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Identification of Small GTPases That Phosphorylate IRF3 through TBK1 Activation Using an Active Mutant Library Screen

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Abstract

Interferon regulatory factor 3 (IRF3) integrates both immunological and non-immunological inputs to control cell survival and death. Small GTPases are versatile functional switches that lie on the very upstream in signal transduction pathways, of which duration of activation is very transient. The large number of homologous proteins and the requirement for site-directed mutagenesis have hindered attempts to investigate the link between small GTPases and IRF3. Here, we constructed a constitutively active mutant expression library for small GTPase expression using Gibson assembly cloning. Small-scale screening identified multiple GTPases capable of promoting IRF3 phosphorylation. Intriguingly, 27 of 152 GTPases, including ARF1, RHEB, RHEBL1, and RAN, were found to increase IRF3 phosphorylation. Unbiased screening enabled us to investigate the sequence-activity relationship between the GTPases and IRF3. We found that the regulation of IRF3 by small GTPases was dependent on TBK1. Our work reveals the significant contribution of GTPases in IRF3 signaling and the potential role of IRF3 in GTPase function, providing a novel therapeutic approach against diseases with GTPase overexpression or active mutations, such as cancer.

Key Words: Small GTPases, IRF3, TBK1, Small-scale library screen

INTRODUCTION

The innate immune response is a rapid cellular reaction to the detection of cytosolic nucleic acids. Activation of cytosolic nucleic acid sensors is important for host defense against viral infection. However, stimulation of these sensors through accumulation of self-nucleic acids may contribute to the pathogenesis of various diseases, including sterile inflammatory diseases such as alcoholic hepatitis and steatohepatitis (Xu *et al*., 2021). Cytosolic nucleic acid sensing is also known to play a role in physiological processes such as organ regeneration (Schulze *et al*., 2018). A major sensing mechanism of cytosolic double-stranded DNA (dsDNA) is the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon gene (STING) pathway. When bound with dsDNA, cGAS produces 2'3'-cyclic GMA-AMP (cGAMP), a second messenger molecule, which activates STING (Ishikawa and Barber, 2008; Gao *et al*., 2013). Signaling through this pathway converges to the activation of interferon regulatory factor 3 (IRF3).

Recent studies have identified IRF3 as a central regulator of non-immune mediated apoptosis (Chattopadhyay *et al*., 2010). IRF3 interacts with the pro-apoptotic protein Bax to induce the mitochondrial apoptotic pathway. Phosphorylation of IRF3 by STING/TBK1 activates apoptosis in association with Bax. IRF3 can also be activated by ER stress without the presence of invading foreign material such as lipopolysaccharide (LPS), RNA, or DNA (Liu *et al*., 2012). Additionally, ER stress induced by ethanol intake has been shown to cause hepatocyte damage in a dose-dependent manner in the presence of IRF3 (Petrasek *et al*., 2013). While both obesity and diabetes can cause mitochondrial dysfunction and ER stress, studies have revealed a direct role of IRF3 in obesity and diabetes (Mao *et al*., 2017; Rizwan *et al*., 2020). Genetic ablation of IRF3 was shown to increase overnutrition-induced hepatic insulin resistance and glucose intolerance (Qiao *et al*., 2018). Another study showed that hepatic IRF3 activates PP2A through Ppp2r1b transactivation, leading to AMPK dephosphorylation, thereby promoting dysglycemia and insulin resistance (Patel

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et al., 2022). Thus, IRF3 is a significant factor in mediating not only the innate immune response but also in modulating signal transduction of local inflammatory responses.

IRF3 is a constitutively expressed transcription factor localized in the cytoplasm in its inactive form. Upon phosphorylation, it undergoes dimerization and translocation to the nucleus (Hiscott, 2007). In the nucleus, IRF3 cooperatively binds with other transcription factors, including nuclear factor-κB (NF-κB), Activating Protein-1 (AP-1), and Interferon regulatory factor 7 (IRF7) to form a multimolecular enhancer that promotes gene transcription. IRF3 can be phosphorylated via multiple pathways including the Toll-like receptors (TLR) signaling pathway, which recognizes pathogen-associated molecules such as LPS (Shinobu *et al*., 2002); the STING pathway, which recognizes cytoplasmic DNA (Ishikawa and Barber, 2008); and the RIG-1 pathway, which recognizes RNA (Yoneyama *et al*., 2004). Multiple serine and threonine residues in IRF3 can be regulated by several upstream kinases, the best-characterized among them being TBK1 (Fitzgerald *et al*., 2003). Most innate immune sensors phosphorylate IRF3 through TBK1. Nevertheless, IRF3 can also be regulated by other upstream kinases. IRF3 phosphorylation by JNK phosphorylates the N-terminal serine 173 residue, which differs from the TBK1-mediated phosphorylation site(s). JNK inhibition reduced the expression of IRF3 target genes such as Rantes and Interferon-stimulated gene 15 (ISG15) (Zhang *et al*., 2009). Other upstream kinases such as, c-Abl and c-Abl-related kinase (Arg), also phosphorylate IRF3 at the tyrosine 292 residue. A c-Abl inhibitor reduced the induction of IFNB by IRF3 (Luo *et al*., 2019). Thus, it is crucial to identify upstream signals that may enhance or repress IRF3 phosphorylation to regulate pathological outcomes caused by IRF3 dysregulation.

Over 150 small GTPases have been identified in humans, comprising five families: ARF, RHO, RAS, RAB, and RAN, based on similarities in their G domain sequences. Cytoplasmic small GTPases regulate a variety of cellular processes by altering their conformational forms. In their active form, small GTPases signal through effector proteins to regulate multiple cellular functions, such as cell proliferation, cell death, microtubule dynamics, vesicle transport, and protein transport between the nucleus and cytosol (Balch, 1990; Boman *et al*., 1992; Etienne-Manneville and Hall, 2002; Kahn *et al*., 2005; Hodge and Ridley, 2016). Aberrant Small GTPases are associated with a multiple of human diseases such as cancer, neurodegenerative diseases, and inflammatory diseases. For instance, activating mutations of Ras subfamily proteins such as KRAS, HRAS, NRAS have been extensively studied as a driver mutation of tumor transformation in a vast majority of tissues (Punekar *et al*., 2022). In addition, role of KRAS has been implicated in inflammatory diseases including Rheumatoid arthritis (Singh *et al*., 2012). In Alzheimer's disease, overactivation of RhoA leads to amyloid β production and accumulation by promoting secretase-dependent cleavage amyloid precursor protein (Guiler *et al*., 2021).

