Post-transcriptional gene expression control by NANOS is up-regulated and functionally important in pRb-deficient cells

Wayne O Miles¹, Michael Korenjak¹, Lyra M Griffiths², Michael A Dyer², Paolo Provero³,⁴ & Nicholas J Dyson¹,*

Abstract

Inactivation of the retinoblastoma tumor suppressor (pRb) is a common oncogenic event that alters the expression of genes important for cell cycle progression, senescence, and apoptosis. However, in many contexts, the properties of pRb-deficient cells are similar to wild-type cells suggesting there may be processes that counterbalance the transcriptional changes associated with pRb inactivation. Therefore, we have looked for sets of evolutionary conserved, functionally related genes that are direct targets of pRb inactivation. Therefore, we have looked for sets of evolutionary conserved, functionally related genes that are direct targets of pRb inactivation. We show that the expression of NANOS, a key facilitator of the Pumilio (PUM) post-transcriptional repressor complex, is directly repressed by pRb/E2F in flies and humans. In both species, NANOS expression increases following inactivation of pRb/RBF1 and becomes important for tissue homeostasis. By analyzing datasets from normal retinal tissue and pRb-null retinoblastomas, we find a strong enrichment for putative PUM substrates among genes de-regulated in tumors. These include pro-apoptotic genes that are transcriptionally down-regulated upon pRb loss, and we characterize two such candidates, MAP2K3 and MAP3K1, as direct PUM substrates. Our data suggest that NANOS increases in importance in pRb-deficient cells and helps to maintain homeostasis by repressing the translation of transcripts containing PUM Regulatory Elements (PRE).

Keywords  Nanos; post-transcriptional gene regulation; pRb; Pumilio; stress response

Subject Categories  Cell Cycle; Chromatin, Epigenetics, Genomics & Functional Genomics

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Introduction

Cell proliferation and apoptosis are fundamental cellular processes that are essential for development, differentiation, and tissue homeostasis. Each cell within eukaryotic organisms has built-in safeguards that limit the tumorigenic potential of cells that lose their normal controls.

The family of E2F transcription factors plays a central role in the regulation of both proliferation and apoptosis. E2F proteins control the expression of genes involved in cell cycle progression, checkpoint activation, and senescence. The term “E2F” is the integrated activity of a family of proteins that contains both activators of transcription (dE2F1 (flies), E2F1-E2F3 (humans)) and repressors of transcription (dE2F2 (flies), E2F4-8 (humans)) (Chen et al., 2009). An additional layer of regulation is provided at cell cycle genes by the pocket protein family of transcriptional repressors (RBF1, RBF2 (flies) pRb, p107, and p130 (humans)) (Burkhart & Sage, 2008; Dick & Rubin, 2013). The pocket proteins bind directly to activator E2F’s and act as molecular scaffolds to repress E2F-mediated transcription (Dimova et al., 2003). Dynamic fluctuations between the activities of E2F and pRb proteins regulate normal cell proliferation (van den Heuvel & Dyson, 2008).

pRb is functionally inactivated in the majority of tumors, and its activity can be compromised by several different types of events that include E2F amplification (Feber et al., 2004), viral infection (E6/E7) (Dyson et al., 1989), CDK4/6 amplification (Khatib et al., 1993), p16 mutation/silencing (Okamoto et al., 1994) or by mutations within the Rb1 gene (Friend et al., 1986). Although pRb inactivation is widespread in cancer, it is evident the loss of pRb function also generates a series of cellular stresses. For example, pRb loss causes dramatic and widespread changes in transcriptional profiles (Herschkowitz et al., 2008), leads to changes in chromatin architecture (Zhang et al., 2012) and undermines genomic integrity (Longworth et al., 2008; Manning et al., 2010). The mechanism(s) that counteract these stresses and enable the oncogenic growth of pRb-deficient cells remains poorly understood. However, elucidating the mechanisms
that allows cells to cope with the pressures associated with pRb loss is important, since this may reveal points of vulnerability that can be exploited therapeutically to target cancer cells. Although pRb is frequently inactivated in cancer cells, analysis of chimeric animals has shown that Rb1 null cells (or rbf1 null cells in Drosophila) typically do not overproliferate and contribute significantly to differentiated tissues that are relatively normal in appearance (Maandag et al., 1994; Du, 2000). There are likely to be multiple reasons why Rb loss, or E2F deregulation, does not typically promote cell proliferation or cell death. Genetic studies show that in some contexts, related proteins may compensate for pRb loss (Bremner et al., 2004) and that other cdk regulators provide redundant levels of control (Park et al., 1999).

In this study, we have explored the idea that there may be additional types of control that act in pRb-deficient cells to counterbalance the changes in gene transcription. To identify novel candidate genes which may counterbalance E2F dys-regulation, we searched for genes that were directly regulated by E2F/pRb in both Drosophila and mammalian cells and were up-regulated in both species following Rb/RBF inactivation. One of the most intriguing genes that met these criteria was the RNA-binding protein, NANOS. NANOS is a conserved and essential single-stranded RNA-binding protein which functionally cooperates with its obligate binding partner, Pumilio (Pum) (Wharton & Struhl, 1991). Together they form the core of the Pumilio post-transcriptional repressor complex and suppress the translation of mRNAs containing a Pumilio Regulatory Motif (PRE) (UGUAXAUA) within their 3’ untranslated regions (UTR) (Asaoka-Taguchi et al., 1999; Sonoda & Wharton, 1999). The PUM complex activity prevents the translation of its substrates via a number of mechanisms including, 5’ decapping (Cao et al., 2010), ribosome stalling ( Friend et al., 2012), miRNA recruitment (Nolde et al., 2007; Kedde et al., 2010; Friend et al., 2012; Miles et al., 2012), and de-adenylation (Van Etten et al., 2012). RNA immunoprecipitation experiments of PUM complexes in multiple systems have identified a significant number of conserved substrates involved in regulating important oncogenic processes including cell cycle progression, differentiation, and apoptosis (Gerber et al., 2004, 2006; Galgano et al., 2008). In support of these findings, tissue-specific disruption of Pumilio/Nanos activity in a variety of tissues and systems has implicated the post-transcriptional regulation of Pum/Nanos as essential for tissue differentiation (Deshpande et al., 1999), stem cell maintenance/pluripotency (Tsuda et al., 2003; Chen et al., 2012; Lai et al., 2012), and preventing p53-mediated apoptosis (Chen et al., 2012; Lai et al., 2012).

