Pumilio facilitates miRNA regulation of the E2F3 oncogene

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E2F transcription factors are important regulators of cell proliferation and are frequently dysregulated in human malignancies. To identify novel regulators of E2F function, we used Drosophila as a model system to screen for mutations that modify phenotypes caused by reduced levels of dE2F1. This screen identified components of the Pumilio translational repressor complex (Pumilio, Nanos, and Brain tumor) as suppressors of dE2F1-RNAi phenotypes. Subsequent experiments provided evidence that Pumilio complexes repress dE2F1 levels and that this mechanism of post-transcriptional regulation is conserved in human cells. The human Pumilio homologs Pum 1 and Pum 2 repress the translation of E2F3 by binding to the E2F3 3’ untranslated region (UTR) and also enhance the activity of multiple E2F3 targeting microRNAs (miRNAs). E2F3 is an oncogene with strong proliferative potential and is regularly dysregulated or overexpressed in cancer. Interestingly, Pumilio/miRNA-mediated regulation of E2F3 is circumvented in cancer cells in several different ways. Bladder carcinomas selectively down-regulate miRNAs that cooperate with Pumilio to target E2F3, and multiple tumor cell lines shorten the 3’ end of the E2F3 mRNA, removing the Pumilio regulatory elements. These studies suggest that Pumilio–miRNA repression of E2F3 translation provides an important level of E2F regulation that is frequently abrogated in cancer cells.

Keywords: cancer; E2F3; Pumilio; miRNA

Supplemental material is available for this article.

Received November 2, 2011; revised version accepted January 10, 2012.
and cell cycle processes and are critical for stem cell maintenance and pluripotency in worms, flies, mice, and humans (Lin and Spradling 1997; Crittenden et al. 2002; Moore et al. 2003; Spassov and Jurecic 2003). In Drosophila, the RNA-binding proteins of the Pumilio complex, Pumilio and Nanos, bind to target mRNAs via distinct motifs (GUUUGU Nanos regulatory element [NRE] and UGUAXAUA Pumilio regulatory element [PRE]) within the 3′ untranslated region (UTR) (Murata and Wharton 1995). The Pumilio complex inhibits translation via a number of mechanisms, including deadenylation (Goldstrohm et al. 2007) and decapping (Coller and Parker 2005), or by recruiting competitors of the translation initiation machinery to the target mRNA (Chagnovich and Lehmann 2001).

Two Pumilio (Pum 1 and Pum 2) and three Nanos (Nos 1–3) proteins have been identified in humans (Spassov and Jurecic 2002, Moore et al. 2003). The human Pumilio proteins have functions that are similar to their Drosophila homologs and have important roles in regulating male germ cell development (Moore et al. 2003; Ginter-Matuszewaska et al. 2009), synaptic function (Vessey et al. 2010), and cell cycle re-entry following quiescence (Kedde et al. 2010). RNA pull-down assays (RPAs) have identified transcripts regulated by the endogenous human Pumilio machinery (Galgano et al. 2008). The mRNAs that associate with Pumilio show an enrichment for microRNA (miRNA) seed sequences (Galgano et al. 2008), and localized cooperation between Pum and miRNAs has previously been identified in C. elegans (Nolde et al. 2007) and human cells (Kedde et al. 2010).

miRNAs are 21- to 23-nucleotide [nt] noncoding RNAs that act as key post-transcriptional regulators and have important roles in the control of various cellular processes. Mature miRNAs associate with Argonaute [Ago] proteins to form RNA-induced silencing complexes [RISCs] (Hutvagner and Zamoere 2002). These complexes interact directly with target mRNAs and are guided by sequence complementarity between the miRNA and the target. Interaction between the RISC and target mRNA inhibits gene expression through site-specific cleavage, mRNA degradation, or translation inhibition (for recent review, see Gu and Kay 2010).

Here we show that Drosophila dE2F1 is regulated by the Pumilio complex and that this mechanism of regulation is conserved in the human ortholog E2F3. Pumilio and Nanos also facilitate the ability of multiple miRNAs to regulate the complex 3′ UTR of the E2F3 mRNA, and the importance of this regulation is underscored by changes that occur in human cancer cells. E2F3 is often dysregulated during tumorigenesis. Amplification or elevated expression from the E2F3 locus (6p22) has been identified in breast (Tordai et al. 2008), prostate (Olsson et al. 2007), and lung (Cooper et al. 2006) cancers. Deregulated E2F3 also appears to be an important driver of proliferation in bladder carcinomas, since amplification of E2F3 is particularly common in this type of cancer (Feber et al. 2004; Oeggerli et al. 2004; Olsson et al. 2007; Hurst et al. 2008). Here we show that several miRNAs that have been shown to be selectively down-regulated in bladder carcinomas in clinical studies (Dyrskjot et al. 2009) target seed sequences within the E2F3 3′ UTR and repress E2F3 in a manner that is strongly cooperative with Pumilio. We found that many cancer cell lines circumvent Pumilio-mediated regulation of E2F3 by shortening the 3′ end of the E2F3 transcript, which eliminates the PRE required for Pumilio regulation. Taken together, these findings suggest that a functional cooperation between the Pumilio complex and miRNAs constrains E2F3 levels and that cancer cells consistently select changes that eliminate this regulation.

Results

Pumilio is a novel post-transcriptional regulator of dE2F1

To identify novel regulators of dE2F1, we used tissue-specific Gal4 drivers to allow UAS-RNAi transgenes targeting dE2F1 to be expressed in a temporally and spatially constrained manner during Drosophila development. Using this strategy, Glass Multimer Reporter-Gal4 (GMR-Gal4) transgenes enabled the levels of dE2F1 to be reduced specifically in the developing eye, generating a rough eye phenotype. Patched-Gal4 (ptc-GAL4)-driven expression of UAS-dE2F1 RNAi lowered levels of dE2F1 in the developing wing, and the resulting reduction in cell proliferation caused a narrowing of the intervein region between the L3 and L4 veins in the adult wing (Morris et al. 2008). To identify novel regulators of dE2F1 function, we examined the capacity of Exelixis deficiencies (Parks et al. 2004; Thibault et al. 2004) to modify these dE2F1-RNAi phenotypes (J Ji, A Herr, and N Dyson, unpubl.). By screening a collection of PiggyBac insertion mutants, we identified alleles that enhance or suppress the effects of dE2F1 depletion in both the eye and the wing (Morris et al. 2008; J Ji and A Herr, unpubl.).

