Report

The Oncogene eIF4E Reprograms the Nuclear Pore Complex to Promote mRNA Export and Oncogenic Transformation

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http://dx.doi.org/10.1016/j.celrep.2012.07.007

SUMMARY

The eukaryotic translation initiation factor eIF4E is a potent oncogene that promotes the nuclear export and translation of specific transcripts. Here, we have discovered that eIF4E alters the cytoplasmic face of the nuclear pore complex (NPC), which leads to enhanced mRNA export of eIF4E target mRNAs. Specifically, eIF4E substantially reduces the major component of the cytoplasmic fibrils of the NPC, RanBP2, relocalizes an associated nucleoporin, Nup214, and elevates RanBP1 and the RNA export factors, Gle1 and DDX19. Genetic or pharmacological inhibition of eIF4E impedes these effects. RanBP2 overexpression specifically inhibits the eIF4E mRNA export pathway and impairs oncogenic transformation by eIF4E. The RanBP2 cytoplasmic fibrils most likely slow the release and/or recycling of critical export factors to the nucleus. eIF4E overcomes this inhibitory mechanism by indirectly reducing levels of RanBP2. More generally, these results suggest that reprogramming the NPC is a means by which oncogenes can harness the proliferative capacity of the cell.

INTRODUCTION

Nuclear export is a highly regulated process where macromolecular complexes transit to the cytoplasm via the nuclear pore complex (NPC). The NPC consists of the nuclear basket, the central channel spanning the nuclear membrane, and the cytoplasmic face characterized by fibrils that extend into the cytoplasm (Hutten and Kehlenbach, 2007; Köhler and Hurt, 2010; Strambio-De-Castillia et al., 2010; Wente and Rout, 2010). Generally, export complexes are formed by nuclear export signal (NES) containing proteins, chromosome region maintenance protein 1 (CRM1), and RanGTP. These enter the NPC via the nuclear basket and transit through the central channel (Hutten and Kehlenbach, 2007; Wente and Rout, 2010). Once at the cytoplasmic side, the cargo is released from the export complex by one of two mechanisms. CRM1-cargo-RanGTP complexes associate with soluble RanBP1 in conjunction with RanGAP

resulting in RanGTP hydrolysis thereby allowing cargo release from CRM1 (Hutten and Kehlenbach, 2007). Alternatively, the RanBP1 homologous domains of the cytoplasmic fibril protein RanBP2/Nucleoporin (Nup) 358 similarly release CRM1 bound cargo through RanGTP hydrolysis in association with RanGAP (Hutten and Kehlenbach, 2007; Kehlenbach et al., 1999). Interestingly, RanBP2 is absent in yeast, whereas RanBP1 is absent in flies (Hutten and Kehlenbach, 2006; Strambio-De-Castillia et al., 2010).

Although bulk mRNA transits the NPC using the TAP/NXF1 nuclear receptor, the export of some mRNAs is CRM1 mediated including eukaryotic translation initiation factor eIF4E-dependent mRNA export (Hutten and Kehlenbach, 2007; Culjkovic et al., 2005, 2006). Although eIF4E plays a key role in translation, it also acts in the nucleus where it promotes the export of specific mRNAs (Borden and Culjkovic-Kraljacic, 2010). eIF4E's mRNA export and translation activities both contribute to its oncogenic potential. Indeed, eIF4E is elevated in 30% of cancers including subtypes of acute myeloid leukemia (AML) (Borden and Culjkovic-Kraljacic, 2010). Here, eIF4E is highly elevated and almost entirely nuclear with substantially upregulated mRNA export activity (Assouline et al., 2009; Topisirovic et al., 2003, 2009). A competitive inhibitor of the m7G cap, ribavirin, impairs the activities of eIF4E in translation, mRNA export, and oncogenic transformation (Assouline et al., 2009; Borden and Culjkovic-Kraljacic, 2010; Kentsis et al., 2004, 2005). In a phase II multicenter clinical trial, ribavirin targeted eIF4E activity, including reducing mRNA export, and this correlated with clinical responses including remissions in some patients with AML (Assouline et al., 2009).

In contrast to translation the mechanics underlying eIF4E-dependent mRNA export are only starting to emerge. In contrast to the cytoplasm, eIF4E associates with a subset of nuclear mRNAs, and importantly, its export activity is independent of ongoing translation (Culjkovic et al., 2005, 2006). To be an eIF4E export target, mRNAs must be capped and contain a 50-nucleotide structural element in their 3′ UTR known as an eIF4E sensitivity element (4E-SE) (Culjkovic et al., 2005, 2006). eIF4E RNA export targets include *c-Myc*, *Hdm2*, *NBS1*, *ODC*, and *Cyclin D1* among others (Culjkovic et al., 2005, 2006). Many target mRNAs have highly structured 5′ UTRs, making these also translation targets of eIF4E. In this way, eIF4E coordinately drives the production of constituents of many proliferative and survival signaling pathways (Borden and Culjkovic-Kraljacic,

2010). Thus, when dysregulated, eIF4E is well positioned to drive oncogenesis.