Here, we show that NANOS, a fundamental component of the Pum complex, is a direct target of pRb regulation and that NANOS expression is strongly induced following pRb inactivation. This elevation in NANOS levels is seen in multiple experimental systems and, as a result, NANOS gains in importance in pRb- or RBF1-deficient cells. One of the consequences of Nanos upregulation is that it suppresses p53-mediated growth. As a result, the elevated levels of NANOS are particularly important for cancer cell lines that retain a functional p53.

Results

To identify conserved E2F/pRb targets genes, we conducted RBF1 and E2F (E2F1 and E2F2) ChIP-chip experiments from wild-type (w1118) Drosophila larvae and compared the results with the lists of classic E2F/DB targets identified in human cells (Bieda et al., 2006). In addition to the expected E2F targets that we have characterized previously (Korenjak et al., 2012), we noted that the novel E2F2 and RBF1 targets included all three components of the Drosophila Pumilio post-transcriptional repressor complex: pumilio, nanos, and brat (Fig 1A). The Pumilio complex is an interesting target of E2F/RBF regulation because it, in turn, reduces the activity of activator E2F’s in both flies (E2F1) and humans (E2F3) (Miles et al., 2012). To confirm our ChIP-chip results, we conducted ChIP-RT-PCR experiments using antibodies targeting RBF1, E2F1, and E2F2 from Drosophila larvae. This analysis confirmed that the promoter of nanos is strongly bound by RBF1 and the repressive E2F (E2F2), but not by the activator E2F (E2F1), (Fig 1B, Supplementary Fig S1A). The remaining components of the complex, pumilio and brat, are weakly bound by RBF1 and E2F2 (Fig 1B, Supplementary Fig S1A). These data suggest that RBF1 and E2F2 directly constrain the activity of the Pum complex by repressing the expression of the rate-limiting component, Nanos, rather than by regulating the expression of all of the components of the Pum complex.

RBF1 and E2F2 are components of the Drosophila, Rb, E2F, and Myb-associated protein (dREAM) complex, a transcriptional silencing complex that represses many E2F target genes (Korenjak et al., 2004). To determine whether components of the Pum complex are targets for dREAM-mediated repression, we analyzed datasets of published genome-wide dREAM ChIP experiments from Drosophila Kc cells (Georlette et al., 2007) and found a strong ChIP enrichment for all of the dREAM components (E2F2, Myb, Mip120, Mip130, and Lin-52) on the pumilio, nanos, and brat genes (Supplementary Fig S1B). To establish the functional significance of E2F2/RBF1 binding to these promoters, we assayed gene expression levels from Drosophila S2 cells and flies containing dsRNA or RNAi sequences targeting E2F/RBF family members. Depletion of RBF1 or E2F2 (but not E2F1) strongly induced the expression of nanos and modestly elevated the levels of pum and brat (Fig 1C, Supplementary Figs S1C and S2A and B). To further assess the contribution of dREAM activity to the regulation of these targets, we analyzed the levels of the Pum complex in E2F2 homozygous mutant flies and microarray studies from Kc cells treated with dsRNA targeting dREAM components (Georlette et al., 2007). E2F2 mutant flies (Supplementary Fig S1D) and dsRNA-treated Kc cells (Supplementary Fig S2C) display elevated expression of the Pum components, suggesting that dREAM activity regulates the expression of the Pum complex. To confirm that these changes in gene expression were due to direct regulation by the dREAM complex, the promoters of the pumilio, nanos, and brat genes were cloned upstream of a luciferase reporter gene. Depletion of RBF1 or E2F2, but not E2F1, by dsRNA in S2 cells strongly up-regulated the expression from the nanos promoter. It also weakly increased the luciferase production from the pumilio and brat promoters (Supplementary Fig S2D). We conclude that the E2F2/RBF1/dREAM complex in Drosophila directly binds the promoters of nanos, pumilio, and brat and that this regulation is important in repressing the expression of the rate-limiting component of the Pum complex, Nanos.

To investigate the role of E2F/pRb regulation of the PUM complex in human cells, we examined the capacity of each pocket protein [pRb, p107 (Rb like 1 (RB1))], and p130 (RB like 2 (RB2))] to regulate PUM/NANOS expression in human fibroblasts. The
pocket proteins were depleted from BJ cells using siRNAs, and the effects on expression and protein levels of the PUM complex were measured. As shown in Fig 1D and Supplementary Fig S3A, reducing the levels of the pocket proteins produced a strong up-regulation in the expression of the NANOS1 and NANOS3 genes, akin to that of the more conventional E2F target, Cyclin A (Cyc A) (Takahashi et al., 2000). Depletion of the pocket proteins induced only slight changes in PUM1 and PUM2 expression and did not affect NANOS2 levels (Fig 1D). Reducing pocket protein function using siRNAs led to elevated levels of PUM1, PUM2, and NANOS1 (NOS1) proteins (Supplementary Fig 3B and C). These findings suggest that NANOS1 protein levels are elevated due to transcriptional up-regulation upon loss of pocket protein activity and that the changes in PUM protein levels are likely due to increased stabilization of the PUM complex, in agreement with previous studies (Sonoda & Wharton, 1999).

