# Correction

**DEVELOPMENTAL BIOLOGY**

Correction for "Pumilio proteins utilize distinct regulatory mechanisms to achieve complementary functions required for pluripotency and embryogenesis," by Katherine E. Uyhazi, Yiying Yang, Na Liu, Hongying Qi, Xiao A. Huang, Winifred Mak, Scott D. Weatherbee, Nicola de Prisco, Vincenzo A. Gennarino, Xiaoling Song, and Haifan Lin, which was first published March 20, 2020; 10.1073/pnas.1916471117 (*Proc. Natl. Acad. Sci. U.S.A.* **117**, 7851–7862).

The authors note that, due to a printer's error, a footnote denoting equal contribution was omitted. The following footnote should have been included for authors Katherine E. Uyhazi and Yiying Yang: "K.E.U. and Y.Y. contributed equally to this work." The online version has been corrected.



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# Pumilio proteins utilize distinct regulatory mechanisms to achieve complementary functions required for pluripotency and embryogenesis

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**Gene regulation in embryonic stem cells (ESCs) has been extensively studied at the epigenetic-transcriptional level, but not at the posttranscriptional level. Pumilio (Pum) proteins are among the few known translational regulators required for stem-cell maintenance in invertebrates and plants. Here we report the essential function of two murine Pum proteins, Pum1 and Pum2, in ESCs and early embryogenesis. Pum1/2 double-mutant ESCs display severely reduced self-renewal and differentiation, and Pum1/2 double-mutant mice are developmentally delayed at the morula stage and lethal by embryonic day 8.5. Remarkably, Pum1-deficient ESCs show increased expression of pluripotency genes but not differentiation genes, whereas Pum2-deficient ESCs show decreased pluripotency markers and accelerated differentiation. Thus, despite their high homology and overlapping target messenger RNAs (mRNAs), Pum1 promotes differentiation while Pum2 promotes self-renewal in ESCs. Pum1 and Pum2 achieve these two complementary aspects of pluripotency by forming a negative interregulatory feedback loop that directly regulates at least 1,486 mRNAs. Pum1 and Pum2 regulate target mRNAs not only by repressing translation, but also by promoting translation and enhancing or reducing mRNA stability of different target mRNAs. Together, these findings reveal distinct roles of individual mammalian Pum proteins in ESCs and their essential functions in ESC pluripotency and embryogenesis.**

Pumilio | translational regulation | embryogenesis | stem cell | mouse

Embryonic stem cells (ESCs) possess dual abilities to self-renew and to differentiate into any cell type in the body. Recent work has identified transcription factors that are essential for stem-cell self-renewal and pluripotency, but the role of posttranscriptional control in ESCs is much less understood. Several lines of evidence indicate that translational regulation provides another important level of control. For example, during mouse ESC differentiation, more than 50% of changes in nuclear protein expression occur without corresponding changes in messenger RNA (mRNA) levels (1). Cells undergoing dynamic state changes such as differentiation may need to quickly modify protein levels of existing mRNAs in response to environmental stimuli (2). Furthermore, such regulation can be effectively achieved by a few key posttranscriptional regulators that coordinate the expression of many target mRNAs, acting as a "regulon" (3). These findings highlight the importance of translational regulation during ESC differentiation and suggest that translational regulators may be key to embryonic development and cell-fate determination.

The *Drosophila* Pumilio (Pum), the founding member of the PUF [for Pumilio and FBF (*fem-3* binding factor)] protein family, has been well characterized as a translational repressor that directly binds to its target mRNAs. The Pumilio-Homology Domain (Pum-HD), consisting of eight tandem imperfect repeats of 36 amino acids (aa) in the C-terminal region of the protein, forms a curved structure in which each repeat contacts one RNA base within the PUF Response Element (PRE), an eight-nucleotide conserved motif in the 3′ untranslated region (UTR) of Pum target mRNAs (4–7). Upon binding, Pum proteins usually repress translation through both poly(A)-dependent (8–10) and poly(A)-independent pathways (11).

PUF proteins have conserved functions in stem-cell proliferation and self-renewal in invertebrates and plants (12, 13). Pum was first identified as a maternal-effect mutant required for embryonic patterning in *Drosophila* (14, 15) and has since been implicated in diverse biological processes. In the *Drosophila* ovary, loss of Pum function results in symmetric, rather than asymmetric, division of germline stem cells that leads to the depletion of the functional stem-cell pool (16–18). The *Caenorhabditis elegans* Pum homolog FBF promotes germline stem-cell proliferation and inhibits

## Significance

This report demonstrates the essential functions of mammalian Pumilio (Pum) proteins for embryonic stem-cell (ESC) pluripotency and embryogenesis. Furthermore, our research reveals the distinct but complementary functions of individual Pum proteins in regulating ESC pluripotency, despite their largely overlapping expression and high homology. Moreover, this report unravels a complex regulatory network in which Pum1 and Pum2 form a negative interregulatory feedback loop that regulates at least 1,486 messenger RNAs (mRNAs). This regulation occurs not only by translational repression, as expected, but also by translational activation and the enhancement or reduction of the stability of different target mRNAs, which effectively demonstrates all four modes of Pum-mediated posttranscriptional control of ESCs.