The association with and dependence of eIF4E ribonuclear particles (RNPs) on CRM1 rather than TAP/NXF1 are major differences between eIF4E-dependent and bulk mRNA export pathways (Culjkovic et al., 2005, 2006; Topisirovic et al., 2009). The eIF4E nuclear RNP consists of eIF4E, the m⁷G-capped 4E-SE mRNA, LRPPRC (a bridging factor binding both 4E-SE and eIF4E), UAP56, hnRNPA1, and others, but not REF/Aly (Topisirovic et al., 2009). Despite these inroads, it is unknown how eIF4E promotes mRNA export. Here, we show that eIF4E alters the NPC. These changes correlate with increased export of 4E-SE mRNAs and increased transformation by eIF4E. These findings suggest that the NPC can be reprogrammed by oncogenes as part of the transformation process.

RESULTS

eIF4E Overexpression Alters the Composition of the NPC

In order to understand the mechanics behind eIF4E-dependent mRNA export, we examined the status of NPC components upon eIF4E overexpression, including constituents of the nuclear basket, central channel, and cytoplasmic face (Figures 1 and S1). We used two overexpression systems to ensure no construct dependence: a bicistronic construct with untagged eIF4E and GFP virally transduced into U2OS cells, and a stable cell line overexpressing FLAG-eIF4E (Figures 1 and S1). In Figure 1, eIF4E overexpression is ~3-fold relative to vector by qRT-PCR. Note that patients with M4/M5 AML generally have eIF4E overexpression ranging from ~3- to 8+-fold relative to healthy volunteers (Assouline et al., 2009).

Observed changes were focused on the cytoplasmic face of the NPC. For instance, eIF4E overexpression leads to ~3-fold reduction in RanBP2, the main constituent of cytoplasmic fibrils (Figure 1B). Furthermore, the remaining RanBP2 is less concentrated at the nuclear rim with increased nucleoplasmic staining (Figures 1C and S1). Another component of the cytoplasmic face, Nup 214, had reduced nuclear rim and increased nucleoplasmic staining with no detectable changes in total levels (Figures 1 and S1). Studies of Nup88 (also a constituent of the cytoplasmic face) were confounded by differences in results depending on the antibody used, precluding analysis at this point. Similar results were observed in mouse embryonic fibroblasts (MEFs) as in U2OS cells; thus, these findings are not restricted to a single cell line (data not shown). Consistent with previous results, levels of eIF4E targets, e.g., Cyclin D1, NBS1, and c-Myc, increase by ~2- to 3-fold (Figure 1B).

We also monitored factors involved in cargo release on the cytoplasmic side of the NPC. Most strikingly, we observed an ~3-fold RanBP1 elevation, with no changes to its localization, upon eIF4E overexpression (Figures 1B and S1). No changes in levels or localization of RanGAP or Ran were observed (Figures 1B and S1). We also observed an ~2- to 3-fold elevation in an RNA helicase DDX19 and Gle1, its activating protein, with no change in distribution by subcellular fractionation (Figures 1B and S1). However, further analyses are needed to determine the extent of DDX19 and Gle1 binding to the NPC in eIF4E-

overexpressing cells. DDX19 and Gle1 release bulk mRNA export cargoes at the cytoplasmic face in conjunction with Nup214 or in its absence, hCG1/NLP1 (Hodge et al., 2011; Kendirgi et al., 2005; Noble et al., 2011). We noted no changes in levels or distribution upon eIF4E expression for bulk mRNA export factors (Nup98, Rae1), nuclear pore components (Nup 153, Nup62, GP210, hCG1/NLP1, and Tpr), CRM1, CAS, Importin β1, Importin α2, Importin α3, LRPPRC, or Lamin A (Figures 1B and S1).

Consistent with the overexpression studies, eIF4E inhibition, via RNAi or ribavirin, leads to RanBP2 elevation, enrichment of RanBP2 and Nup214 at the nuclear rim, and reduction in Gle1, DDX19, and RanBP1 (Figures 2 and S2). eIF4E knockdown substantially increases RanBP2 levels, and increases RanBP2 in the nuclear rim and nucleus, likely due to saturation of NPC binding sites. eIF4E knockdown also leads to enrichment of Nup214 in the nucleus and the rim. Conversely, Gle1, DDX19, and RanBP1 levels were reduced by ~3-fold with no change in distribution by fractionation. Similarly, established eIF4E targets, e.g., Cyclin D1, Hdm2, and c-Myc, were reduced by ~3- to 10-fold, whereas nontargets, e.g., CRM1 or β-Actin, were unchanged.

Ribavirin treatment also leads to RanBP2 elevation, and reduction in RanBP1, Gle1, and DDX19 (by ~2- to 10-fold) in both control and eIF4E-overexpressing cells (Figures 2 and S2), and enrichment of Nup214 and RanBP2 in the perinuclear and nuclear fractions. As expected, ribavirin represses eIF4E-dependent mRNA export as observed by reduction of Cyclin D1, Hdm2, and c-Myc levels. Ribavirin did not modulate other factors examined, e.g., Nup153, Rae, Ran, or Lamin A.