Using this approach, we discovered that mutant alleles that affect subunits of the Pumilio translational repressor complex are strong suppressors of dE2F1-RNAi phenotypes. Mutations affecting pumilio (pum), nanos (nos), or brain tumor (brat) suppressed both the GMR-dE2F1-RNAi and ptc-dE2F1-RNAi phenotypes (Fig. 1A; Supplemental Fig. S1). A search of the 3′ UTRs of the Drosophila E2F-Rbf components for putative NREs [GUUUGU] and PREs [UGUAXAUA] revealed the presence of a putative PRE and NREs within the 3′ UTR of dE2F1 [Fig. 1B] but not in other E2F/Rbf mRNAs. To test whether the endogenous dE2F1 mRNA is a target of Pumilio regulation, we conducted TAP tag RPAs from transgenic flies expressing the Pum RNA-binding motif (Gerber et al. 2006). dE2F1 transcripts were strongly enriched in Pum RPAs compared with controls (w1118 and TAP-tagged dNeurofibromin-1; gift from James Walker) in a manner that resembled the previously characterized Pum target CG8414 (Fig. 1C, Gerber et al. 2006). In contrast, no other members of the E2F-Rbf family were enriched in the TAP-Pum RPAs, although they all interacted with Poly-A-binding protein [PABP] (Fig. 1C; Supplemental Fig. S1C). These results suggest that there is a specific interaction between dE2F1 and Pumilio.
To assess the influence of Pumilio on the levels of dE2F1 expression, RNAi was used to deplete Pum from Drosophila S2 cells [Supplemental Fig. S2A]. Knockdown of Pumilio elevated the levels of dE2F1 protein without changing transcript levels [Fig. 1D] and also increased expression of the E2F target genes [Supplemental Fig. S2B]. Depletion of Culinaa, a known regulator of dE2F1 ubiquitination and degradation [Shibutani et al. 2008], similarly increased dE2F1 levels, in contrast, RNAi of belle [bel], an RNA-binding protein that has previously been reported not to regulate dE2F1 [Ambrus et al. 2007], had no effect [Fig. 1D]. These data suggest that Pumilio may be a novel regulator of dE2F1 translation. As Rhi1 is a known repressor of transcription of dE2F1-regulated genes, we tested the prediction that Pumilio regulation of dE2F1 is distinct from regulation by Rhi1. To do this, we depleted Pumilio, Rhi1, or both [Supplemental Fig. S2C] and measured the effect on E2F target gene expression. Knocking down either Pumilio or Rhi1 elevated E2F target gene expression. The codepletion of both proteins had an additive effect on E2F target gene expression, suggesting that Pumilio and Rhi1 act in parallel to suppress the activity of dE2F1 via separate mechanisms [Supplemental Fig. S2D], although due to the fact that there is residual protein remaining after RNAi, we cannot completely rule out the possibility that they act cooperatively. Taken together, these results suggest that Pumilio and Rhi provide different mechanisms of dE2F1 regulation and that the cumulative activity of both proteins is required to constrain dE2F1 activity.

To determine whether Pumilio regulation of dE2F1 is dependent on the putative PRE, we generated a GFP reporter fused to the 3’ UTR of dE2F1 (GFP-dE2F1-3’ UTR). To test whether the putative PRE is functional, we mutated the sequence encoding the PRE (UGUAAXAUA) to give a nonfunctional element (CCCCXXAUA; GFP-mut). GFP-dE2F1-3’ UTR and GFP-mut-3’ UTR expression constructs were transfected into Drosophila S2 cells and treated with dsRNA targeting Pumilio, Bel, and tubulin [control]. Depletion of Pum increased the level of GFP-dE2F1-3’ UTR translation compared with both the Bel and tubulin controls, however, this effect was abolished when the PRE was mutated (GFP-mut) [Fig. 1E]. In addition, disruption of the PRE (GFP-mut) elevated the expression of GFP in cells treated with control dsRNAs [T7 or bel] [Fig. 1E], suggesting the putative PRE is functional. To confirm that Pumilio regulates dE2F1 in vivo, we overexpressed Pum in the Drosophila ovary using the Nos-Gal4 driver. Elevated levels of Pumilio strongly reduced the levels of endogenous dE2F1 and decreased the expression of dE2F target genes (CycE, Orc5, and Nebb) [Fig. 1F]. Together, these results strongly suggest that Pumilio is an important regulator of endogenous dE2F1 activity in Drosophila.

Pumilio regulation of activator E2F levels is conserved in human cells

To ask whether Pumilio regulation of E2F translation might be conserved in humans, we searched the 3’ UTRs of the
human activator E2Fs (E2F1–3) for putative NRE and PRE sequences. Potential NRE/PREs were found within the 3′ UTRs of E2F2 and E2F3 [Fig. 2A] but not E2F1 (or the pocket proteins [pRb, p107, and p130]). To test whether Pumilio regulation of these activator E2Fs is conserved in human cells, we conducted Rpas using antibodies against endogenous Pum 1 and Pum 2 from primary human fibroblasts (IMR90). E2F3 transcripts were strongly enriched in immunoprecipitates of both Pum proteins [Fig. 2B] as compared with E2F1 or pRb mRNAs, suggesting that Pumilio regulation of activator E2F levels is conserved. The level of E2F2 mRNA in these cells was below the level of detection prior to RPA analysis and was not studied further. To evaluate the role of the endogenous Pum proteins in regulating the levels of E2F3, we depleted Pum from primary human fibroblasts (IMR90) using shRNAs specific to each Pumilio protein [cell cycle profiles] [Supplemental Fig. S3A]. Knockdown of Pum 1 or Pum 2 specifically increased the levels of E2F3 [Fig. 2C, data not shown]. The levels of E2F1 and the pocket proteins were not increased [Supplemental Fig. S3A–C], suggesting that Pumilio regulation is specific to E2F3.