Some small GTPases have been reported to suppress innate immune responses. For instance, ARL5B and ARL16 inhibit RIG-I and MDA5, respectively, in the RNA-sensing RLR pathway and suppress the production of type I interferon against viral RNA sensing (Yang *et al*., 2011; Kitai *et al*., 2015). In contrast, some small GTPases activate innate immune responses. For example, RAB1B binds to TRAF3 to promote the formation of MAVS-TRAF2/3 complex, thereby facilitating innate immune response through TBK1-IRF3 signaling (Beachboard *et al*., 2019). Another small GTPase, RAB2B, forms a complex with GARIL5 to regulate the cGAS-STING signaling axis to promote IFN responses to DNA viruses (Takahama *et al*., 2017). Rac1 transactivates NF-κB during TLR2 stimulation upon bacterial invasion (Arbibe *et al*., 2000). RAB11a is also involved in the recruitment of TLR4 and TRAM to the phagosome during bacterial invasion and induces the expression of *IFNB* through IRF3 signaling (Husebye *et al*., 2010). Although several small GTPases are involved in innate immune responses, the link between small GTPases and IRF3 remains unknown.

Small GTPases utilize GDP/GTP alternation to actuate functional switches (Cherfils and Zeghouf, 2013). As they lie upstream in signal transduction pathways, GTPases only remain transiently active. In the basal state, small GTPases remain inactive in their GDP-bound conformation. After activation, they quickly return to an inactive state by intrinsic hydrolysis of GTP. Thus, overexpression of individual GTPases in an unedited form does not ensure proper screening validity. To investigate the role of small GTPase activation by ectopic expression, it is crucial to utilize constitutively active mutants by deleting the intrinsic GTPase domain, which lacks an autoinhibitory function. Attempts to comprehensively investigate the role of GTPases have been unsuccessful due to the need for labor- and time-intensive site-directed mutagenesis and the large number of homologous proteins. Here, we compared small GTPases in an unbiased manner using a small-scale constitutively active mutant expression library. We discovered multiple GTPases that increase IRF3 phosphorylation and investigated the sequence-activity relationship. In addition, our study revealed that the regulation of IRF3 by small GTPases is generally dependent on TBK1, emphasizing the role of the kinase in the link between GTPase signaling and innate immunity or other IRF3-mediated functions.

MATERIALS AND METHODS

Active mutant expression library construction

The pRK7 vector (Addgene plasmid #10883) was modified to have a HA-tag on either N- or C-terminus of each gene to be cloned. The tagged empty vectors were then linearized with two different restriction enzymes. The small GTPase inserts were amplified using purified human cDNA which was obtained from HEK293 or MCF7 cell line according to respective mRNA abundance. To simultaneously introduce constitutively active mutation, each gene was amplified in two parts utilizing mutagenic primers on the joining side. Gibson assembly reactions were done at 50°C for 1 h using NEBuilder HiFi DNA assembly master mix (New England Biolabs, Ipswich, MA, USA). After purification, all resultant plasmids were further verified by Sanger sequencing. The introduced mutations are listed in Table 1.

Cell culture, DNA transfection and treatment

HEK293 and MCF7 cell lines were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 ug/mL streptomycin Invitrogen (Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO_2. For DNA trans-

Table 1. Amino acid residues with induced mutations in small GTPases

Symbol	Mutation	Symbol	Mutation	Symbol	Mutation
Arf subfamily		Rrad	P100V	Rab27A	Q78L
Arl3	Q71L	Rem1	P89V	Rab27B	Q78L
Arl2	Q70L	Rem2	Q173L	Rab28	Q72L
Arf5	Q71L	Diras1	G16V	Rab30	Q68L
Arf4	Q71L	Diras2	G16V	Rab32	Q85L
Arf3	Q71L	Diras3	Q95L	Rab33A	Q95L
Arf1	Q71L	RasD1	S33V	Rab33B	Q92L
Arf6	Q67L	RasD2	S30V	Rab34	Q111L
TRIM23	K458I	RasL10B	I63L	Rab35	Q67L
Arl1	Q71L	RasL10A	P13V	Rab36	Q182L
Arl5B	Q70L	NkiRas1	WT	Rab37	Q82L
Arl5A	Q70L	NkiRas2	WT	Rab38	Q69L
Arl14	Q68L	Rab subfamily		Rab39A	Q72L
Arl11	Q67L	Rab1A	Q70L	Rab39B	Q68L
Arl4A	Q79L	Rab1B	Q67L	Rab40A	Q73L
Arl4C	Q72L	Rab2A	Q65L	Rab40B	Q73L
Arl4D	Q80L	Rab2B	Q65L	Rab40C	Q73L
ArfRP1	Q79L	Rab3A	Q81L	Rab41	Q90L
Arl6	Q73L	Rab3B	Q81L	Rab42	H74L
Arl13B	G75L	Rab3C	Q89L	IFT27	P14V
Sar1a	H79G	Rab3D	Q81L	RasEF	Q600L
Sar1b	H79G	Rab4A	Q72L	Rho subfamily	
Arl15	A86L	Rab4B	Q67L	Rac3	Q61L
Arl16	C86L	Rab5A	Q79L	Rac1	Q61L
Arl8A	Q75L	Rab5B	Q79L	Rac2	Q61L
Arl8B	Q75L	Rab5C	Q80L	RhoG	Q61L
Arl10	S132L	Rab6A	Q72L	Cdc42	Q61L
Arl9	S9L	Rab6B	Q72L	RhoJ	Q79L
Ras subfamily		Rab6C	Q72L	RhoQ	Q67L
Rit1	G30V	Rab7A	Q67L	RhoU	Q107L
Rit2	G29V	Rab7B	Q67L	RhoV	Q89L
Rap2C	G12V	Rab7L1	Q67L	RhoB	Q63L
Rap2A	G12V	Rab8A	Q67L	RhoC	Q63L
Rap2B	G12V	Rab8B	Q67L	RhoA	Q63L
Rap1B	G12V	Rab9A	Q66L	RhoF	Q77L
Rap1A	G12V	Rab9B	Q66L	RhoD	Q75L
Rras2	G23V	Rab10	Q68L	Rnd2	WT
Rras	G38V	Rab11A	S20V	Rnd3	WT
Mras	G22V	Rab11B	S20V	Rnd1	WT
Kras	G12V	Rab12	Q101L	RhoH	WT
Nras	G12V	Rab13	Q67L	RhoBTB2	WT
Hras	G12V	Rab14	Q70L	RhoBTB1	WT
RalB	G23V	Rab15	Q67L	Ran/unclassified	
RalA	G23V	Rab17	Q77L	Ran	Q69L
Eras	Q99L	Rab18	Q67L	IFT22	C12V
RhebL1	Q64L	Rab19	Q76L	SRPRB	C73V
Rheb	Q64L	Rab21	Q78L	RhoT1	P13V
Rerg	Q64L	Rab22A	Q64L	RhoT2	A13V
RasL12	R29V	Rab22B	Q65L	RabL3	S15V
RasL11A	G36V	Rab23	Q68L	RabL2A	Q80L
RasL11B	S42V	Rab24	S67L	RabL2B	Q80L
RergL	Q62L	Rab25	S21V	Rab20	R59L
Gem	Q84V	Rab26	Q123L		

fection, the cells were transfected with plasmids using PolyJet *in vitro* transfection reagent Signagen (Frederick, MD, USA) for 24-48 h according to the manufacturer's protocol.