RanBP2 Specifically Impairs 4E-SE mRNA Export

To investigate if RanBP2 inhibits eIF4E-dependent mRNA export, we used the zinc fingers (ZFs) of RanBP2, which binds CRM1 (aa 1,314–1,963) and Nup-4 (aa 2,500–3,224), a C-terminal RanBP2 fragment that does not bind CRM1 (Singh et al., 1999; Yaseen and Blobel, 1999). Nup-4 contains cyclophilin homology and RanBP1 homology 4 domains. We analyzed nuclear and cytoplasmic RNA content in eIF4E-overexpressing cells, and compared the effects of RanBP2-ZF overexpression on *LacZ* and *LacZ-4E-SE* mRNA export, where only the latter is eIF4E dependent (Figure 3). We also monitored the export of *c-Myc*, an endogenous eIF4E target. *tRNA^lys* and *U6* snRNA served as controls for the cytoplasmic and nuclear fractions, respectively (Figure S3). As previously shown, *LacZ-4E-SE* mRNA export and protein levels are higher compared to *LacZ* only in eIF4E-overexpressing cells. Note that eIF4E overexpression does not change the nuclear stability, or total RNA levels of eIF4E targets (Figures 3 and S3; Culjkovic et al., 2006).

RanBP2-ZF impaired the export of LacZ-4E-SE mRNAs by ~3-fold with no effect on *LacZ* (Figure 3). Consistently, LacZ-4E-SE protein levels were reduced by over 2-fold in RanBP2-ZF cells relative to vector. RanBP2-ZF impeded *c-Myc* mRNA export up to 5-fold relative to vector in both *LacZ* and *LacZ-4E-SE*-expressing cells, and lowered c-Myc protein levels ~3-fold. RanBP2-ZF overexpression did not alter eIF4E levels. In contrast, Nup-4 had no effect on eIF4E-dependent or bulk
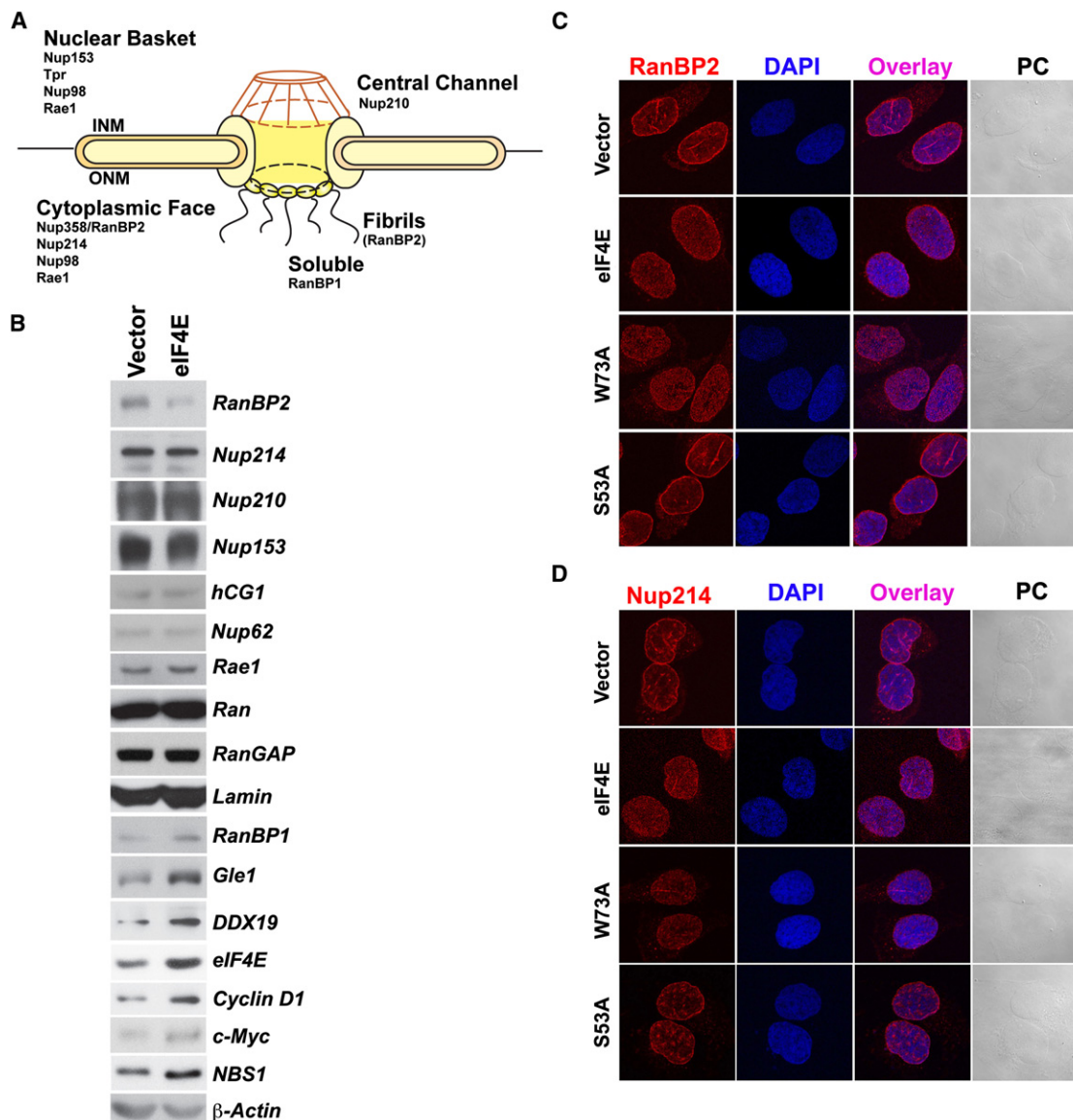
Figure 1. eIF4E Overexpression Modulates the Cytoplasmic Face of the NPC
(A) Cartoon of the NPC.
(B–D) U2OS cells were examined by western blot (B), or immunofluorescence in conjunction with confocal microscopy (C and D) with indicated antibodies to assess the effects of eIF4E wild-type or mutant expression. Magnification is 200×. DAPI is in blue. PC, phase contrast.
Additional data are shown in Figure S1.

mRNA export (Figure 3). Thus, RanBP2-ZF specifically impairs eIF4E-dependent mRNA export.