To evaluate the functionality of the Pum and Nos proteins on E2F3, IMR90 cells were transfected with different combinations of Pum and Nos overexpression constructs. Elevated levels of Pum and Nos dramatically diminished the levels of E2F3 protein (compared with E2F1) [Fig. 2D], and this reduction in E2F3 was accompanied by decreased expression of E2F target genes [Fig. 2E; Supplemental Fig. S3D] or E2F-responsive reporter constructs [E2F4-Luciferase] [Supplemental Fig. S3E]. None of the E2F target genes analyzed in these experiments contain PREs. The suppression of target gene expression by Pum/Nos expression was rescued by the cotransfections of an E2F3 overexpression construct that lacked the 3′ UTR sequence [Supplemental Fig. S3D]. We conclude from these results that Pumilio regulation of activator E2F activity is conserved between Drosophila and [non-transformed] human cells.

The E2F3 3′ UTR contains two PREs

To identify the specific elements that mediate Pumilio regulation of E2F3, we characterized the PREs in the 3′ UTR of its mRNA. Sequence analysis suggested that the E2F3 3′ UTR contains two putative PRE sequences at the 3′ distal end of the transcript. To determine whether these elements are functional, we cloned the 3′ UTR containing both motifs downstream from Luciferase and used site-directed mutagenesis to mutate the motifs [which we designate as A and B, respectively] from the canonical sequence [UGUAXAUA] to an inactive form [UCCAXAUA] [Fig. 3A]. We then tested the capacity of the putative PREs to regulate the translation of Luciferase in TCCSUP bladder carcinoma cells. Previously published constructs containing the E2F1 3′ UTR [Sylvestre et al. 2007], which does not contain a PRE, served as a negative control. Mutations compromising all PRE activity [AB] elevated Luciferase’s levels compared with the wild-type E2F3 (F3) sequence [Fig. 3B]. Mutation of single PRE sequences [A or B] produced only minor increases in Luciferase levels, suggesting that both PREs are functional. To confirm that these changes in Luciferase levels are dependent on Pumilio activity, we depleted Pum using shRNAs. Knockdown of Pumilio significantly increased Luciferase production from all constructs containing an intact PRE (F3, A, and B), while the double PRE mutant [AB] remained unresponsive [Fig. 3C]. Pum knockdown had no effect on Luciferase production from control vectors [Gl3 or E2F1 3′ UTR sequence (Supplemental Fig. S3D). We conclude from these results that Pumilio enables miRNA regulation of E2F3.

Figure 2. Pumilio regulation of activator E2F levels is conserved in human cells. (A) Schematic of the human activator E2Fs [E2F1–3] demonstrating the relative positions and sequences of the PRE and distal polyadenylation sites [Poly-A signals]. (B) RT-qPCR results of gapsdh (GAPDH), e2f1 [E2F1], e2f3 [E2F3], e2f4 [E2F4], and retinoblastoma [Rb] from RPAs from IMR90 cells using human Pum 1 and Pum 2 antibodies compared with HA controls [Mock]. Note that insufficient e2f2 transcript was detected in IMR90 cells, so it was excluded from further analysis. (Relative input) Input levels set at 1. (C) Western blots of Pumilio 2 (Pum 2), E2F3, and Tubulin [Tub] from IMR90s infected with lentiviral shRNA targeting Scrambled controls [Scr] or Pumilio 2 [Pum 2]. (D) Western blots of HA, E2F3, E2F1, and Tub from primary human fibroblast [IMR90s] transfected with HA-Pum and Myc-Nos overexpression constructs. (E) RT-qPCR results for the E2F target genes [e2f1, e2f3, cdc6, cdc25, cyc-A, and mcm5] and the pocket proteins [pRb, p107, and p130] from IMR90s overexpressing Pum 2 and Nos 3. (*) P < 0.01.
Nos overexpression constructs. Luciferase expression without Pum cotransfection was set to 1. (bled shRNA was set at 1. ({[50x472]are down-regulated in bladder carcinomas compared with filing experiments have identified a set of miRNAs that are important driver in bladder cancer, and amplifications of the miRNA processing enzyme Dicer 1 (Dcr 1) increased the levels of E2F3 protein (Fig. 4A), indicating that both components of the complex are required in stoichiometric concentrations for repression (NRE schematic in Supplemental Fig. S4A). These results show that there are two functional PREs 161 and 482 base pairs (bp) from the distal end of the E2F3 transcript.

PREs enhance miRNA regulation of E2F3

Previous studies have suggested that there may be cross-talk between Pumilio-binding sites and miRNA regulation. Indeed, the 3’ UTR of E2F3 contains a substantial number of putative target sites for miRNAs (TargetScan) [Supplemental Fig. S4A], and siRNA-mediated depletion of the miRNA processing enzyme Dicer 1 (Dcr 1) increased the levels of E2F3 protein [Fig. 4A], indicating that at least some of these sites are likely to be functional. To identify miRNAs that impact E2F3 levels, we took advantage of the knowledge that miRNAs are known to be selectively down-regulated in human tumors. E2F3 is an important regulator of proliferation, and gains in E2F3 gene copy number have been identified in a diverse assortment of human cancers. However, E2F3 is a particularly important driver in bladder cancer, and amplifications of the E2F3 locus have been found in a substantial proportion of bladder carcinomas (Veltman et al. 2003; Oeggerli et al. 2004; Olsson et al. 2007). Genomic profiling experiments have identified a set of miRNAs that are down-regulated in bladder carcinomas compared with normal patient tissue [Dyrskjot et al. 2009]. Remarkably, E2F3 is a predicted target for three of the five miRNAs that show a greater than twofold decrease in levels in bladder carcinomas compared with normal tissue and is a predicted target for three of the four miRNAs that decrease in expression with increasing tumor grade [TargetScan] [Supplemental Fig. S5B]. These observations suggest that the process of bladder carcinogenesis selects for the down-regulation of miRNAs that target E2F3.