Antibodies and reagents

Anti-Flag (#F1804) and anti-vinculin (#V9131) antibodies were from Sigma-Aldrich (St. Louis, MO, USA). Phos-tag acrylamide (#AAL-107) was from Wako Chemicals (Richmond, VA, USA). BX795 (#14932) was from Cayman (Ann Arbor, MI, USA). Rapamycin (#5318893) was from Peprotech (East Windsor, NJ, USA).

Immunoblot analysis

Cells were lysed with a denaturing buffer containing SDS and β-mercaptoethanol. After boiling for 5 min, proteins were separated by polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunoblotting was then performed using specific antibodies and the blots were further visualized by chemiluminescence using a digital imager. For phos-tag assay, 7.5% polyacrylamide gels were polymerized in presence of Phos-tag acrylamide and $MnCl_2$. Phos-tag gels separate phosphorylated proteins non-specifically for serine, threonine, tyrosine, and histidine phosphorylation.

Phylogenetic tree construction

Protein sequences of small GTPases were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/protein/). The sequences were compared using the Molecular Evolution Genetic Analysis (MEGA 11) software (https://www.megasoftware.net/) by multiple sequence alignment through ClustalW algorithm. The tree was constructed using maximum likelihood analysis. The confidence levels of nodes were tested by bootstrapping 100 times (Hillis and Bull, 1993).

Analysis of protein sequence identity and similarity

Sequence identities and similarities were calculated with Sequence Identities and Similarities (SIAS) software (http://imed.med.ucm.es/Tools/sias.html). For protein similarity calculation, all positively charged amino acids (Arg, Lys, and His), all negatively charged amino acids (Asp and Glu), and all aliphatic amino acids (Val, Iso, and Leu) were considered as respectively similar. Additionally, the aromatic amino acids Phe, Tyr, and Trp, the polar amino acids Asn and Gln, and the small amino acids Ala, Thr, and Ser were treated as similar, respectively. To calculate the normalized similarity score, the BLOSUM62 matrix was used.

RESULTS

Gibson assembly cloning enables efficient construction of an expression library comprising constitutively active mutant small GTPases

To investigate the role of small GTPases in IRF3 activation, we constructed an expression library comprising clones encoding individual GTPases with constitutively active mutations. Conventional site-directed mutagenesis is time- and labor-intensive and severely limits the number of constructed clones. Gibson assembly cloning using overlapping primers with variant sequences allowed us to directly clone active mutant small GTPases from wild-type cDNA (Fig. 1). Target mutation sequences were designed to ablate intrinsic GTPase activity and to increase affinity for GTP, based on previous reports and predictions according to their sequence homology with K-RAS (e.g., mutation of amino acid residues that correspond to G12 or Q61 in K-RAS). GTPases that are intrinsically deficient in GTP-hydrolyzing activity or constitutively bound to GTP were used in their native sequences (Table 1). Small GTPases were N-terminal HA-tagged as they generally undergo C-terminal isoprenylation. However, Arf GTPases were C-terminally HA-tagged because they are post-translationally modified by C-terminal myristoylation (Prakash and Gorfe, 2013). A library comprising of 152 expression clones encoding active mutant small GTPases was constructed and individually expressed with IRF3 for unbiased evaluation of their contribution to IRF3 signaling.

ARF1/3/5/6 increase IRF3 phosphorylation

The Arf family of GTPases regulates vesicular traffic and organelle structure. Recently, some members of the Arf protein family have been found to modulate molecular signaling pathways involving IRF3. For instance, ARF6 promotes IRF3 activation to induce IRF3-dependent genes that interfere with TLR4 signaling (Van Acker *et al.*, 2014). However, the role of other Arf proteins in IRF3 signaling remains largely unknown. We examined whether Arf family proteins regulate