Consistent with the idea that the dREAM complex represses NANOS1 expression, chromatin immunoprecipitation (ChIP) experiments using antibodies targeting the dREAM complex components, E2F4, p107, and p130, confirmed that all three proteins bind directly to the promoter of NANOS1 (NOS1) in human fibroblasts (BJ cells) (Supplementary Fig S4A). Interestingly, ChIP experiments showed that E2F4 and p107 were completely absent from the NANOS1 promoter in Y79 retinoblastoma cells that completely lack pRb (Supplementary Fig S4B), and the binding of these dREAM components to the NANOS1 promoter was dramatically reduced by knockdown
of pRb from BJ cells (Supplementary Fig S4A). These observations suggest that pRb stabilizes dREAM-binding to the NANOS1 promoter, a conclusion that agrees with previous studies linking pRb function to dREAM-mediated repression (Tschoep et al, 2011).

The functional inactivation of Rb family members is a widespread phenomenon in cancer as these proteins regulate important oncogenic pathways including cell cycle progression, senescence, differentiation, and apoptosis (for review (Di Fiore et al, 2013)). Commonly, cancer cells constitutively inactivate the pocket proteins by overexpressing the cyclin-dependent kinases which target pRb (Khatib et al, 1993) or by disrupting the upstream regulators of CDK activity (p16INK4A) (Okamoto et al, 1994). To determine how these regular oncogenic events modify pRb’s capacity to regulate the expression of the PUM complex, we depleted p16 from BJ cells or treated HCT116 cells which lack p16, with CDK4/6 inhibitors. Knockdown of p16 in BJ cells stimulated the expression of the entire PUM complex except NANOS2 (Supplementary Fig SSA). Conversely, re-activating pRb by treating HCT116 cells with the CDK4/6 inhibitor (PD0332991) reduced cell number and NANOS expression (Supplementary Fig SSB and C). To examine whether there is a link between pocket proteins and the expression of PUM complex components in cancer cells, we compared the expression of each PUM and NANOS gene to that of each pocket protein (Rb1, Rb1l, and Rbl2) across a broad panel of tumor cell lines (Barretina et al, 2012). In agreement with our Drosophila data linking RBF1 to the repression of Nanos, this analysis revealed a strongly significant anti-correlation between pRb and NANOS1 expression ($P = 1.03 \times 10^{-18}$) and a weaker anti-correlation between pRb and NANOS3 levels ($P = 1.22 \times 10^{-5}$) (Fig 1E, Supplementary Fig S6A and B). Consistent with our fly experiments, we did not find a negative correlation between pRb and PUM1, PUM2, or the poorly characterized Nanos homolog, NANOS2 (Fig 1E).

Previous studies have identified a gene expression signature associated with pRb loss in tumors (Herschkowitz et al, 2008). When we compared the pRb loss signature with the expression pattern of the PUM complex components, we found that NANOS1 expression is correlated with the Rb1 loss signature (Supplementary Figs S6C-F and S7). As an additional test of pRb’s role in regulating NANOS1 expression, we compared the expression profiles of the PUM complex in primary retinoblastoma tumors (that which contain homozygous mutations in the Rb1 gene) with control retina tissue. NANOS1 expression is up-regulated in the primary retinoblastoma tumor cells (3/3) and retinoblastoma tumor cells grown (2/3) as orthotopic xenografts in mice (Fig 1F). We conclude that NANOS1 expression is up-regulated in cells deficient for pRb activity.

Collectively, these data show that RBF1/pRb controls Nanos/NANOS1/3 expression and that this regulation is conserved between Drosophila and humans. Next, we investigated the importance of this interaction. To examine how elevated levels of the Pumilio complex contributed to the cellular homeostasis of tissue with reduced dREAM activity, we tested how reducing the expression of the Pum complex affected Drosophila wings sensitized by RNAi transgenes that depleted E2F2/RBF1/dREAM (Mip120/Mip130) components (Dietzl et al, 2007). Expression of the Pum/Nos/Brat RNAi transgenes alone produced no visible phenotype (Fig 2A, Supplementary Fig S8). Reducing the levels of the Pumilio complex using RNAi in the wing pouch of Drosophila sensitized by depletion of dREAM proteins caused mis-shaped and blistered wings (Fig 2A and B, Supplementary Fig S8, and Supplementary Table S1). A detailed description of how this assay was scored can be found in the Materials and Methods section. These results show that Nanos levels are not only up-regulated when dREAM function is reduced, but that the elevated activity of the Pum complex is also important in Drosophila tissues with compromised E2F/RBF regulation.