To test whether these miRNAs cooperate with Pum to regulate E2F3, we first focused on miR-503, the miRNA that is a predicted target for three of the five miRNAs that are down-regulated in bladder cancer [Supplemental Fig S5C]. We transfected cells with both scrambled controls [miR-Scr or anti-Scr], miR-503, or the anti-miR of miR-503 (anti-503) and measured the effect on E2F3-Luciferase expression. Addition of miR-503 repressed translation of F3-Luciferase, confirming that E2F3 is a bona fide miR-503 target [Fig. 4C]. In complementary experiments, anti-503 enhanced F3 translation [Fig. 4C]. To test whether Pum activity contributes to the capacity of miR-503 to suppress E2F3, we assayed the capacity of miR-503 to regulate translation from Luciferase plasmids containing single PREs or the AB-Luciferase plasmid that lacks a functional PRE. Addition of either miR-503 or anti-503 modifies expression from plasmids containing single PREs (A and B) [Supplemental Fig. S5E] but had little effect on AB-Luciferase levels [Fig. 4C].

To determine whether Pum cooperates solely with mir-503, we examined other miRNAs that contain seed sequences proximal to mir-503. mir-20a and mir-125b were selected for further characterization because they have been linked to E2F regulation [miR-20a] or are down-regulated in bladder cancer [miR-125b]. mir-20a has
Pumilio enables miRNA regulation of E2F3

With Luciferase constructs and miR-503. Knockdown of Pum strongly suppressed the capacity of miR-503 to repress F3-Luciferase translation, however, it did not affect the translation of AB-Luciferase [Fig. 5A]. These findings suggested to us that it may be possible to enhance the repressive effect of miR-503 by elevating the levels of Pumilio. To test this, we overexpressed Pum/Nos and miR-503 and assayed the effect on F3 and AB translation. Consistent with this prediction, cotransfection of Pum/Nos and miR-503 strongly enhanced the repression of F3 compared with either alone [Fig. 5B], while AB remained unresponsive.

To determine whether cooperation between Pum and miR-503 regulates the levels of endogenous E2F3 in bladder carcinoma cells, we first titrated the levels of transfected Pum/Nos plasmids to find a concentration that was insufficient to reduce the level of E2F3 and then assayed the effect of adding miR-503. miR-503 alone only weakly reduced the levels of E2F3, but this effect was dramatically enhanced by the addition of exogenous Pum/Nos [Fig. 5C]. As expected, the suppression of E2F3 levels by Pum/Nos/miR-503 was rescued by cotransfection of an E2F3 expression construct lacking a 3′ UTR sequence. To test whether miR-503 influences the levels of endogenous E2F3 in bladder cancer cells, we transfected TCCSUP cells with anti-503. Addition of anti-503 increased E2F3 protein levels and also impaired the ability of exogenous Pum to repress E2F3 [Fig. 5D]. We conclude that miR-503 is a physiological regulator of E2F3 and that it acts in cooperation with Pumilio.

Taken together, these results show that both the PREs and Pumilio are important for multiple miRNAs to target E2F3 and this regulation affects at least two miRNAs (503 and 125b) whose expression is suppressed in bladder carcinoma cells, to escape Pumilio-mediated regulation of E2F3. miRNA silencing is a common feature of cancer cells, and our findings raise the possibility that the changes in miRNA levels may not only reduce miRNA regulation of their targets but also compromise the capacity of Pumilio to repress translation of specific targets.

Pumilio-mediated regulation of E2F3 in cancer cells

Pumilio is an essential gene, and, to date, genome-wide cancer studies have not identified changes in Pumilio expression level or activity in cancer cells. We focused, therefore, on the more specific question of whether Pumilio-dependent regulation of E2F3 is compromised in cancer cells. As bladder cancer cells represent a physiological cellular context in which the control of E2F3 is particularly meaningful, we used these to examine Pumilio regulation of E2F3.

As a first step, we surveyed a panel of bladder cancer cell lines that reflect the variety of E2F3 copy numbers seen in human tumors. Quantification of E2F3 genomic amplification, RNA, and protein levels for these cell lines has been previously published [Feber et al. 2004]. Real-time quantitative PCR (RT-qPCR) was used to compare

Figure 4. miRNA regulation of E2F3 is enhanced by the PREs. [A] Western blots of Dcr-1, E2F3, and Tub from TCCSUP cells depleted with a pool of Scrambled siRNAs (Scr) or Dicer-1 siRNAs [Dcr 1]. [B] Schematic of the E2F3 3′ UTR outlining the relative position of the seed sequences for miR-503, miR-20a, and miR-125b relative to PRE 1. [C] Relative Luciferase assays from TCCSUP cells transfected the E2F3 or AB constructs, testing the effect of adding miR-503 or the anti-mer inhibitor [anti-503]. [*] * P < 0.05. (D) Relative Luciferase readings from TCCSUP cells comparing the capacity of miR-20a to regulate F3 and AB translation with scrambled controls (miR-Scr). [**] ** P = 0.01. (E) Relative Luciferase readings from TCCSUP cells comparing the capacity of miR-125b to regulate F3 and AB translation with scrambled controls (miR-Scr). [*] P < 0.01.

highly conserved binding sites within all three human activator E2Fs [Supplemental Fig. S6A] and has been shown to weakly regulate E2F3 [Sylvestre et al. 2007]. To test whether Pum actively cooperates with miR-20a to repress E2F3, we analyzed the effect of miR-20a on the translation of F3- or AB-Luciferase constructs. miR-20a strongly repressed F3 levels but had only weak effects on AB translation [Fig. 4D], indicating that the PREs enhance the repressive capacity of miR-20a on E2F3. Reduced expression of miR-125b has also been linked to the initiation and progression of bladder carcinogenesis [Supplemental Fig. S5B,C; Dyrsjkot et al. 2009], and we therefore tested its ability to regulate E2F3 and whether this requires the PRE sequence. As seen with miR-503, the addition of miR-125b dramatically repressed F3 expression compared with AB [Fig. 4E]. Our findings suggest that PREs are important for miRNA activity on the E2F3 3′ UTR at seed sequences proximal to the PREs.