RanBP2 Reduction Increases eIF4E-Dependent mRNA Export

We examined if reduction in RanBP2 levels increased eIF4E-dependent mRNA export using RNAi-mediated knockdown of *RanBP2*, or by comparing wild-type and *RanBP2* hypomorph MEFs (Figure S4). In either case, RanBP2 reduction resulted in increased mRNA export for *LacZ-4E-SE* and endogenous eIF4E target mRNAs, compared to controls. Consistently,

LacZ-4E-SE and endogenous target protein levels were elevated. Importantly, we observed no difference in eIF4E levels or localization as a function of *RanBP2* expression (data not shown). However, the effects are more modest here (~1.5- to 2-fold) relative to eIF4E overexpression. Thus, all of the effects of eIF4E on the NPC, in their totality, likely contribute to its mRNA export phenotype.

No changes were observed in *RanBP2* mRNA export or total mRNA levels upon eIF4E overexpression (Figure S5; data not shown). Consistent with RanBP2 protein stability studies (Um et al., 2006), RanBP2 protein levels are stabilized in vector
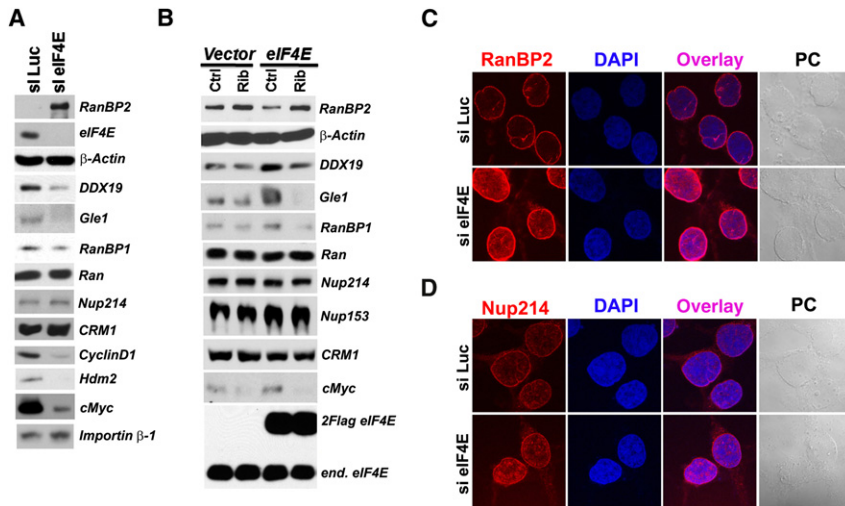
controls treated with the proteosomal inhibitor MG132 (Figure S5). However, eIF4E overexpression abrogated the stabilizing effects of MG132. Thus, eIF4E appears to indirectly reduce the protein stability of RanBP2.

RanBP1, Gle1, and DDX19 Are mRNA Export Targets of eIF4E

We examined whether *RanBP1*, *Gle1*, and *DDX19* are direct targets of eIF4E-dependent mRNA export (Figures 4 and S5). RNA immunoprecipitations (RIPs) from nuclear fractions indicate that endogenous eIF4E binds *Gle1*, *DDX19*, and *RanBP1* transcripts with 5- to 10-fold enrichment relative to IgG using qRT-PCR or sqPCR (Figure 4 and data not shown). To assess if nuclear RIP correlated with export, the cytoplasmic to nuclear (C/N) ratio of mRNAs was examined by qRT-PCR upon eIF4E overexpression (Figure 4). eIF4E promoted export up to 3-fold for all three mRNAs relative to vector. The C/N ratio for non-eIF4E export targets, e.g., *VEGF* and *Ubiquitin*, was not altered. Consistently, increased mRNA export corresponded to increased protein levels with no changes in total mRNA levels (Figures 4 and S5). Bioinformatics analysis identified putative 4E-SE elements in the 3′ UTR of these mRNAs (data not shown). Thus, *RanBP1*, *Gle1*, and *DDX19* mRNAs are direct export targets of eIF4E.

Linking NPC Reprogramming, mRNA Export, and Oncogenic Activities of eIF4E

To dissect features of eIF4E required for modulating the NPC, we monitored the effects of two previously described eIF4E mutants, W56A and W73A (Cohen et al., 2001; Culjkovic et al., 2005, 2006, 2008), and of a third mutant that we further characterize here, S53A. The W56A mutation impairs cap binding and thus renders eIF4E inactive in mRNA export, translation, transformation, and apoptotic rescue. The W73A mutant is active in mRNA export, transformation, and apoptotic rescue but does not promote translation due to impaired eIF4G binding. The S53A mutant is active in translation (Kaufman et al., 1993; Zhang et al., 1995) but does not transform cells (Lazaris-Karatzas et al., 1990).