We next wanted to determine whether the activity of the Pum complex that is required to maintain tissue homeostasis in RBF1/ E2F2/dREAM compromised cells is conserved in mammalian cells. To do this, we assayed the effect of depleting Pum1, Pum2, and Nanos1 using shRNA from 3T3 lines derived from mouse embryonic fibroblasts (MEFs) containing mutations in the pocket proteins (Rb1, p107, and p130) (Clason et al, 2000). We did not examine the effects of Nanos2 and Nanos3 knockdown because we were unable to find shRNAs that gave efficient depletion of these targets. As shown in Fig 3A and Supplementary Fig S9A–E, depletion of Nanos1 reduced the number of cells in Rb1 null and triple-negative (Rb1, p107, and p130$^{−/−}$) 3T3s, suggesting that Nanos1 is a critical component in Rb1-deficient cells. Knockdown of Nanos1 did not affect 3T3s solely lacking p107 or p130. Depletion of either Pum1 or Pum2 did not affect the viability of any of the 3T3s. To understand why Nanos1 depletion reduced the number of only Rb1 null 3T3s, we measured the relative expression levels of p16 and the Pum complex components in each of the 3T3 genotypes. All of the 3T3s except the p107 nulls expressed p16 (Supplementary Fig S10B). The levels of the Pum genes varied little between genotypes and remained high compared to the non-E2F-regulated E2F3 gene; however, Nanos1 and Nanos3 levels were strongly elevated in the 3T3s lacking Rb1 alone or the triple-negative cells (Supplementary Fig S10A). These results show that Nanos1 and Nanos3 are specifically up-regulated following the inactivation of pRb and that depletion of Nanos1 levels reduces the numbers of cells.

To test the idea that NANOS1 contributes to the growth of human pRb-deficient cells, we examined the effects of NANOS1 depletion on (Rb1 null) human retinoblastoma cancer cell lines. Knockdown of NANOS1 using shRNA significantly reduced the number (Alamar Blue) of Y79 retinoblastoma cells compared to scrambled controls (SCR) (Fig 3B). To examine the hypothesis that co-depletion of pRb and NANOS1 may reduce cell number, we treated human cell lines, BJ (Fibroblasts) and Calu-1 cells (non-small cell lung carcinoma cells (NSCLC)), with shRNAs targeting pRb and NANOS1, and assayed cell number using crystal violet staining. Excitingly, lowering the levels of pRb and NANOS1 reduced the number of cells in both fibroblasts (Fig 3C, Supplementary Fig S10C) and NSCLC cells (Supplementary Fig S10D). This reduction in cell number could be rescued by the transfection of plasmid containing a shRNA insensitive pRb (Fig 3C and D).

To examine the role of NANOS1 in pRb-deficient cells, we depleted NANOS1 or scrambled control sequences using shRNAs and assessed cell number in 18 cancer cell lines of diverse tissue origins and mutational profiles. The depletion of NANOS1 reduced cell number in a subset of lines (for quantification of cell staining, Supplementary Fig S11A), and we noticed that these lines have the shared property and that they are compromised for pRb function (mutant for either Rb1 or p16INK4a) and retain an intact p53 (Fig 4A and B, Supplementary Fig S11B). To determine the consequence of NANOS1 loss from pRb-deficient cells, we depleted NANOS1 using siRNA from Y79 retinoblastoma and NCI-H1666...
NSCLC cell lines and counted cells over time. As shown in Supplementary Fig S12A–C, depletion of NANOS1 resulted in reduced cell number from both cell lines after 5 days, suggesting NANOS1 functions to inhibit cellular expansion of pRb-deficient cells. To evaluate the contribution of p53 to this interaction, we examined HCT116 cells, a p16INK4a mutant cell line that is sensitive to NANOS1 depletion, and compared the effects of NANOS1 depletion in isogenic lines that either lack or retain p53. HCT116 cells retaining p53 activity display a strong reduction in cell number upon NANOS1 depletion; however, HCT116 p53 null cells were unaffected (Fig 4C, Supplementary Fig S11C (quantification of staining)). These results suggest that NANOS1 prevents p53-mediated inhibition of cellular growth in cells that are deficient for normal pRb activity.

To investigate how NANOS1-mediated post-translational regulation contributes to the growth of pRb-deficient cells, we examined gene expression profiles from normal retina tissue and retinoblastoma tumors (Ganguly & Shields, 2010). We observed a striking percentage of PUM substrates among the transcripts that were up-regulated (19%, 208/1,083) and down-regulated (22%, 171/770) in retinoblastomas (Fig 5A). To determine how dys-regulation of the E2F transcription factors would affect these putative PUM substrates, we analyzed the promoters of these genes for E2F binding motifs. E2F motifs were identified in 51% of the up-regulated and 7% of the down-regulated PUM substrates in retinoblastomas (Fig 5A). This suggested that the up-regulation of the E2F transcriptional program may be sufficient to counterbalance and override the

### Figure 2. The Pumilio and dREAM complexes genetically interact.

**A** Phenotypes produced from genetic interaction experiments using RNAi driven by Nub-Gal4 to reduce the levels of dREAM components (e2f2, mip120, mip130, and rbf1) and the pumilio complex members (pum, nos, and brat) in the Drosophila wing pouch. RNAi constructs used in this experiment were PUM RNAi (36676), NANOS RNAi (28300), BRAT RNAi, and luciferase RNAi as a control. Genetic interaction analysis of wings was scored as follows: no phenotype (−), variable minor phenotype (−/+), minor extra wing vein (+), minor blistering (++), and severely blistered and deformed wings (+++).

**B** Table outlining the genetic interaction between PUM complex members and dREAM components.

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post-transcriptional regulation of the PUM complex. We therefore focused on the genes that are down-regulated in retinoblastomas and that did not contain E2F motifs. Interestingly, gene ontology classification of the putative PUM targets among these down-regulated genes showed a strong enrichment for kinases and regulators of apoptosis, an enrichment that was not evident when the overall group of down-regulated transcripts was examined (Fig 5B). To determine whether this reduction in the mRNA levels of PRE-containing transcripts in tumors was due to PUM-mediated mRNA instability, we conducted RNA-stability assays on PUM substrates. To do this, we treated Y79 retinoblastoma cells depleted of PUM by shRNA with Actinomycin D, to poison RNA polymerase II. As shown in Supplementary Fig S13A–D, reducing the levels of PUM in retinoblastoma cells significantly increased the stability of the PRE-containing transcripts (MAP3K1 and MAP2K3) compared to a control non-PUM substrate (E2F4), suggesting that elevated PUM activity in these cells may contribute to the reduced transcript levels of MAP3K1 and MAP2K3 in retinoblastoma cells.