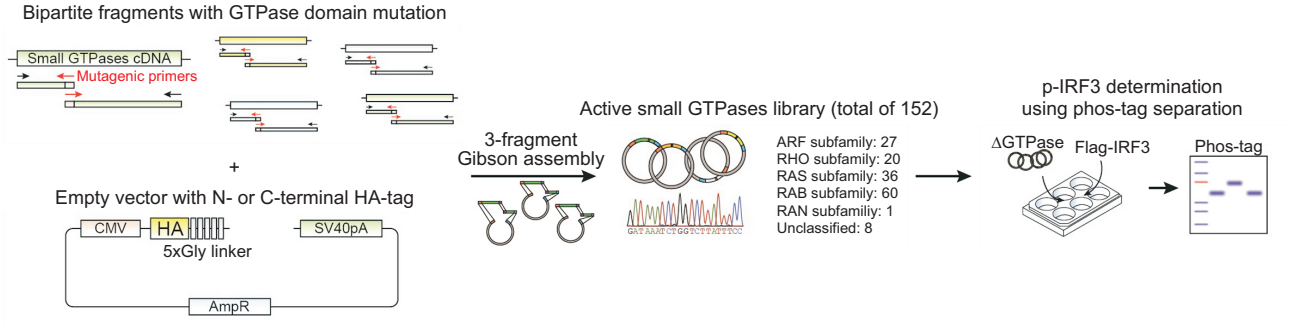
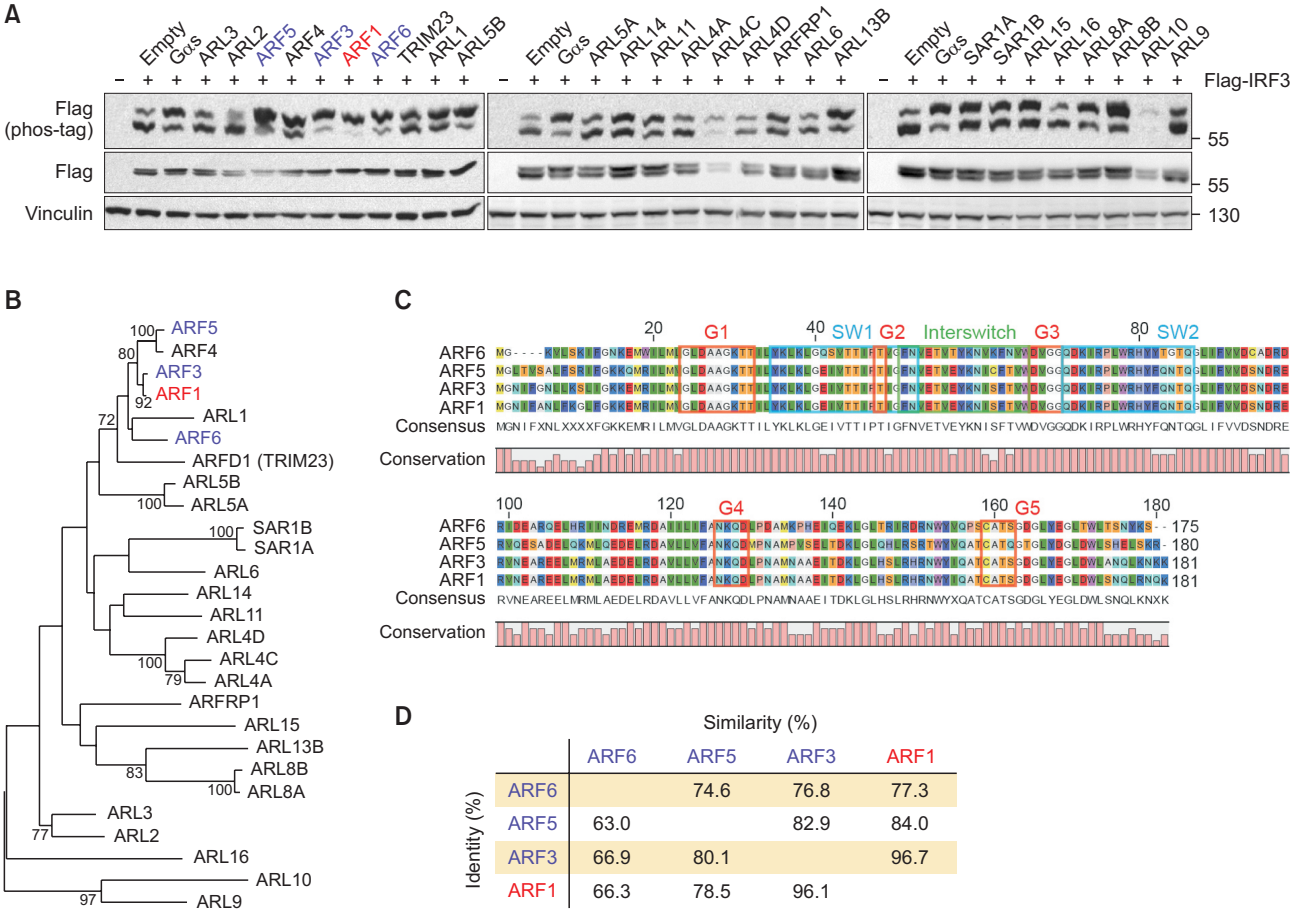


Fig. 1. Construction of an expression library comprising constitutively active mutant small GTPases. Cloning scheme for the constitutively active mutant small GTPase library. Each GTPase was amplified into two fragments by using mutagenic primers in the GTPase domain. The PCR products were then fused to the linearized backbone vector with an HA-tag on either the N- or C-terminal side of the insert site simultaneously through 3-fragment Gibson assembly reaction.

IRF3 phosphorylation, which in turn activates the innate immune response. Phosphorylation of IRF3 was examined using a phos-tag gel after co-transfection with FLAG-IRF3 and individual Arf GTPases. Among the Arf subfamily members, ARF1 robustly increased the phosphorylation of IRF3, with almost no non-phosphorylated protein. Other proteins, such as ARF3, ARF5, and ARF6, also activated IRF3, as observed by IRF3 phosphorylation (Fig. 2A). We generated a phylogenetic tree to ascertain evolutionary redundancy among ARF proteins that positively regulate IRF3 phosphorylation. Of the 27 Arf subfamily genes, 4 genes that activate IRF3 share high protein sequence homology with shared amino acid sequences and domain structures (Fig. 2B). In particular, the ARF proteins that phosphorylated IRF3 shared a very high homology throughout the entire protein, suggesting that both the core G domain and other domains are responsible for IRF3 regulation. (Fig. 2C, Supplementary Fig. 1A). The two positive Arf protein sequences showed a 62-96% match with functional similarities such as serine and threonine amino acid matching of 74-96%, implying their structure-activity relationship. (Fig. 2D, Supplementary Fig. 1B) Our results showed that the control of IRF3 by these Arf proteins may be evolutionarily conserved.