First, we examined the effects of the S53A mutant on mRNA export (Figure 4). This mutant does not increase export of endogenous eIF4E targets, e.g., *Cyclin D1*, *NBS1*, *Hdm2*, or *c-Myc*. In contrast, overexpression of wild-type eIF4E, or the W73A mutant, increases mRNA export and protein levels of targets (by up to 3-fold). Consistent with c-Myc being both a translation and export target of eIF4E, its levels are slightly elevated in S53A cells relative to vector or W56A cells, but not to the same extent as in wild-type or W73A cells. Importantly, all constructs expressed eIF4E to similar levels and did not affect total RNA levels for targets (Figures 4 and S5). Thus, the S53A mutant is impaired in mRNA export.

Given these findings, we determined if the S53A mutant formed a nuclear RNP (Figure 4). RIP studies indicate that wild-type eIF4E binds specific mRNAs in the nucleus as observed previously (Culjkovic et al., 2006). For instance, wild-type eIF4E binds *ODC* mRNA in the nucleus and cytoplasm, whereas it only binds *VEGF* in the cytoplasm. In contrast the S53A mutant does not bind mRNAs in the nucleus but does bind mRNAs, including *VEGF*, in the cytoplasm with similar efficiency to wild-type eIF4E (Figure 4), consistent with its ability to act in translation. FLAG IPs were of equivalent efficiency for S53A and wild-type eIF4E (Figure 4). Thus, the S53A mutant does not bind mRNAs in the nucleus but does so in the cytoplasm.

Cap binding was not altered by the S53A mutation (Figure S6). ^{1}H-^{15}N HSQC NMR studies indicated that the S53A mutant has a fold and cap binding activity indistinguishable from wild-type eIF4E. Furthermore, the subcellular localization of the S53A mutant was not altered relative to wild-type eIF4E (data not shown).

We next examined the effects of these mutants on the NPC (Figures 1 and 4). The W73A export-competent mutant mirrors wild-type eIF4E: reducing RanBP2 levels, altering Nup214 and RanBP2 localization, and increasing mRNA export of *Gle1*, *DDX19*, and *RanBP1*. In contrast the S53A and W56A mutants are completely impaired in these activities. These data suggest a functional link between remodeling the NPC and eIF4E-dependent mRNA export.

We examined whether activities in mRNA export and NPC reprogramming were linked to the oncogenic potential of eIF4E. eIF4E overexpression leads to the formation of
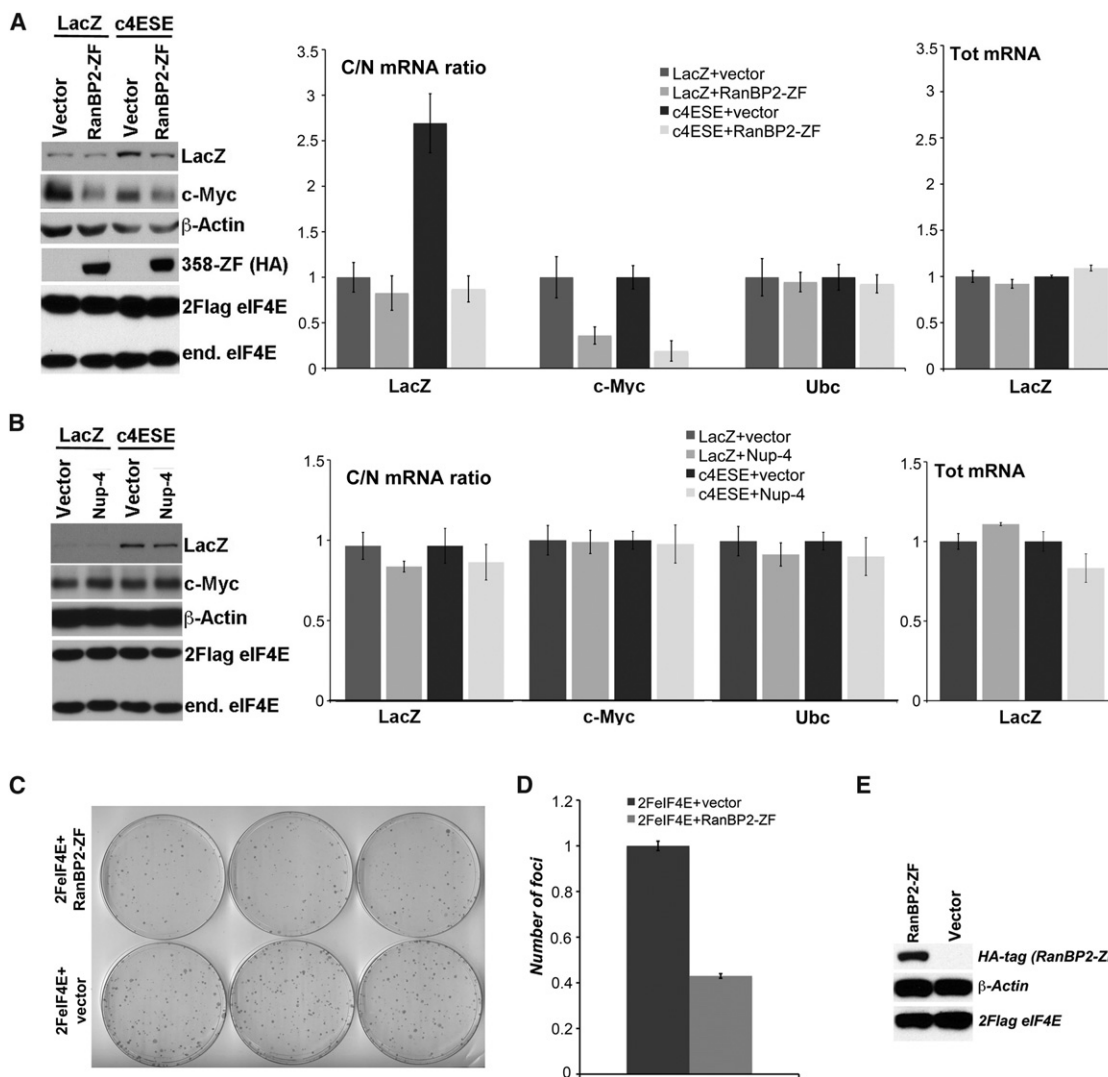
Figure 3. RanBP2 Suppresses eIF4E-Dependent mRNA Export and Transformation