These observations raised the possibility that the increased levels of NANOS1 may down-regulate the translation of a set of kinases and pro-apoptotic proteins in pRb-deficient cells. To test this idea, we selected one of the kinases within the list of down-regulated genes, MAP2K3, a kinase that is known to help activate p53, and asked whether it was indeed controlled by NANOS and PUM. MAP2K3 contains a putative PRE sequence (UGUAXAUA) (Galgano et al., 2008) and 3 putative NANOS regulatory elements (NRE) (GUUGU) (Sonoda & Wharton, 1999) within its 3'UTR (Fig 6A). To determine whether MAP2K3 is a substrate for PUM/NANOS repression, we created point mutations within each PRE and NRE motif of the MAP2K3 3'UTR downstream of the luciferase gene and assayed their affect on luciferase production. As shown in Fig 6B, mutation of singular NRE elements or the PRE produced modest changes in
expression; however, disruption of NANOS1 regulation by mutating all of the putative NRE sites (NRE ALL) induced a robust increase in luciferase levels. To ascertain the contribution of PUM and NANOS in regulating MAP2K3 translation, we depleted PUM1, PUM2, and NANOS1 from cells and assayed the affect on luciferase production from the wild-type 3'UTR, PRE mut 3'UTR, and NRE all mutant 3'UTR. Silencing of either PUM increased the levels of both the wild-type and NRE mut all constructs, while a reduction in NANOS1 levels contributed to an up-regulation in the wild-type and PRE mutant 3'UTRs (Fig 6C, Supplementary Fig S14A). Studies from Pumilio-1 mutant mice have implicated aberrant upstream translation of the mouse homologs of MAP2K3 and MAP3K1, as regulators of apoptosis during spermatogenesis (Chen et al., 2012). To examine whether MAP3K1 activity was sufficient to reduce cell number upon NANOS1 loss, we depleted NANOS1 and MAP3K1 from HCT116 cells and assayed cell number. Loss of MAP3K1 activity is unable to rescue cells with inactive pRb from the affects of NANOS1 silencing (Supplementary Fig S14D and E). To determine whether loss of NANOS1 activity in pRb-deficient cells contributed to the activation of the p38 kinase, downstream of MAP3K1 and MAP2K3, we silenced NANOS1 using shRNA and assayed p38 status. As shown in Fig 6E, depletion of NANOS1 stimulates the phosphorylation and activation of the p38 kinase upstream of p53.

These findings show that NANOS1 suppresses the expression of MAP kinases that function upstream of p53. Elevated NANOS1 levels contribute to increased PUM complex activity, and this is one of the mechanisms which repress the cellular stress response in pRb-activate cells.

Figure 4. p53 activity is necessary for the reduction in cell number upon silencing of Rb1 and NANOS1.
A Crystal violet staining of cancer cell lines from diverse tissue types (fibroblast, retina, head and neck, kidney, bone, bladder, breast, non-small cell lung carcinoma (NSCLC), and colorectal) infected with shRNAs targeting NANOS1 (hNOS1-1 and hNOS1-3) or scrambled sequences.
B Summary table of the pRb and p53 status of the cells tested in the panel above and a description of the consequence of NANOS1 and scrambled sequence depletion.
C Crystal violet staining of isogenic HCT116 cells with (p53 wt) and without (p53 mut) p53 infected with shRNAs targeting NANOS1.
Discussion

This study shows that *nanos* is a direct target of RBF1/E2F2/dREAM in *Drosophila* and that this regulation is important for restricting Nanos expression. We additionally find that the regulation of NANOS1 by pRb is conserved in mammalian cells and that upon pRb-inactivation NANOS levels are strongly up-regulated. *In vitro* cell culture and *in vivo Drosophila* data demonstrate that elevated Nanos levels in flies produces genetic interaction phenotypes in RBF1 mutant wings and depletion of Nanos1/NANOS1 in mouse or human cells lacking pRb reduces cell number. To understand how NANOS1 functions to support the growth of pRb-deficient tumors, we compared gene expression profiles from normal retinal tissue and retinoblastoma tumors (*Rb1/C0/C0*) and identified a strong enrichment for PRE-containing genes in signaling and apoptotic pathways as being down-regulated in these tumors.

Collectively, these data strongly support a model where pRb inactivation stimulates the up-regulation of NANOS1 expression and protein levels, which in turn increases NANOS-mediated transcript degradation (Bhandari *et al*, 2014) but also the overall efficiency of the PUM post-transcriptional repressor. NANOS levels have previously been shown to be important for the activity and stability of the PUM complex (Sonoda & Wharton, 1999), and *in vitro* studies in *Drosophila* show that stoichiometric cooperation between PUM and NANOS is important for efficient repression (Wharton *et al*, 1998). These findings raise the hypothesis that by elevating the levels of NANOS, cells may be able to increase the overall efficiency of the PUM complex. Our data suggest that upon pRb-inactivation cells stimulate the expression of NANOS1 to similar levels with both PUM1 and PUM2 (Fig 1D, Supplementary Fig S3) and that this elevation in NANOS1 levels increases the levels of both PUM proteins by stabilizing the PUM complex (Supplementary Fig S3B and C). By elevating the levels and activity of the PUM complex, cells deficient for pRb can suppress apoptotic signals and maintain cell homeostasis.