Pumilio facilitates miRNA regulation of E2F3

Having demonstrated that the PREs are important for miRNA regulation of E2F3, we next asked whether Pumilio is the critical factor acting on these sites. To do this, we depleted both Pumilio proteins (Pum 1/2 shRNA) [Supplemental Fig. S6C,D] from cells before transfecting them
the relative expression levels of E2F1, E2F3, Pum, and various E2F target genes. While the levels of E2F1 and Pum transcripts changed little between cell lines, large differences were evident in the level of E2F3 mRNA and, to a lesser degree, in the expression of E2F target genes (Cdc6, Cdc25, CycA, and Mcm5) [Fig. 6A]. Interestingly, E2F3 mRNA correlates poorly with E2F3 protein levels [Fig. 6A]. For subsequent experiments, we selected TCCSUP cells to represent one end of the spectrum: These cells have amplified E2F3, a high level of E2F3 mRNA, and a high expression of E2F targets [Feber et al. 2004; Hurst et al. 2008]. At the other end of the spectrum, we examined T24 bladder cancer cells. T24 cells lack E2F3 amplification, have much lower levels of E2F3 mRNA, and express the E2F target genes to a lower level than that of TCCSUP cells.

To assess whether Pumilio regulates E2F3 protein levels in bladder carcinoma cells, we manipulated Pum levels. Depletion of Pum 1 or Pum 2 caused a substantial increase in E2F3 levels in TCCSUP cells, but had only minor effects in T24 cells [Fig. 6B; Supplemental Fig. S6]. In converse experiments, the ectopic expression of Pum...
and Nos again had only modest effects in T24 cells (Fig. 6C), but significantly reduced E2F3 protein levels in TCCSUP cells (Fig. 6D). This reduction in E2F3 levels in TCCSUP cells dramatically suppressed the expression of E2F target genes (Fig. 6E). Taken together, both sets of data suggest that the level of E2F3 in TCCSUP cells is sensitive to Pumilio regulation. The amplification of the E2F3 loci in TCCSUP cells appears to produce sufficient mRNA to exceed the regulatory capacity of the endogenous Pumilio protein, since additional Pum/Nos complex significantly reduces the amount of E2F3. To understand why T24 cells are largely insensitive to Pum when compared with both TCCSUP cells and primary human cells, we examined the E2F3 mRNA present in these cells. Analysis of the E2F3 transcript using 3′ rapid amplification of cDNA ends [RACE] and RT-qPCR revealed that the mRNA present in T24 bladder cancer cells has a shortened 3′ UTR that lacks both of the putative PRE sequences [Fig. 6F]. In contrast, this region is retained in the E2F3 transcripts expressed in TCCSUP cells. Our findings suggest that 3′ end shortening [seen in T24 cells] and gene amplification [seen in TCCSUP cells] may represent two distinct mechanisms by which E2F3 can escape Pum regulation.

To determine whether 3′ end shortening of the E2F3 transcript was unique to bladder cancers or is a more widespread mechanism to evade Pumilio translational regulation, we expanded the 3′ UTR that compromises regulation via the PRE end shortening of the E2F3 transcript that removes the distal PRE sequence in the majority of cancer cell lines. A smaller subset of cell lines had shortened the 3′ UTR sufficiently to remove both PREs [BT4NY, SW480, and T24]. By way of comparison, no shortening of the 3′ UTRs for E2F4 and E2F5 [two ubiquitously expressed repressor E2Fs] was observed in any of the cells tested. Taken together, these findings suggest that Pumilio regulation of E2F3 is circumvented in many cancer cells by a shortening the 3′ UTR that compromises regulation via the PRE. Given that Pum-binding sites affect the ability of multiple miRNAs to suppress E2F3, this shortening may not only affect regulation by Pum, but also reduce the capacity of miRNA to limit the expression of E2F3.

**Discussion**

We found that E2F proteins are subject to a regulatory pathway that is conserved between flies and humans, in which the Pumilio repressor complex interacts with defined motifs within the 3′ UTRs of activator E2Fs and, by regulating their translation, limits the synthesis of these key drivers of cell proliferation. The human E2F3 mRNA contains multiple regulatory features within a long 3′ UTR (3.3 kb). These include a large number of miRNA seed sequences, two PRE motifs, two NRE motifs, and several alternative polyadenylation signals. The results described here show that Pumilio plays a critical role in the regulation of E2F3 and has a major impact on the ability of multiple miRNAs to target the 3′ UTR. While previous studies in *C. elegans* [Nolde et al. 2007] and human primary cells [Kedde et al. 2010] have shown that Pum proteins can influence the effects of specific miRNAs on nearby seed sequences, these results show that in the regulation of E2F3, Pumilio proteins can modulate miRNA regulation within the context of a complex 3′ UTR. Pumilio interaction has been shown to disrupt the secondary folding of RNA stem–loop structures and to open up the surrounding RNA [Kedde et al. 2010]. In a similar way, an interaction between the Pumilio complex and the E2F3 transcript may promote conformational changes in the secondary structure of the mRNA, enabling multiple miRNAs to gain access to seed sequences throughout the 3′ UTR and enhancing the repression of E2F3 [Fig. 7B]. Additionally, the rat homolog in mice, Trim 32, can increase the activity of miRNAs [including miR-503] by interacting with Argonaute 1 [Schwamborn et al. 2009]. This suggests that Pumilio complexes may also influence the recruitment of miRNAs to their targets via Trim 32. We note, however, that Pumilio repressor components were not identified in mass spectrometry analysis of the RISC [Meister et al. 2005]. A close relationship between Pumilio regulation and miRNA function has broad implications, as previous in silico studies have noted that Pumilio targets are especially enriched with miRNA seed sequences [Galgano et al. 2008]. Potentially, the functional interaction between Pumilio and miRNAs as described here on E2F3 may be a widespread phenomenon.