(A and B) Western blot analysis of protein levels in U2OS cells stably expressing 2FLAG-eIF4E and LacZ or LacZ-4E-SE (Xpress tag) upon RanBP2-ZF (A) or Nup-4 (B) overexpression; end., endogenous eIF4E. The C/N ratio of *lacZ* (±4E-SE) or endogenous *c-Myc* target mRNAs was determined using qRT-PCR. Values represent relative fold difference ±SD (error bars). Averaged values were normalized to *β-Actin* and to vector control (set to 1).

(C) Foci assays in U2OS cells stably transfected as indicated.

(D) Values are number of foci ±SD (error bars).

(E) Western analysis as indicated with β-actin as a loading control. Foci assays were carried out in triplicate three independent times.

See also Figures S3 and S4.

anchorage-dependent foci due to the loss of contact inhibition, a hallmark of transformation (Lazaris-Karatzas et al., 1990), Importantly, the S53A mutant is devoid of the ability to form these foci (Figure S5; Lazaris-Karatzas et al., 1990). Furthermore, we observe that RanBP2-ZF, which repressed eIF4E-dependent mRNA export, represses foci formation in eIF4E-overexpressing cells, with a 60% reduction versus controls (Figure 3). Thus, RanBP2-ZF inhibits both eIF4E-dependent mRNA export and transformation. Together, these findings functionally link the mRNA export, NPC reprogramming, and transformation activities of eIF4E.

DISCUSSION

We demonstrate that eIF4E overexpression leads to major alterations to the cytoplasmic face of the NPC, and this is linked to both its mRNA export and oncogenic activities. These effects on the NPC are due to both indirect effects on Nup214 and RanBP2, as well as direct effects on *RanBP1*, *Gle1*, and *DDX19* mRNAs. Moreover, RanBP2 is a potent and specific inhibitor of the mRNA export function of eIF4E. Although RanBP2 depletion leads to a loss of cytoplasmic fibrils, it does not lead to substantial defects in the export of specific NES-containing cargoes or
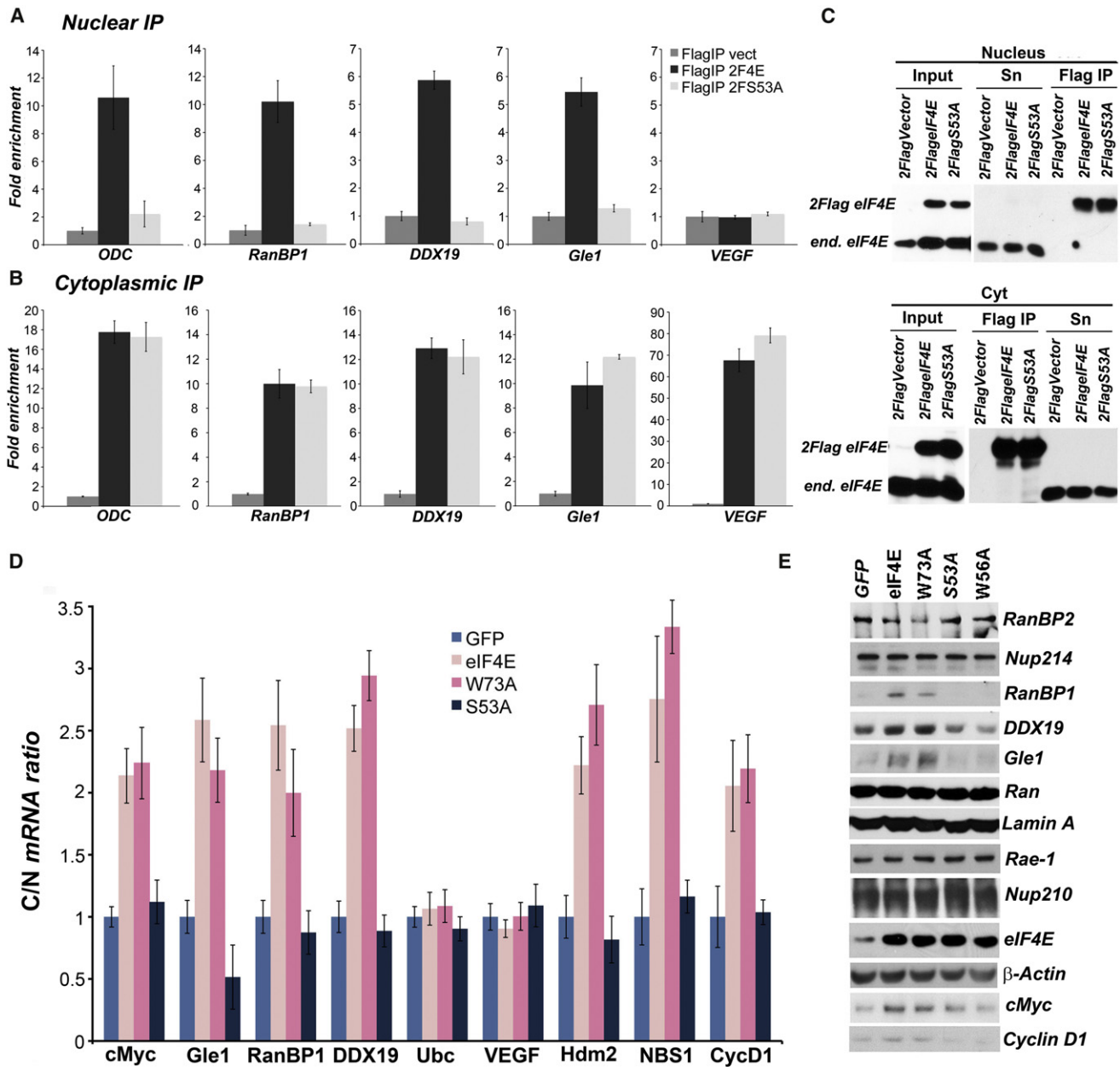
Figure 4. Link between NPC, mRNA Export and Oncogenic Transformation