Evidence supporting the hypothesis that increased PUM activity is important in pRb-deficient cells comes from our comparison of gene expression profiles from normal retinal tissue and Rb1 null retinoblastoma tumors. This analysis identified equal proportions of PRE-containing transcripts in genes both up- and down-regulated in tumor samples, suggesting no transcriptional bias toward putative PUM substrates. However, when the PUM substrates which contain
E2F motifs within their promoter regions were analyzed, we found that the majority of the PUM substrates that are up-regulated in retinoblastomas are E2F targets (51%), while only a small subset of down-regulated PUM targets contain E2F motifs (7%) (Fig 5A). The up-regulated PUM substrates have fundamental roles in E2F-driven processes including cell cycle progression, DNA replication and transcription, whereas the down-regulated PUM targets are involved in stress and apoptotic responses (Figs 5 and 6E).

These results provide important insights into how pRb-inactive tumor cells respond to the cellular stress associated with the loss of pRb activity. The retinoblastoma gene expression profiles suggest the direct transcriptional up-regulation of E2F upon loss of pRb enables E2F-regulated genes to overcome elevated PUM-mediated repression and promote E2F-driven processes; however, increased NANOS1 expression enables pRb-deficient cells to post-translationally suppress the synthesis many non-E2F regulated products, including those that control deleterious events for cancer cells (Fig 6F). In support of this hypothesis, we find that depleting PUM from retinoblastoma cells stabilizes the transcripts of the PRE-containing mRNAs, such as MAP3K1 and MAP2K3, compared to non-PUM substrates (Supplementary Fig S13).

We then analyzed the capacity of the PUM complex to post-transcriptionally regulate two of the pro-apoptotic genes which were down-regulated in retinoblastoma tumors. By utilizing shRNA knockdown and luciferase reporter assays, we confirmed that the candidate genes, MAP2K3 and MAP3K1, are both direct targets of E2F (Fig 6A).

Figure 6. NANOS1 post-translationally inhibits Map3K1 and Map2K3 levels preventing p38 activation.
A Schematic of Map2K3 and MAP3K1 3’UTRs detailing PRE and NRE positions.
B Relative luciferase levels testing how mutations within the PRE and NREs affect the control of the Map2K3 3’UTR on a downstream luciferase reporter construct transfected into HCT116 p53 mut cells (mean ± SD, n = 3).
C Relative luciferase levels testing the affect of depleting PUM1 (PUM1-15, PUM1-19), PUM2 (PUM2-1, PUM2-4), and NANOS1 (NOS1-1, NOS1-3) on luciferase constructs containing the MAP2K3 3’UTR, PRE mutant 3’UTR (PRE mut), or triple NRE mutant 3’UTR (NRE all) (mean ± SD, n = 3).
D Western blots from HCT116 p53 mut cells of MAP3K1, MAP2K3, NANOS1, and tubulin (TUB) after treatment with shRNAs targeting NANOS1 (N1-1, N1-3) and scrambled sequences (Scr).
E Western blots from HCT116 p53 mut cells of p38 and phospho-p38 (Pho-p38) after treatment with shRNAs targeting NANOS1 (N1-1, N1-3) and scrambled sequences (Scr).
F Schematic depicting the model of how NANOS1 contributes to the survival of Rb1-deficient cells by preventing the activation of p53-mediated apoptosis.

Source data are available online for this figure.
PUM/NANOS1 and E2F is an important regulatory loop that has been maintained during evolution. The conservation of this interaction between flies and humans indicates that the interplay between these pathways, these proteins not only dampen the effects of E2F deregulation, but they also suppress signals that lead to the activation of pRb which is not sensitive to NANOS1 depletion (MCF7) contains a number of mutations within closely related MAP kinases which may suppress stress responses in a NANOS1-independent manner (Forbes et al, 2008, 2011).

Because PUM- and NANOS1-regulated substrates impact multiple pathways, these proteins not only dampen the effects of E2F deregulation, but they also suppress signals that lead to the activation of p53. In these tumor cells, the activity of the PUM complex becomes important to counterbalance cellular stress and to maintain cell number; as such PUM and NANOS1 represent exciting targets for novel therapeutic intervention. The conservation of this interaction between flies and humans indicates that the interplay between PUM/NANOS1 and E2F is an important regulatory loop that has been maintained during evolution.

Materials and Methods

Fly stocks, genetic crosses, and modifier screen

The following stocks were used for these studies: wild-type (w1118) (Bloomington), NUB-Gal4 (Bloomington), Ptc-Gal4 (Bloomington), En-Gal4 (Bloomington), UAS-DICER (Bloomington). The mutant alleles or UAS-RNAi constructs used in this study include: brat (brat1, brat31, brat6038, brat441333 (VDRC)), Pumilio (pum5, pum13, pum3, pum900010, pum445815 (VDRC), pum46676 (TRIP)), nanos (nos117, nos241, nos210890 (VDRC), nos922663 (VDRC), nos6320 (TRIP), nos58975 (TRIP)), e2f2 (e2f276Q, e2f276Q, e2f276Q), rbl1 (rbl16744 (TRIP), rbl120), dp (dp51), mmp120 (mmp120102463), and mmp130 (mmp130102463). Control lines used in this project include luciferase RNAi, Ga4 RNAi and GFP RNAi. Variation in the genetic interaction between flies and humans indicates that the interplay between PUM/NANOS1 and E2F is an important regulatory loop that has been maintained during evolution.