Activator E2Fs, such as E2F3 and dE2F1, are potent regulators of cell cycle progression, with the capacity to stimulate quiescent cells to proliferate. Cells have evolved multiple tiers of regulation to limit the potentially damaging consequences of inappropriate E2F activation, and these mechanisms are often perturbed in cancer cells. Mammalian E2F activity is the integrated function of a large family of proteins, with different family members playing key roles in specific cellular contexts. Although elevated levels of E2F3 have been found in a variety of human cancers, including breast [Tordai et al. 2008; Bild et al. 2009], prostate [Olsson et al. 2007], and lung [Cooper et al. 2006], the activity of E2F3 is known to be especially important in bladder carcinoma, where amplification of the E2F3 locus (6p22) has been identified as a driver mutation [Feber et al. 2004; Oeggerli et al. 2004; Olsson et al. 2007]. Interestingly, genomic studies have shown that tumors that amplify E2F3 often also acquire mutations in *RB1* [Hurst et al. 2008], suggesting that there may be continuous selection in these tumors for changes that sequentially elevate E2F3 activity.

There are several types of changes in bladder cancer cells that undermine Pumilio/miRNA regulation of E2F3. Amplification of the *E2F3* locus and increased mRNA synthesis may enable E2F3 to overwhelm the effects of Pumilio/miRNA repression. We found that TCCSUP cells, which contain amplified E2F3, remain highly sensitive to elevated levels of Pumilio (Fig. 6D). The PREs within E2F3 are located at the very distal end of the 3′ UTR. 3′ UTR shortening has been linked to accelerated...
cellular growth rates [Sandberg et al. 2008] and cancer [Mayr and Bartel 2009]. We found shortened E2F3 transcripts [lacking PRE sequences] in T24 bladder carcinoma cells that lack E2F3 amplification and in a large number of additional cancer cell lines [Fig. 7A]. This suggests that 3’ UTR shortening frequently eliminates Pumilio regulation of E2F3, potentially contributing to elevated E2F3 levels and increased cancer cell proliferation. In contrast, we saw little change in the 3’ UTR of the highly expressed, nononcogenic repressor E2F4, E2F4 and E2F5.

Previously published clinical studies have also identified a set of miRNAs that consistently show reduced levels of expression in bladder carcinomas [Dyrsjkot et al. 2009]. Interestingly, E2F3 is a predicted target of many of these miRNAs, and it is striking that several of the miRNAs that are selectively down-regulated in bladder cancers target E2F3 and act in a manner that is strongly dependent on Pumilio [Figs. 4, 5]. Together, these observations suggest that the normal regulation of E2F3 is compromised in bladder carcinomas by a combination of changes that include gene amplification, RB1 mutation, 3’ UTR shortening, and miRNA silencing [Fig. 7B]. Since individual tumors use multiple avenues to compromise the regulation of E2F3, these alternative mechanisms may provide opportunities for therapeutic intervention in specific subsets of cancer cells. For example, by enhancing Pumilio/miRNA regulation, it may be possible to suppress the activity of E2F3 in bladder carcinomas that have amplified E2F3 and/or mutated RB1. E2F3 protein levels are generally elevated in transformed cells compared with primary cells, even though the changes in mRNA levels are often very modest [Supplemental Fig. S7]. Disruption of Pumilio/miRNA regulation is likely to be one contributory factor, but E2F3 may also be subject to additional mechanisms of post-translation deregulation that are altered by oncogenesis. The complete elimination of Pumilio regulation is unlikely to promote tumor cell proliferation. Pum proteins are thought to have hundreds, perhaps more than a thousand, potential targets [Gallego et al. 2008], and Pum genes are essential for cell survival and proliferation [Barker et al. 1992]. To date, mutations or changes in Pumilio expression levels have not been identified in cancer genomic studies, but these results raise the possibility that 3’ UTR shortening is one mechanism by which Pumilio targets that have important roles in the control of proliferation and differentiation can circumvent Pumilio/miRNA regulation.

The overall effects of Puf proteins are likely to be narrower than miRNAs. While over half of the human genome contains seed sequences for miRNAs [Lewis et al. 2005; Friedman et al. 2009], the number of identified Pumilio targets is much smaller [Gallego et al. 2008], indicating that only a subset of miRNA targets are likely to be influenced by Pumilio. Pumilio binding may not be advantageous in 3’ UTRs with relaxed secondary structures or high-quality miRNA seed sequences. Alternatively, Pumilio interaction with the 3’ UTR may be necessary to dislodge RNA-binding proteins that inhibit miRNA function [e.g., HuR/Dnd1] [Bhattacharyya et al. 2006; Kedde et al. 2007]. Another possibility is that other RNA-binding proteins may cooperate with miRNAs to promote the regulation of other transcripts. Interestingly, seed sequences for miR-20a are found within the 3’ UTRs of all three activator E2Fs [E2F1–3]. Although miR-20a regulation of E2F3 is dependent on Pumilio, miR-20a is far more efficient at regulating E2F1 that lacks a PRE [Sylvestre et al. 2007]. These findings suggest that cooperation between Pumilio and the miRNA is transcript-specific rather than miRNA-specific.

Bioinformatic studies suggest that the vast majority of Pumilio targets contain miRNA seeds [Gallego et al. 2008], raising the possibility that cooperation with miRNAs
may be a general feature of Pumilio-mediated regulation. We speculate that cooperation between Pumilio and miRNA may be especially relevant on key regulators of cell proliferation (such as E2F3 and p27) that need to provide a graded response to multiple types of regulatory inputs. The importance of this control is underscored by the fact that tumor cells that are driven by E2F3 select changes that bypass this regulation to generate the high levels of E2F3 activity that promote tumorigenesis. In human malignancies like these, where a key driver of cell proliferation can be controlled by the cooperative effects of Pumilio and miRNAs, manipulating Pumilio activity may provide a novel opportunity to re-establish/strengthen the regulatory potential of miRNAs and suppress tumor cell proliferation.