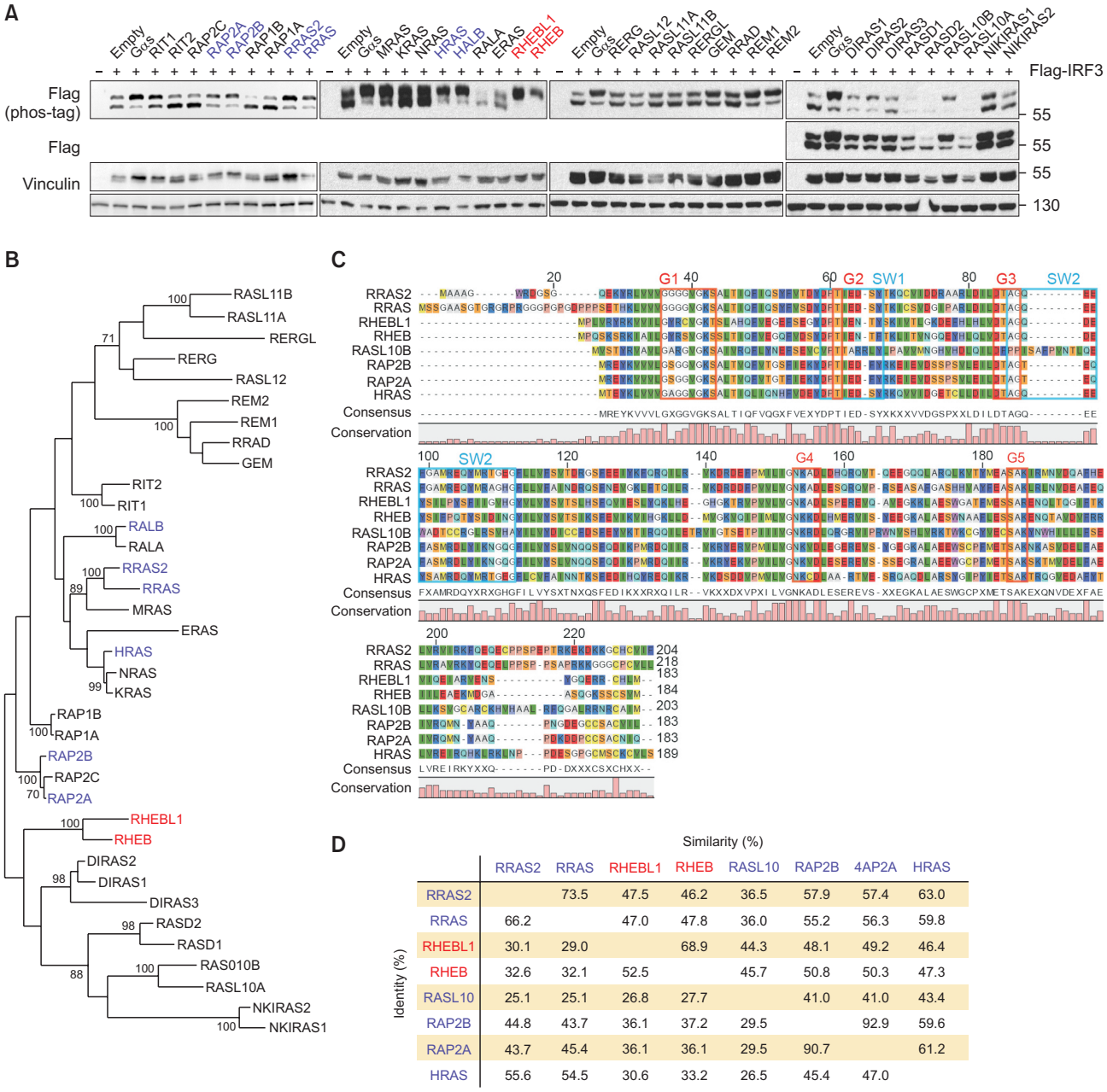
Multiple Ras proteins activate IRF3

The Ras subfamily of small GTPases is involved in cell proliferation, differentiation and apoptosis. Recently, some members of the RAS family have been shown to modulate innate immune responses. For instance, knockdown of HRAS reduces virus-induced IRF3 phosphorylation, and RLR signaling is differentially propagated according to HRAS activity (Chen *et al*., 2017). Despite the various cellular functions modulated by Ras GTPases, information on the link between RAS GTPases and cytosolic DNA sensing is limited. Therefore, we examined the role of the Ras subfamily GTPases in IRF3 activation. The expression of individual Ras GTPases along with IRF3 and their parallel comparison enabled the identification of the members that control IRF3 signaling. Phos-tag analysis clearly showed that 9 out of 36 Ras subfamilies regulate IRF3 (Fig. 3A). Notably, the effects of RHEB and RHEBL1



	ARF6	ARF5	ARF3	ARF1
ARF6		74.6	76.8	77.3
ARF5	63.0		82.9	84.0
ARF3	66.9	80.1		96.7
ARF1	66.3	78.5	96.1	

Fig. 2. ARF1/3/5/6 increases IRF3 phosphorylation. (A) HEK293 cells were co-transfected with FLAG-IRF3 and individual Arf GTPases. 24 h post transfection, the migration shift of IRF3 was determined by phos-tag gel electrophoresis. Red, strong phosphorylation; blue, weak phosphorylation. (B) Phylogenetic comparison between Arf family proteins. The sequences were aligned using ClustalW. Horizontal distance represents the proportion of amino acid difference and the branch values denote the bootstrap confidence values. (C) Alignment of amino acid sequences for Arf GTPases that positively regulate IRF3 phosphorylation. Common domain structure of Arf GTPases is shown above. (D) Protein sequence identity among Arf GTPases that increase phosphorylation of IRF3. Identity, percentage of identical residues; similarities, percentage of similar functional residues.



	RRAS2	RRAS	RHEBL1	RHEB	RASL10	RAP2B	4AP2A	HRAS
RRAS2		73.5	47.5	46.2	36.5	57.9	57.4	63.0
RRAS	66.2		47.0	47.8	36.0	55.2	56.3	59.8
RHEBL1	30.1	29.0		68.9	44.3	48.1	49.2	46.4
RHEB	32.6	32.1	52.5		45.7	50.8	50.3	47.3
RASL10	25.1	25.1	26.8	27.7		41.0	41.0	43.4
RAP2B	44.8	43.7	36.1	37.2	29.5		92.9	59.6
RAP2A	43.7	45.4	36.1	36.1	29.5	90.7		61.2
HRAS	55.6	54.5	30.6	33.2	26.5	45.4	47.0	

Fig. 3. Multiple Ras proteins activate IRF3. (A) HEK293 cells were co-transfected with FLAG-IRF3 and individual Ras GTPases. 24 h post transfection, the migration shift of IRF3 was determined by Phos-tag gel electrophoresis. Red, strong phosphorylation; blue, weak phosphorylation. (B) Phylogenetic comparison between Ras family proteins. The sequences were aligned using ClustalW. Horizontal distance represents the proportion of amino acid difference and the branch values denote the bootstrap confidence values. (C) Alignment of amino acid sequences for Ras GTPases that positively regulate IRF3 phosphorylation. Common domain structure of Ras GTPases is shown above. (D) Protein sequence identity among Ras GTPases that increase phosphorylation of IRF3. Identity, percentage of identical residues; similarities, percentage of similar functional residues.

were particularly robust. However, protein sequence comparison showed a lack of correlation between sequence homology and IRF3 phosphorylation activity, unlike the Arf GTPases (Fig. 3B). RHEB and RHEBL1 differ from other small Ras GTPases in their G1 domain sequence, which may contribute to their strong phosphorylation of IRF3. As expected, the overall sequence identity was as low as 25% among the positive hits, and the similarity varied from 35% to 92% (Fig. 3C, 3D). Our data suggest that specific functions exerted by RHEB and RHEBL1, such as controlling protein synthesis through mTOR regulation, may be related to IRF3 signaling and cytosolic DNA sensing.