RNAi in Drosophila S2 cells

Double-stranded RNA for RNAi experiments was generated using the RiboMax large scale RNA production system (Promega) following the manufacturer’s instructions. Drosophila S2 cells were incubated with 50 μg of dsRNA for 4 days and performed as previously described (Dimova et al, 2003). All RNAi experiments used in this paper were conducted in triplicate, and averages and standard deviations are displayed in this paper.

siRNA transfection

Human BJ fibroblast cells were transfected with 50 nM of siRNAs for targeting Rbl1, p107, p130 and a scrambled control (Dharmacon Smart pool siRNA) using the Lipofectamine RNAiMAX (Invitrogen) as per the manufacturer’s specifications. Cells were lysed and analyzed using RT-PCR and Western blotting 2 days post-transfection, all data included within this paper represent biological triplicates (Westerns) and biological triplicates and technical duplicates (RT-PCR).

Chromatin Immunoprecipitation and ChIP-chip from Drosophila larvae

ChIP experiments from Drosophila 3rd instar larvae were performed using a previously published method (Negre et al, 2006). Wild-type or mutant animals were homogenized, cross-linked, sonicated, and immunoprecipitated as described elsewhere (Negre et al, 2006).

Chromatin immunoprecipitation from human cells

ChIP experiments from human fibroblasts BJ cells and Y79 retinoblastoma cancer cell lines were conducted using a previously published method (Black et al, 2013). BJ cells were depleted using siRNA as detailed above.

Real-time quantitative PCR (RT-PCR)

Total RNA was purified using the RNeasy Extraction Kit (Qiagen). Reverse Transcription was performed using the Taq Man Reverse Transcription (PE Applied Biosystems) according to the manufacturer’s specifications. RT-PCR was performed for 50 cycles using an ABI prism 7900 HD Sequence Detection system. mRNA levels were measured using SYBR Green detection chemistry (Applied Biosystems). Quantification was performed using the comparative ΔCt method as described by the manufacturer. Tubulin, Actin, GAPDH, and Rsp26 were used a control for normalization. All RT-PCR experiments were conducted in biological triplicates and technical duplicates. Graphs representing RT-PCR data contain averages and standard deviations.

Cell culture, transfections, and luciferase expression constructs

pGL4 (Promega), pGL3 (Promega), pGL3-pum promoter region, pGL3-nanos promoter region, pGL3-brat promoter region, psi-CHECK2 (Promega), psi-CHECK-Map2K3-3UTR (kind gift from Francois Houle), psi-CHECK-Map2K3-3UTR PRE MUT (GT1690/1CC), psi-CHECK-Map2K3-3UTR NRE 1 MUT (GT1687/8CC), psi-CHECK-Map2K3-3UTR NRE 2 MUT (GT1793/4CC), psi-CHECK-Map2K3-3UTR NRE 3 MUT (GT2190/1CC), psi-CHECK-Map2K3-3UTR NRE all MUT (GT1687/8, 1793/4, 2190/1CC), pCMV-Rb. Drosophila S2 cells were transfected for 48 h using X-tremeGENE HP transfection reagent.
reagent (Roche) as per the manufacturer’s instructions. Human cells: HCT116 (p53−/−), were transfected for 48 h with X-tremeGENE transfection reagent (Roche) and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. All transfection experiments were conducted in biological triplicates.

**Antibodies**

Antibodies used in this study include: de2F1 (polyclonal anti-rabbit, C. Seum, kind gift of Pierre Spierer), de2F2 (Dimova et al, 2003), RB1 (Du et al, 1996), Tubulin (Developmental Studies Hybridoma Bank, E7), Pumilio-1 (PUM1) (Bethyl, A300-201A), Pumilio-2 (PUM2) (Bethyl, A300-202A), E2F1 (Santa Cruz, sc-193), E2F3 (pg-30), Rb1 (Santa Cruz, sc-50), p107 (Santa Cruz, sc-318), p130 (Santa Cruz, sc-317), NANO1 (Abcam, ab3417), anti-HA epitope (Covance, 16B12), p38 (Cell Signaling, 9212), and phosphor-p38 (Cell Signaling, 9211).

**Luciferase assays**

Luciferase assays in S2 *Drosophila* cells were transfected in 12-well plates with 100 ng of pG4 and 150 ng of the pGL3-pumilio, pGL3-nanos, and pGL3-brat promoter luciferase constructs. For Map2K3 luciferase experiments, HCT116 cells (p53−/−) were transfected in 12-well plates with 150 ng of psi-CHECK-Map2K3-3’UTR (or variations of). Unless otherwise stated, luciferase levels were measured 48 h post-transfection (data are expressed as mean ± s.e., n = 3). Luciferase readings were taken using the Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer’s instructions. All luciferase assays were conducted in biological triplicate and technical duplicate. Luciferase readings in the paper are averages and standard deviations of these measurements.

**Lentiviral shRNA**

The DNA preparation, transfections, and virus preparation methods have been published elsewhere (Pearlberg et al, 2005). LKO.1 shRNA vectors targeting the human PUM complex were as follows: PUM1 (sh#1 TRCN0000147347, sh#16 TRCN0000148785, sh#17 TRCN0000148491, sh#18 TRCN0000148263, sh#19 TRCN0000146945), PUM2 (NM_015317; sh#1 TRCN0000061858, sh#2 TRCN0000061859, sh#3 TRCN0000061860, sh#4 TRCN0000061861, sh#5 TRCN0000061862), NANO1 (NM_199461; sh#1 TRCN0000118075, sh#2 TRCN0000118076, sh#3 TRCN0000118072, sh#4 TRCN0000118073, sh#5 TRCN0000118074), and NANO2 (NM_01029861; sh#1 TRCN0000118123, sh#2 TRCN0000118122, sh#3 TRCN0000118124, sh#4 TRCN0000118125, sh#5 TRCN0000118126). Rb1 targeting shRNAs were E3 TRCN000004163, D4 TRCN0000010418, and D5 TRCN0000010419. MAP3K1 targeting shRNAs were sh#1 TRCN0000197225 and sh#2 TRCN0000196318.