**Materials and methods**

*Fly stocks, genetic crosses, and modifier screens*

The following stocks were used for these studies: wild-type (w^{1118} [Bloomington Stock Center], GMR-Gal4 [Bloomington Stock Center], Ptc-Gal4 [Bloomington Stock Center], nos-Gal4 [Bloomington Stock Center], UAS-TAP-PumHD [Gerber et al. 2006], UAS-TAP-NF1 [kind gift of James Walker], UAS-TAP-PABP [Gerber et al. 2006], and UAS-Pum [Scheuwers et al. 2002]). The UAS-de2F2 transgenic lines contain a 650-bp (2690–3340) region of the de2F2 gene within a pWIZ vector [Dimova et al. 2003, Morris et al. 2008]. The GMR-Gal4, UAS-de2F2 RNAi #10 [Morris et al. 2008] and ptc-Gal4, UAS-de2F2 RNAi #3 [Morris et al. 2008] lines were used to screen against Exelixis deficiencies or PiggyBac modifications for the de2F2-RNAi phenotype. Exelixis PiggyBac insertions that affected the Pumilio complex, which suppressed these phenotypes, were c01494, c05844, and c01497. Mutant alleles or UAS-RNAi constructs used to validate this interaction were as follows: brat [brat^{18} [Bloomington Stock Center], brat^{627} [Bloomington Stock Center], brat^{N133} [Vienna Drosophila Stock Center (VDRC)], Pumilio [pum^{13} [Bloomington Stock Center], pum^{6088} [Bloomington Stock Center], pum^{6815} [VDRC]], and nanos [nos^{L7} [Bloomington Stock Center], nos^{1089900} [VDRC], and nos^{92943} [VDRC]].

**Tag tag RPAs**

Wild-type (w^{1118}, nos-Gal4/UAS-TAP-PumHD, nos-Gal4/UAS-TAP-NF1, and nos-Gal4/UAS-TAP-PABP) adult females were grown on standard cornmeal–molasses fly food. The adults were then frozen in liquid nitrogen and stored at −80°C. Adult flies [2.5 g] were suspended in 15 mL of buffer 1 [20 mM Tris-HCl at pH 8, 150 mM NaCl, 10 mM EDTA, 0.2% Nonidet P-40, 0.02 mg/mL heparin, 1.5 mM DTT, 1 mM PMSF, 0.5 μg/mL of leupeptin, 20 U/mL DNase I, 10 U/mL RNasin] and ground to a powder with a pestle. The powder was then dounced and centrifuged twice at 10,000 g for 10 min. Cleared extracts were then incubated with 500 μL of slurry [50% [v/v] of IgG-agarose beads for 90 min at 4°C]. The beads were then washed once in buffer 1 for 15 min before being washed three more times in buffer 2 [20 mM Tris-HCL at pH 8, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 1 mM DTT, 10 U/mL RNasin]. Tag-tagged proteins were removed from the beads by incubating 150 U of ActEVE protease [Invitrogen] for 2 h. RNA was then isolated using TRIzol reagent [Invitrogen], followed by RNeasy [Qiagen] purification as per the manufacturer’s instructions [Gerber et al. 2006]. Each RNA Tap tag pull-down in Figure 1 was conducted in triplicate, and averages and standard deviations from RT-qPCR experiments were used in this study.

**RNAi in Drosophila S2 cells**

dsRNA for RNAi experiments was generated using the RibonMax large-scale RNA production system [Promega] following the manufacturer’s instruction. *Drosophila* S2 cells were incubated with 50 μg of dsRNA for 4 d and performed as previously described [Dimova et al. 2003]. T+ and belle dsRNA were used as controls in our experiments, as neither affected de2F1 transcription or translation. All RNAi experiments used in Figure 1 were conducted in triplicate, and averages and standard deviations are displayed in this study.

**RT-qPCR**

Total RNA or immunoprecipitated RNA was purified using the RNeasy extraction kit (Qiagen). Reverse transcription was performed using TaqMan 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 as a control for normalization. Gene-specific primers are available on request. All RT-qPCR experiments used in Figures 1–6 were conducted in biological triplicates and technical duplicates. Graphs representing RT-qPCR data contain averages and standard deviations.

**Cell culture, expression constructs, and miRNA**

Plasmids used in this study include pAC-GFP [Invitrogen], pAC-GFP-de2F1-3’ UTR, pAC-GFP-mut-3’ UTR, pDEST-HA-Pum 1, pDEST-HA-Pum 2, pMyc-Nos 1 [kind gift from Frans Van Roy], pDEST-MyC-Nos 3, pCMV-E2F3, pCMV-E2F4-Luciferase, pGL4 [Promega], pGL3 [Promega], pGL3-E2F1 3’ UTR, pGL3-E2F3 3’ UTR [2527–4940] [F3], pGL3-E2F3 3’ UTR Mut A [2527–4940], pGL3-E2F3 3’ UTR [2527–4940] Mut B, and pGL3-E2F3 3’ UTR [2527–4940] Mut AB. *Drosophila* S2 cells were transfected for 48 h using FuGene 6 transfection reagent [Roche] as per the manufacturer’s instructions. Human cells [IMR90, HeLa, T24, SW780, J28, HT1376, and TCCSUP cells] were transfected for 48 h with FuGene 6 transfection reagent [Roche] and Lipofectamine 2000 [Invitrogen] according to the manufacturer’s instructions. miRNA were transfected using Lipofectamine 2000, mir-503 [Ambion, AM17100], anti-503 [Ambion AM17000], miR-20a [pTMP-20a; kind gift from Pascal Chartand], and mir-125b [Dharmacon, C-300595-03]. All transfection experiments in Figures 1–7 were conducted in biological triplicate.

**Antibodies**

Antibodies used in this study include de2F1 [polyclonal anti-rabbit; C. Seum], GFP [Santa Cruz Biotechnology, sc-8334], Tubulin [Developmental Studies Hybridoma Bank, E7], Pumilio 1 [Bethyl Laboratories, A300-201A], Pumilio 2 [Bethyl Laboratories, A300-202A], E2F1 [Santa Cruz Biotechnology, sc-193], E2F3 [pp-30], Rb [Santa Cruz Biotechnology, sc-50], p107 [Santa Cruz Biotechnology, sc-318], p130 [Santa Cruz, sc-317], Dicer 1 [Abcam, ab4735], and anti-HA epitope [Covance, 16B12].
RNA affinity isolations were performed as described previously (Galgaño et al. 2008). HeLa cells were grown to 90% confluency, then washed in 1× PBS and collected by centrifugation at 2000 g for 10 min. Cells were then lysed in Polysome lysis buffer (10 mM HEPES-KOH at pH 7.5, 50 mM KCl, 10 mM MgCl$_2$, 5 mM MgCl$_2$, 25 mM EDTA, 0.5% Nonidet P-40, 2 mM DTT, 0.2 mg/mL heparin, 50 U/mL RNase OUT [Invitrogen], 50 U/mL Superase IN, 1× complete protease inhibitor tablet [Roche]). This lysate was then spun three times at 14,000 g for 10 min, and any cellular debris was removed. Aliquots were then flash-frozen and stored at −80°C.

