (version 3.52). The mass tolerance of precursor and sequence ions was set to 7 ppm and 0.3 Da, respectively. Methionine oxidation and the acrylamide modification of cysteine were used as variable modifications. False discovery rates were $< 1\%$, based on matches to reversed sequences in the concatenated target decoy database. Labeling efficiency with $^{13}C_6$ L-lysine and $^{13}C_6$, $^{15}N_4$ L-arginine was determined to be 99%.

Data extraction

Protein hits for the microbeads (BeadsC, BeadsMA), nanotrap (NanoC, NanoMA) and negative control (GFP) screens were obtained in the form of NCBI gi numbers and EMBL/EBI IPI identifiers. Corresponding sequence records were fetched from NCBI or EBI and parsed to extract the NCBI Entrez Gene taxon and GeneID, if present. CTAP and negative control (TAP) results were mined from the web pages generated by Mascot (Matrix Science Ltd., 2008). Protein sequence identifiers in the form of NCBI gi numbers, NCBI RefSeq, NCBI GenPept, PIR and UniProtKB identifiers were extracted for all proteins found. All extracted identifiers were looked up at NCBI (gi numbers, RefSeq, GenPept identifiers) or at ExPASy (PIR, UniProtKB identifiers). For each of Beads, Nano and CTAP, only human accessions (NCBI taxon 9606) with a GeneID were retained. Hits were then filtered to remove proteins with fewer than 3 peptides, as well as any hits found in the corresponding negative control, regardless of number of peptides in the latter. SILAC mass spectrometry data obtained using MaxQuant were processed to extract sequence identifiers. The corresponding taxon and GeneID were determined for each RefSeq, UniProt and KEGG (Kanehisa and Goto, 2000) identifier

present in each MaxQuant hit. Human proteins with a GeneID were retained. Significant hits were selected satisfying the criteria H/L ratio >5.0 and ≥ 3 peptides identified. All extracted screening data were stored in a relational database (PostgreSQL) along with a reference collection of human loci with all historical GeneID obtained from NCBI Gene (data as of 2010-08-20). Every protein in the screens or in published papers with an associated GeneID could be cross referenced through the common locus and associated with other data, such as current and obsolete HGNC (Eyre et al., 2006) gene symbols and synonyms, pathway information, RNA interference data and protein–protein interaction data (see Börner et al., 2010 for more information).

Contaminant and artifact filtering

The mass spectrometry analysis software identifies and reports proteins based on matching peptides in sequence databases. These are predominantly machine generated translations of predicted gene products rather than experimentally confirmed protein sequences. Accordingly, some are actually pseudogenes and a simple filter based on the Entrez annotation of each sequence accession was used to exclude this kind of data processing artifact. More problematic are contaminants and artifacts arising from the experimental procedures. MaxQuant output for SILAC indicates some peptide profiles as being likely due to contaminants from material handling: mostly keratins, actins, tubulins, tropomyosins and non-human proteins including caseins and trypsin. Additional lists of common AP/MS contaminants were obtained from the Global Proteome Machine Organization (cRAP) and the Max Planck Institute of Biochemistry, Martinsried. Hits from all screens were annotated using the combined contaminant list. A comprehensive set of probable artifacts was constructed based upon the findings of Trinkle-Mulcahy et al. for Protein G-conjugated Sepharose, agarose and magnetic bead screens (Trinkle-Mulcahy et al., 2008) yielding the following classes: cytoskeletal, structural and motility proteins (actin, myosin, tubulin, tropomyosin, cofilin, filamin, desmoplakin, epiplakin, plectin), intermediate filaments (including desmin, peripherin, vimentin, keratin), DEAD/H Box proteins, translation elongation and initiation factors, heat shock proteins, histones, ribonucleoproteins (hnRNP), ribosomal proteins. Trinkle-Mulcahy et al. also present a list of 222 additional non-specifically binding proteins which was extracted and assigned to a miscellaneous class. Specific filters for these classes of protein were prepared using PANTHER protein family and subfamily terms (version 6.1.1 as of 2009-02-09, Thomas et al., 2003), NCBI filtered GO terms (data as of 2010-08-20, Ashburner et al., 2000) and official protein names (Eyre et al., 2006). Hits from all screens were then annotated using these artifact classes.

Authors' contributions

C.E.E., G.A.M. and H.G.K. conceived of and designed the experiments. C.E.E. performed the affinity purification screens. C.E.E. and G.A.M. carried out the SILAC experiment. M.S. and E.K. performed quantitative mass spectrometry for the SILAC screen. K.B. carried out the siRNA screen. N.P.B. and C.E.E. extracted, filtered and analyzed the screening data. L.K. performed statistical analysis of the SILAC data. C.E.E., N.P.B. and H.G.K. wrote the paper. All authors read and approved the final paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.04.038.

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