LKO.1 shRNA vectors targeting the mouse Pum complex were as follows: Pum1 (NM_014767; sh1 TRCN0000148785, sh2 TRCN0000148785, sh3 TRCN0000146945, sh4 TRCN0000148263, sh5 TRCN0000148491), Pum2 (NM_030723; sh1 TRCN0000102260, sh2 TRCN0000102261, sh3 TRCN0000102262, sh4 TRCN0000102263, sh5 TRCN0000102264), NANO1 (NM_179421.2; sh1 TRCN0000096769, sh2 TRCN0000096770, sh3 TRCN0000096771, sh4 TRCN0000096772, sh5 TRCN0000096773) were obtained from the RNAi Consortium (Boston, MA). The effect of each shRNA was tested in triplicate, and representative samples are displayed.

**Crystal Violet stain**

Cells were washed in PBS and then fixed in PBS containing 10% acetic acid and 10% methanol for 15 min. The fixative was then removed, and the cells stained in PBS containing 0.4% Crystal Violet and 20% ethanol for 30 min. The stain was removed, and the cells washed with water before being air-dried. Cell numbers quantified using ImageJ software.

**Alamar Blue assay**

Cells were stained with 100 mg/ml of Alamar Blue (Resazurin, Sigma) for 4 h at 37°C. Emissions were read Perkin Elmer 2103 multi-label reader using Wallac EnVision Manager software. Scramble control set to 1.

**Cell lines**

The cell lines used in this study are as follows: *Drosophila* S2 cells, Mouse Embryonic Fibroblasts containing mutations within the Rb1, Rb1 and Rb2 genes. The human-derived cells used were: BJ (fibroblast), RPE (retinal), Y79, WERI (retinoblastoma), FADU, JHU029 (head and neck), 293T (kidney), RPE (retinal), Y79, WERI (retinoblastoma), FADU, JHU029 (head and neck), Calu-1, CorL-105, SW1573, NCI-H1563, NCI-H1666 (non-small cell lung cancer) and HCT116 colorectal (p53−/− kind gift from Bert Vogelstein).

**RNA stability assays**

Y79 retinoblastoma cells were depleted using shRNA specific to PUM1, PUM2, and scrambled controls and placed under puromycin selection for 4 days. Knockdown was validated by Western blot. Cells were then treated with Actinomycin D (5 μM) and collected every hour for the next 5 h. RNA was then extracted from each time point, and the relative amounts of remaining transcript assayed using RT-PCR. Graphs shown represent two independent shRNAs per gene and were conducted in triplicate.

**CDK 4/6 inhibitor treatment**

HCT116 cells were treated with increasing concentrations of CDK4/6 inhibitor PD0332991 and DMSO (35 nM–20 μM) for 4 days and cell number assayed using Alamar Blue. Assay was conducted in biological and technical triplicates. For RT-PCR analysis of the NANO and PUM genes, HCT116 cells were treated with 1 μM or 100 nM of PD0332991 or DMSO for 2 days before the cells were harvested for RNA and mRNA quantified using RT-PCR.

**Computational informatics**

We used publicly available gene expression data from the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al, 2012) to compute
the correlation in gene expression between RB1, RBL1, RBL2 and five genes of the Pumilio complex (PUM1, PUM2, NANOS1, NANOS2, NANOS3). Gene expression data were obtained in normalized form from the Gene Expression Omnibus, under accession GSE36133. The expression values are already summarized in terms of Entrez Gene IDs so that no conversion between probe sets and genes was necessary. We computed Pearson’s correlation coefficients and the associated $P$-values, obtained from a standard correlation test, over all the 917 cell lines represented in the dataset.

In the same way, we computed the correlations between the five genes of the Pumilio complex and the genes included in the Rb1 loss signature (Herschkowitz et al., 2008). One hundred and forty-seven of the 159 signature genes were represented in the CCLE dataset. For each Pumilio gene, we then compared the correlation with the RB signature genes vs. the correlation with all other genes in the array, using a Mann–Whitney $U$-test to determine the statistical significance of the difference.

The ChIP-Chip and microarray datasets used to analyze the dream regulation of the pum complex in Drosophila was taken then from (Georlette et al., 2007). The retinoblastoma and normal retina expression array datasets were taken from (Ganguly & Shields, 2010). Gene ontology was conducted using the DAVID annotation program (Huang da et al., 2009a,b).

DNA manipulation

The promoter regions of the pumilio, nanos, and brat genes and a control region of the same size were cloned into the pGL3-promoter plasmid using KpnI (for primers see primer list below). Site-directed mutagenesis of the Map2K3 luciferase 3'UTR was conducted as per the Stratagene PFU Quick-change manufacturers’ specifications.

**Primer list**

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The EMBO Journal

NANOS upregulation and role in cells lacking pRb

Wayne O Miles et al
### Mouse experiments

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### Human experiments

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### Supplementary information for this article is available online:

http://emboj.embopress.org

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### Author contributions

The experiments in this paper were designed and conducted primarily by W.O.M. and N.J.D. The ChIP experiments were designed, conducted, and analyzed by M.K. The retinoblastoma tumor and xenografts experiments were designed and conducted by L.M.G. and M.A.D. P.P. conducted the bioinformatics analysis outlined in this paper. The manuscript has been read and approved by all of the authors and the manuscript was designed and written by W.O.M. and N.J.D.

### Conflict of interest

The authors declare that they have no conflict of interest.
References


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