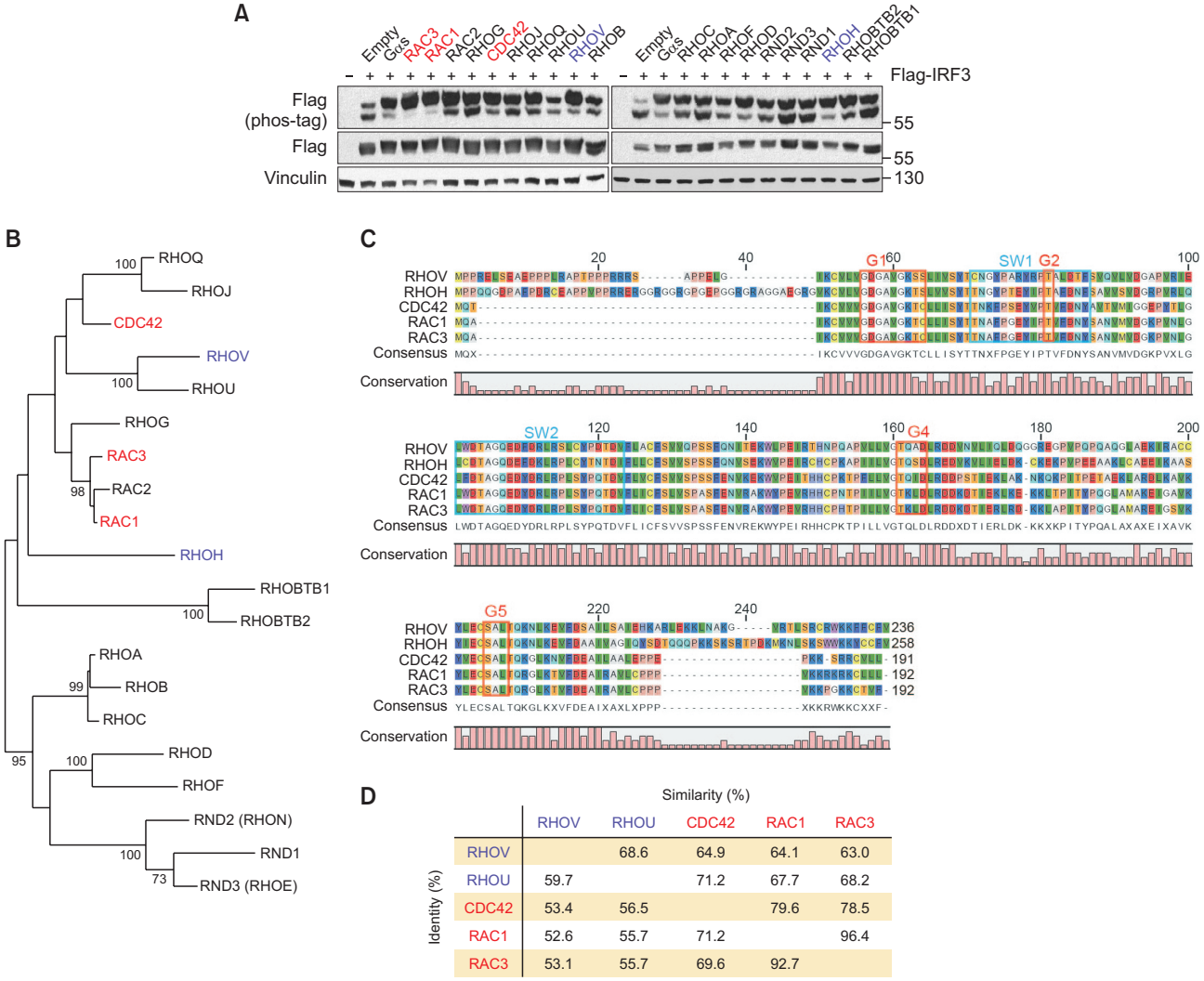
RAC1/3 and CDC42 phosphorylate IRF3

Rho GTPases are primarily responsible for regulating actin organization, cell morphology, and polarity. RAC1 is activated

by viral infection, and inhibition of RAC1 reduces IRF3 phosphorylation and IFNB promoter activity (Ehrhardt *et al*., 2004). The link between other Rho GTPases and IRF3 has not yet been elucidated. We found that five out of 20 Rho GTPases, namely RAC1/3, CDC42, RHOH, and RHOV, upregulated phospho-IRF3 (Fig. 4A). The most robust signaling GTPases were RAC1/3 and CDC42, whereas most of the other RhoA-related proteins had little or no effect on IRF. Intriguingly, RHOH and RHOV, which possess higher sequence homology with RAC1/3 and CDC42 than RHOA, also had some effects on IRF3 phosphorylation (Fig. 4B). As expected, the five positive Rho GTPases were also similar in domain structure and amino acid sequences. The identity of the RHO proteins that phosphorylate IRF3 was approximately 52-92%, and the similarity was approximately 63-96% (Fig. 4C, 4D). It is noteworthy that RAC1/3 and CDC42 play a key role in the positive regulation of cell motility and protrusion, whereas RHOA and others similar members exert the opposite effect. Based on our observations, we hypothesize that cellular movement or actin polymerization may be closely linked to IRF3 signaling.

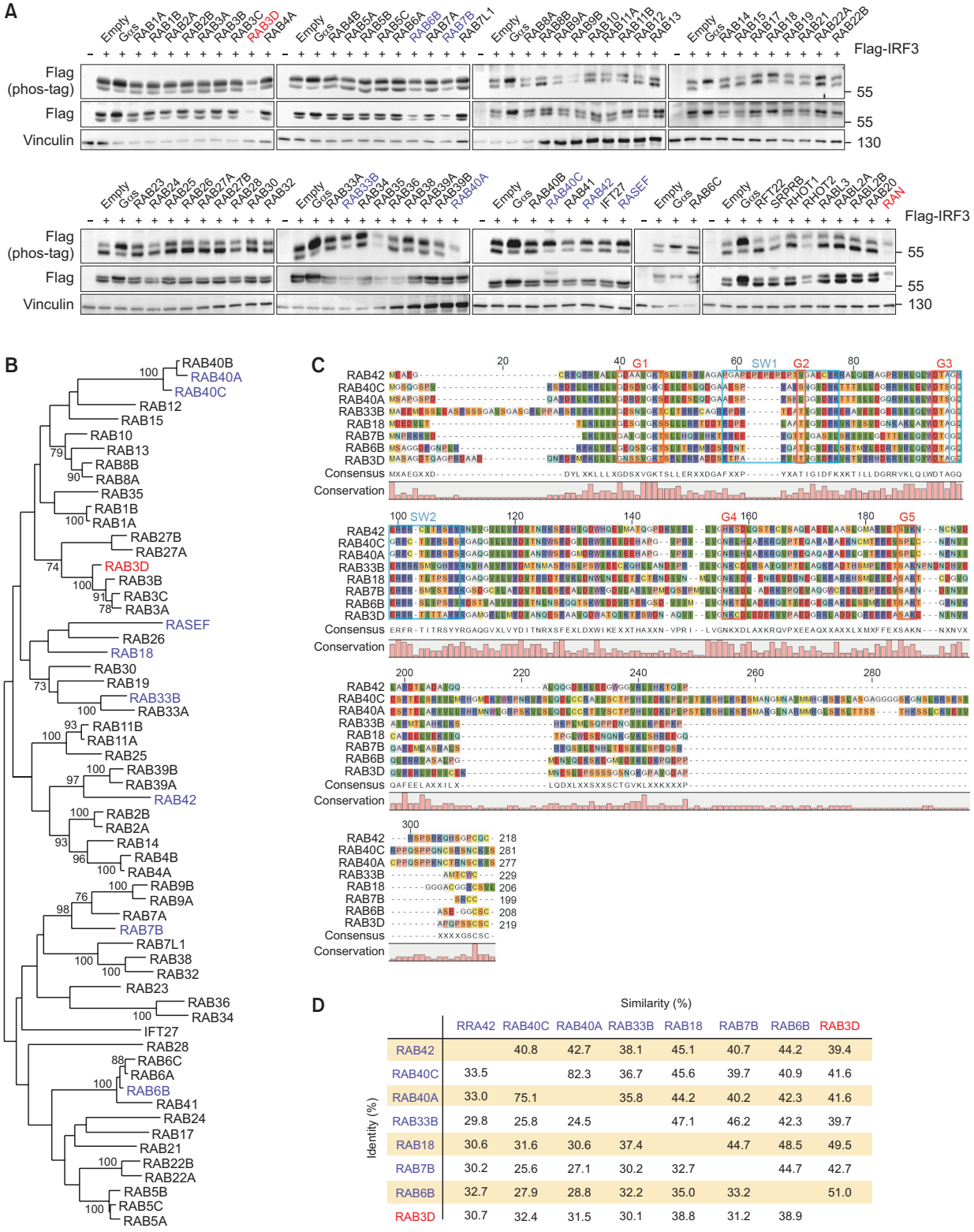
Multiple RAB GTPases and RAN stimulate IRF3 phosphorylation