Fifty microliters of protein G or protein A beads [Amersham] was equilibrated in NT2 buffer (50 mM Tris-HCL at pH 7.5, 150 mM NaCl, 1 mM MgCl$_2$, 0.05% Nonidet P-40, 0.2 mg/mL heparin [Sigma], 0.02 mg/mL heparin). Twenty microliters of Pum 1 antibody and 50 μg of Pum 2 were then coupled to the beads and incubated for 12 h at 4°C. The beads were then washed three times in NT2 buffer. Twenty milligrams of lysate was then added to the bead-coupled antibody and mixed for 6 h at 4°C. The beads were then used four times in NT2 buffer, and RNP was eluted in SDS-EDTA buffer (50 mM Tris at pH 8, 100 mM NaCl, 10 mM EDTA, 1% SDS). Purified RNA from these pull-downs was analyzed using RT-qPCR. Each RPA was done in duplicate, and the RT-qPCR of SDS). Purified RNA from these pull-downs was analyzed using RT-qPCR. Each RPA was done in duplicate, and the RT-qPCR of these pull-downs was performed in triplicate. RT-qPCR data from these experiments are displayed as averages and standard deviations.

**3’ RACE and genomic DNA preparations**

RNA was extracted as described above and used in 3’ RACE assays in the Invitrogen SuperScript II protocol. One microgram of RNA was used in the first strand cDNA synthesis before 1 μL of RNase H [Invitrogen, 18021-14] was added to remove the remaining RNA. Two microliters of the initial cDNA reaction was then used in a standard PCR reaction using a E2F3 3’ UTR-specific primer (GGCACCTCAACTCATATGT) and amplification-specific primer (GGCCACGCCTGCACTAGTAC). DNA from this secondary record of PCR was purified using the PCR purification kit [Qiagen], and 2 μL of this was used in another PCR reaction using the amplification-specific primer and another E2F3 primer (GGAATACTAATAAGTCTT) distal from the original primer. Ten microliters of this PCR reaction was run out on a 2% agarose gel. The RNA used in the 3’ RACE experiments was extracted in duplicate. The 3’ RACE experiments to map the 3’ end of the transcript in Figures 6 and 7 were conducted in biological triplicate and technical duplicate.

** Luciferase assays**

In E2F4-Luciferase assays, IMB90 cells were transfected in six-well plates with 100 ng of pGGL and 150 ng of the E2F4-Luciferase constructs. For E2F3-based Luciferase experiments, TCCSUP cells were transfected in six-well plates with 100 ng of pGGL and 150 ng of pGGL-E2F3 (or variations thereof). Unless otherwise stated, Luciferase levels were measured 48 h post-transfection (data are expressed as mean ± SE, n = 3).

Luciferase readings were taken using the Dual-Luciferase Reporter Assay system (Promega) as per the manufacturer’s instructions. All Luciferase assays in Figures 3–5 were conducted in biological triplicate and technical duplicate. Luciferase readings in this study 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 PUM 1 [sh#1, TRCN0000147347, sh#2, TRCN0000147875, sh#3, TRCN0000148491, sh#4, TRCN0000148263, sh#5, TRCN0000146945], PUM 2 [NM_015317; sh#1, TRCN0000061858, sh#2, TRCN0000061859, sh#3, TRCN0000061860, sh#4, TRCN0000061861, sh#5, TRCN0000061862], NANOS 1 [NM_1999461; sh#1, TRCN0000118075, sh#2, TRCN0000118076, sh#3, TRCN0000118073], and SHOS1 [NM_00129861; sh#1, TRCN0000118123, sh#2, TRCN0000118122, sh#3, TRCN0000118124, sh#4, TRCN0000118125, sh#5, TRCN0000118126] were obtained from the RNAi Consortium. The effect of each shRNA was tested in triplicate, and representative samples are displayed.

** FACS**

Flow cytometry was performed as described earlier [Binne et al. 2007; Tschöp et al. 2011]. Briefly, cells were labeled for 1 h with BrdU [GE], fixed in 75% ethanol, denatured in 2 M HCl and 0.5% Triton X-100, and neutralized in 0.1 M borate [pH 8.5]. Cells were incubated with anti-BrdU antibody [BD; 1:80 in PBS, 0.5% Tween-20, 1% BSA] and secondary anti-mouse FITC-conjugated antibody [1:400, BD]. Cells were stained with 5 μg/mL propidium iodide in PBS, 1% BSA, and 250 μg/mL RNase A. Cells labeled with Edu were detected using the Click-iT Edu Flow Cytometry Assay kit [Invitrogen] with modification of the protocol by fixation of cells in 75% ethanol. Cell morphology [cell size and granularity] was determined using flow cytometry on cells fixed in 1% paraformaldehyde/PBS. All samples were analyzed in triplicate using a BD FACSCalibur flow cytometer and CellQuest software.

**Acknowledgments**

We thank Dr. Spyros Artanavis-Tsakonas for providing fly stocks. We also thank A. Gladden, A. Walker, H. Najafi-Shoushtari, S. Vasudevan, A. Naar, and J. Walker for careful reading of the manuscript, and all members of the Naar and Dyson laboratories for helpful comments and discussions. This work was supported by National Institutes of Health (NIH) grant R01GM053203 to N.J.D. N.J.D. is the Saltonstall Scholar of the Massachusetts General Hospital Cancer Center.

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Pumilio facilitates miRNA regulation of the E2F3 oncogene

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*Genes Dev.* 2012, 26:
Access the most recent version at doi:10.1101/gad.182568.111