(A and B) RNAs isolated by RIP with an anti-Flag antibody from nuclear (N) or cytoplasmic (C) fractions of U2OS cells stably expressing with indicated constructs and analyzed using qRT-PCR. Values represent relative fold difference ±SD (error bars).

(C) Western blot analysis of IP efficiency.

(D) The C/N ratio of target transcripts as a function of wild-type or mutant eIF4E overexpression relative to β-Actin.

(E) Western blot analysis of the effects of eIF4E mutants on the NPC. Note that mutants are expressed to similar levels.

Additional data are shown in Figures S3 and S5.

bulk mRNA export (Dawlaty et al., 2008; Hutten and Kehlenbach, 2006; Walther et al., 2002). In fact, and consistent with the eIF4E phenotype, *RanBP2* hypomorph mice have substantially higher rates of spontaneous tumor formation than littermates (Dawlaty et al., 2008). In contrast, deletion of *RanBP2* is lethal, and *RanBP2*^{−/−} MEFs harbor substantial bulk mRNA export defects

(Hamada et al., 2011). Importantly, the regions of RanBP2 involved in eIF4E-dependent and bulk mRNA export are distinct, involving the ZFs and leucine-rich domains, respectively (Figure 3; Hamada et al., 2011).

Why reduce RanBP2 and increase RanBP1 levels given that they play similar roles in cargo disassembly? RanBP2 at

358 kDa forms the cytoplasmic fibrils of the NPC, whereas RanBP1 is a soluble 23 kDa protein. RanBP2 may provide docking sites for the sequestration of 4E-SE-specific export factors. Reduction in RanBP2 may enable faster release/recycling of specific rate-limiting factors back to the nucleus, leading to increased export of eIF4E-sensitive mRNAs (Figure S7). Here, elevated RanBP1 compensates for reduced RanBP2. Consistently, studies in RanBP2$^{-/-}$ cells suggest that docking of CRM1 to RanBP2 is not critical for CRM1 cargo disassembly (Hamada et al., 2011), presumably due to RanBP1. In summary, eIF4E overexpression favors a RanBP1 release pathway. These changes likely enhance export by promoting release and/or recycling of export complexes (Figure S7).

Why deplete Nup214 and RanBP2 from the nuclear rim? Importantly, Nup 214 localization is independent of RanBP2, and vice versa (Hutten and Kehlenbach, 2006; Walther et al., 2002). This suggests that the relocalization of Nup214 and RanBP2 upon eIF4E overexpression is a targeted event. The factors controlling their localization, and thus the associated mechanism, are unknown. Notably, Nup214 is found on nuclear and cytoplasmic sides of the NPC and may bind multiple sites within the NPC (Boer et al., 1997). Mechanistically, Nup214 directly binds CRM1 and forms a GTPase-resistant Nup214-CRM1-RanGTP complex (Hutten and Kehlenbach, 2006; Walther et al., 2002). Thus, reducing Nup214 at the NPC may enhance release of 4E-SE RNA cargoes. In the absence of Nup214, hCG1/NLP1 controls the localization of Gle1 and enables mRNA export (Kendirgi et al., 2005). Importantly, hCG1 also directly binds CRM1, RanGTP, and cargoes, and here, RanBP1 dissociates this complex (Kendirgi et al., 2005; Waldmann et al., 2012). Given that hCG1 does not change upon eIF4E overexpression (Figures 1 and S1), it is tempting to speculate that perhaps a hCG1, CRM1, Ran, 4E-SE cargo (plus accessory factors) complex forms. Additionally, it is possible that modulation of Nup214 or RanBP2 enables unknown functions that promote export of eIF4E target mRNAs.

eIF4E overexpression may not only change CRM1-dependent export of 4E-SE RNAs but also modulate the export of a subset of mRNAs using the TAP/NXF1-Gle1-DDX19 pathway. Thus, there could be crosstalk between these RNA export pathways. Alternatively, the RNA helicase activity of DDX19 may be important for remodeling 4E-SE export RNPs. Additionally, DDX19 and Gle1 play independent roles in the initiation and termination of translation for some mRNAs (Alcázar-Román et al., 2010; Bolger et al., 2008; Gross et al., 2007), and it may be these activities that are relevant. Thus, in addition to its direct effects on translation, eIF4E could impact on translation indirectly via its effects on DDX19 and Gle1. In all, these findings strongly suggest that substantial interplay exists between the bulk and eIF4E-dependent mRNA export machinery and, perhaps, between these mRNA export and translation pathways.