Rab family G proteins control vesicular trafficking and endocytosis. Despite the large number of members, only RAB7 has been shown to block TBK1-mediated phosphorylation of IRF3 (Yang *et al*., 2016). Here, we examined the effects of the Rab subfamily and other unclassified GTPases, including RAN on IRF3 phosphorylation. We found that Rab GTPases, including RAB7, were less potent in phosphorylating IRF3, with the



	RHOV	RHOU	CDC42	RAC1	RAC3
RHOV		68.6	64.9	64.1	63.0
RHOU	59.7		71.2	67.7	68.2
CDC42	53.4	56.5		79.6	78.5
RAC1	52.6	55.7	71.2		96.4
RAC3	53.1	55.7	69.6	92.7	

Fig. 4. RAC1/3 and CDC42 phosphorylate IRF3. (A) HEK293 cells were co-transfected with FLAG-IRF3 and individual Rho GTPases. 24 h post transfection, the migration shift of IRF3 was determined by Phos-tag gel electrophoresis. Red, strong phosphorylation; blue, weak phosphorylation. (B) Phylogenetic comparison between Rho family proteins. The sequences were multiply aligned by using ClustalW. Horizontal distance represents the proportion of amino acid difference and the branch values denote the bootstrap confidence values. (C) Alignment of amino acid sequences for Rho GTPases that positively regulate IRF3 phosphorylation. Common domain structure of Rho GTPases is shown above. (D) Protein sequence identity among Rho GTPases that increase phosphorylation of IRF3. Identity, percentage of identical residues; similarities, percentage of similar functional residues.



	RRA42	RAB40C	RAB40A	RAB33B	RAB18	RAB7B	RAB6B	RAB3D
RAB42		40.8	42.7	38.1	45.1	40.7	44.2	39.4
RAB40C	33.5		82.3	36.7	45.6	39.7	40.9	41.6
RAB40A	33.0	75.1		35.8	44.2	40.2	42.3	41.6
RAB33B	29.8	25.8	24.5		47.1	46.2	42.3	39.7
RAB18	30.6	31.6	30.6	37.4		44.7	48.5	49.5
RAB7B	30.2	25.6	27.1	30.2	32.7		44.7	42.7
RAB6B	32.7	27.9	28.8	32.2	35.0	33.2		51.0
RAB3D	30.7	32.4	31.5	30.1	38.8	31.2	38.9	

Fig. 5. Multiple RAB GTPases and RAN stimulate IRF3 phosphorylation. (A) HEK293 cells were co-transfected with FLAG-IRF3 and GTPases of Rab family, unclassified GTPases, or Ran. 24 h post transfection, the migration shift of IRF3 was determined by Phos-tag gel electrophoresis. Red, strong phosphorylation; blue, weak phosphorylation. (B) Phylogenetic comparison between Rab family proteins. The sequences were multiply aligned by using ClustalW. Horizontal distance represents the proportion of amino acid difference and the branch values denote the bootstrap confidence values. (C) Alignment of amino acid sequences for Rab GTPases that positively regulate IRF3 phosphorylation. Common domain structure of Rab GTPases is shown above. (D) Protein sequence identity among Rab GTPases that increase phosphorylation of IRF3. Identity, percentage of identical residues; similarities, percentage of similar functional residues.

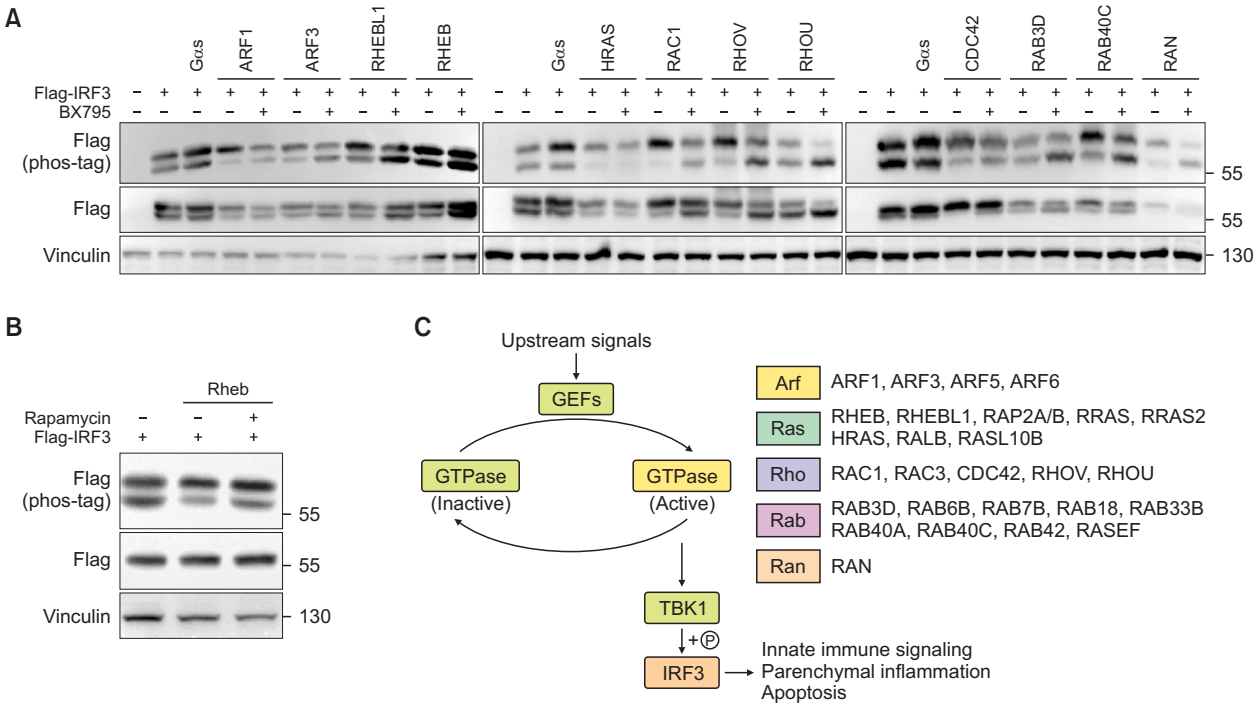


Fig. 6. IRF3 phosphorylation mediated by small GTPases require TBK1. (A) HEK293 cells were co-transfected with 0.2 μg of FLAG-IRF3 and 0.2 μg of GTPases, respectively. 24 h post transfection, the cells were treated with BX795 for 3 h. (B) HEK293 cells were transfected with 0.2 μg of FLAG-IRF3 and 0.2 μg of HA-RHEB-Q64L or empty plasmid. 24 h post transfection, the cells were treated with Rapamycin or vehicle for 3 h. (C) Overall scheme of this study.

exception of RAB3D (Fig. 5A). Intriguingly, RAB3A/B/C, which share protein homology with RAB3D, did not show a similar effect (Fig. 5B-5D).

Notably, RAN significantly increased IRF3 phosphorylation. Unlike other GTPases, RAN shuttles between the nucleus and cytoplasm to facilitate intracellular protein relocation. We speculate that at least one of the crucial upstream kinases of IRF3 is a nuclear-cytoplasmic shuttling kinase(s) that requires RAN in the cytoplasm.

Most IRF3 phosphorylation by small GTPase overexpression occurs via TBK1

Next, we investigated whether TBK1 is required for small GTPase-mediated phosphorylation of IRF3 by using BX795, a well-known TBK1 inhibitor that inhibits of IRF3 phosphorylation (Fig. 6A). Surprisingly, every GTPases we have tested required TBK1 activity to regulate IRF3 except for Rheb/RhebL1. Although the contribution of TBK1 in the GTPase-mediated functional alterations is to be identified, the data clearly shows TBK1 as an important link between IRF3 and GTPase signaling. To identify how Rheb/RhebL1 can signal through IRF3, we have tested the role of mTOR on downstream IRF3 phosphorylation. Inhibition of mTOR by rapamycin partially diminished phosphorylation of IRF3 in cells overexpressed with constitutively active Rheb (Fig. 6B). In summary, we demonstrated that multiple small GTPases can phosphorylate IRF3, and the phosphorylation is largely dependent on TBK1 (Fig. 6C).

DISCUSSION

In this study, we showed that (1) multiple small GTPases have the potential to phosphorylate and regulate IRF3; (2) this phosphorylation occurs through TBK1 and is inhibited by BX795; and (3) small GTPases that phosphorylate IRF3 showed protein domain homology in each GTPase family, with some phylogenetic distance.

Our study demonstrates that the small GTPase ARF1 is a potent inducer of IRF3 phosphorylation. A recent study demonstrated that cGAMP stimulation activated ARF1 by enhancing its binding to GGA3 (Gui *et al*., 2019). Thus, it is possible that ARF1 controls cytosolic DNA sensing in cells by controlling IRF3 phosphorylation. ARF1 expression is elevated in breast, colon/colorectal, gastric and liver cancers (Casalou *et al*., 2020). Since cytosolic DNA is significantly increased in cancer patients (Qin *et al*., 2016), this mechanism may play a role in cancer proliferation. Furthermore, the inhibition of IRF3 may provide a new therapeutic strategy to repress ARF1-mediated cancer proliferation.

We showed that nine out of 36 RAS small GTPases phosphorylate IRF3. Among these, RHEB and RHEBL1 were the strongest stimulators of IRF3. Rheb/RhebL1 are distinct from other RAS GTPases in their activation of mTOR, which is involved in cell proliferation, autophagy, and apoptosis. It has been recently reported that activation of mTOR complex has a potential to promote IRF3 nuclear translocation and target gene expression (Öhman *et al*., 2015; Bodur *et al*., 2018). Our data adds more value to these findings by suggesting Rheb as an important upstream mTOR regulator to control IRF3 activity. Rheb/RhebL1 act as cellular sensors of nutrients, energy

levels, and growth factors (MacKeigan and Krueger, 2015). Our data expand the current knowledge that Rheb/RhebL1 may serve as an innate immunity trigger by integrating cellular inputs other than cytosolic DNA into the IRF3 transcriptome. For other Ras proteins that phosphorylate IRF3, the sequence homology is low. However, in spite of the low homology they converge on downstream signaling which involves RAF, MEK, ERK and others. Therefore, one of the signaling molecules commonly activated by RAS signaling might interact closely with IRF3.

Another key finding of our study was that the expression of mutant RAN activated IRF3. RAN shuttles between the nucleus and cytoplasm to facilitate the intracellular relocation of other proteins by binding to its cargo targets and importins and does not signal through downstream effectors (Stewart, 2007). After GDP-bound RAN translocates into the nucleus, Ran-GEF replaces RAN-bound GDP with GTP. GTP-bound RAN then shuttles back to the cytosol along with its cargo, translocating the target proteins from the nucleus. The mutant RAN from our expression library does not shuttle through the nuclear membrane since we induced mutations in the GTPase domain. It is designed to be cytosol-localized and thus inhibit nuclear trafficking of proteins. Therefore, our observation that mutant RAN increased IRF3 phosphorylation indicates the existence of crucial upstream regulator(s) of IRF3 that have RAN-dependent translocation between the cytosol and nucleus. Furthermore, IRF3 phosphorylation by mutant RAN was dependent on TBK1, as shown by the inhibition of phosphorylation after BX795 treatment. Since TBK1 is not nuclear-localized, our results warrant further investigation into the upstream regulator(s) of TBK1.

In summary, we have identified small GTPases that phosphorylate IRF3 in an unbiased manner for the first time and revealed a sequence-activity correlation followed by the identification of the necessity of TBK1 as a key link. IRF3 is an emerging target that integrates various cellular inputs. Therefore, our results warrant further studies to determine how a specific GTPase triggers IRF3 transcriptome changes and the involved cellular functions. Furthermore, since most small GTPases signal through TBK1 to phosphorylate IRF3, TBK1 inhibition may serve as a potential therapeutic strategy against diseases with GTPase overexpression or active mutations, such as neoplastic malignancies.

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