Our studies provide a link between eIF4E's ability to reprogram the NPC, promote mRNA export, and transform cells. The S53A mutant is impaired in reducing and relocalizing RanBP2, increasing RanBP1, DDX19, and Gle1, relocalizing Nup214, and in mRNA export and transformation, but not in translation. Although the S53A mutant rescues yeast null in eIF4E and acts in global translation (Kaufman et al., 1993; Zhang et al., 1995),

we cannot rule out the possibility that some mRNAs are more sensitive to this mutation, and this reduces the oncogenic activity of eIF4E, without altering viability.

Interesting parallels can be drawn between targeting the NPC by the oncogene eIF4E and by viruses. For instance, vesicular stomatitis virus inhibits cellular mRNA export by interaction of the viral M protein with Nup98 and Rae1 (Enninga et al., 2002; Faria et al., 2005). Poliovirus impairs cellular nuclear import by degrading Nup153 and Nup62 (Gustin and Sarnow, 2001). In some cancers, CRM1 and Nup88 are overexpressed (Köhler and Hurt, 2010), but whether this drives transformation, or is a side product of it, is not known. eIF4E targets the NPC in a distinct manner from those described above. These observations suggest that there will not be a single oncogenic NPC phenotype but, rather, that just as viruses co-opt the NPC using different strategies, oncogenes will modulate the NPC in specific fashions.

In conclusion these studies show that an oncogene alters the composition of the NPC. Here, drastic modification of the cytoplasmic face of the NPC leads to enhanced mRNA export and increased oncogenic potential. We hypothesize that this activity will not be restricted to eIF4E so other oncogenes likely reprogram the NPC to subvert normal growth control.

EXPERIMENTAL PROCEDURES

Reagents and Constructs

pcDNA2Flag-eIF4E, *MSCV-pgk-GFP-eIF4E* (or mutants), *pcDNA3.1-lacZ*, *lacZ-4E-SE* (from *Cyclin D1*), and bacterial expression constructs are from Culjkovic et al. (2006). Human *RanBP2-ZF* and *Nup358-4* (Yaseen and Blobel, 1999) were subcloned into pKH and confirmed by sequencing. MG132 was obtained from Sigma-Aldrich and ribavirin from Kemprotec.

Cell Culture

U2OS cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). *2Flag-eIF4E* wild-type or mutant and *LacZ/LacZ-4E-SE* ± 2Flag-*eIF4E* U2OS cell lines were generated as in Culjkovic et al. (2006). *MSCV-pgk-GFP-eIF4E* wild-type or mutants were used for retroviral transduction of U2OS cells as in Culjkovic et al. (2008). For siRNA experiments, U2OS cells were transfected with Lipofectamine 2000 (Invitrogen) and 20 nM siRNA duplexes and analyzed 96 hr after transfection. Transient transfections were carried out using *Trans*IT-LT1 Transfection Reagent (Mirus). Cells were 50%–70% confluent when used for protein and RNA preparation. See Extended Experimental Procedures for antibodies, siRNAs, and subcellular fractionation.

Anti-FLAG M2 agarose affinity beads were used to purify 2FLAG eIF4E as in Culjkovic et al. (2005). For RIP, complexes were heated to 95°C for 5 min in Tris-EDTA containing 1% SDS and isolated using TRIzol. DNase-treated RNA samples (TurboDNase, Ambion) were reverse transcribed using MMLV reverse transcriptase (Invitrogen). qRT-PCR analyses were performed using EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen) in AB StepOne thermal cycler using the relative standard curve method (Applied Biosystems User Bulletin #2). List of primers used in this study is shown in Table S1.

Anchorage-Dependent Foci Assays

A total of 500 cells were seeded per 10 cm plate for 14 days, then stained with Giemsa (Sigma-Aldrich).

Immunofluorescence and Laser-Scanning Confocal Microscopy

U2OS cells were grown on coverslips, fixed in 2% paraformaldehyde for 15 min at RT, washed three times with PBS, and permeabilized with 0.2% (v/v) Triton X-100 for 10 min at RT. Upon permeabilization, cells were washed

three times, blocked for 1 hr, and incubated with 1° antibodies (1:1,000 dilution) overnight at 4°C, followed by three washes in blocking solution. Cells were then incubated with 2° donkey anti-rabbit IgG-Texas red antibody (Jackson ImmunoResearch; diluted 1:150 in blocking solution), washed four times with 1xPBS (7.4), and mounted in VECTASHIELD with DAPI (Vector Laboratories). Analysis was carried out using a laser-scanning confocal microscope (LSM510; Carl Zeiss), exciting 405 and 543 nm with a 100× objective and 2× digital magnification. Channels were detected separately, with no crosstalk observed. Confocal micrographs represent single sections through the plane of the cell.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.07.007.

LICENSING INFORMATION

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