Author contributions: H.L. designed research; K.E.U., Y.Y., H.Q., W.M., S.D.W., N.d.P., and H.L. performed research; K.E.U. and Y.Y. contributed new reagents/analytic tools; K.E.U., Y.Y., N.L., H.Q., X.A.H., W.M., S.D.W., N.d.P., V.A.G., X.S., and H.L. analyzed data; and K.E.U., Y.Y., and H.L. wrote the paper.

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The authors declare no competing interest.



Data deposition: The ribosomal profiling data and mRNA-seq data have been deposited in National Center for Biotechnology Information Sequence Read Archive (accession no. PRJNA602837).

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differentiation (19). PufA, a Pum homolog in *Dyctiostelium*, sustains growth and inhibits differentiation (20), while in *Planaria*, knockdown of *DjPum* dramatically reduces the number of totipotent stem cells (21). Even in plants, Pum homologs are involved in the regulation of mRNAs involved in shoot stem-cell maintenance (22).

Although PUF proteins are highly evolutionarily conserved (23), our understanding of the function of mammalian PUF proteins remains limited. The mouse genome encodes two PUF proteins, Pumilio1 (Pum1) and Pumilio2 (Pum2). *Pum2* mutant ESCs were reported to have no obvious defects in self-renewal or differentiation (24). Correspondingly, *Pum2* mutant mice were viable and fertile with only smaller testes (25) and subtle neurological defects in memory, nesting behavior, and an increased propensity to seizures (24, 26). Similarly, little is known about the function of Pum1. Conditional knockout of Pum1 in the mouse testis results in decreased fertility (27). Pum1 has also been implicated in human neurodegeneration and motor ataxia (28, 29) and in the maintenance of genomic stability in mice (30). Recent work showed that decreasing the number of $Pum1^{+}$ and/or $Pum2^{+}$ alleles reduces body size in a dose-dependent manner, partly due to translational derepression of the cell-cycle inhibitor CDKN1B (26) and that complete deficiency of *Pum1* and *Pum2* leads to embryonic lethality (31). Conditional knockout of Pum1 and Pum2 in the nervous system affects neural stem-cell maintenance by derepressing the translation of many mRNAs (32, 33). Despite the well-recognized importance of Pum1 and Pum2 in development, the function of Pum1 and Pum2 proteins in ESCs, either individually or collectively, has not been systematically studied. At the molecular level, Pum1 and Pum2 mRNA targets in ESCs are not known, and the mechanisms whereby Pum proteins regulate gene expression other than translational repression have not been reported.

Here we report the distinct but complementary functions of *Pum1* and *Pum2* in ESCs that are essential for mouse ESC pluripotency and embryogenesis. We provide evidence that Pum1 and Pum2 form an interregulatory loop to coordinately control the expression of at least 1,486 mRNAs involved in diverse cellular processes. Furthermore, we demonstrate that such control occurs not only by translation repression—the well-known mode of Pum regulation—but also by promoting translation and enhancing or reducing the stability of different target mRNAs. These findings reveal distinct roles of individual Pum proteins in higher eukaryotes and support mechanisms of Pum-mediated regulation of gene expression.

## Results

**Pum1 and Pum2 Are Expressed in Mouse Embryonic Stem Cells and Early Mouse Embryos.** To explore Pum function in ESCs and embryos, we first examined the expression of Pum1 and Pum2 in ESCs and embryos by immunofluorescence staining. Pum1 is diffusely cytoplasmic in interphase and mitotic ESCs and is also present in the nucleus at low levels (Fig. 1*A*). In metaphase cells, Pum1 appears to be particularly abundant around the spindle periphery (Fig. 1*A*, arrows). In embryonic day 2.5 (e2.5) morulae and e3.5 blastocysts, Pum1 is expressed in the cytoplasm (Fig. 1*B*), with the highest expression in the inner cell mass (ICM) of blastocysts and lower expression levels in trophoblast cells. Pum2 showed nearly identical expression patterns in ESCs (*SI Appendix*, Fig. S1*A*) and e2.5 to e3.5 embryos (*SI Appendix*, Fig. S1*B*). In e8.5 and e9.5 embryos, Pum1 and Pum2 are primarily expressed in the midbrain, developing somites, and the tail bud, as revealed by whole-mount in situ RNA hybridization (*SI Appendix*, Fig. S1 *C* and *D*). The presence of Pum1 and Pum2 in ESCs and early embryos indicates their possible function in ESCs and embryogenesis.

***Pum1*$^{-/-}$ Mice Are Compromised in Viability, Prenatal Development, and Postnatal Growth.** To determine the function of Pum1 in embryogenesis, we generated $Pum1^{-/-}$ mice for phenotypic analysis by breeding $Pum1^{Flox/+}$ mice with *EIIa-Cre* mice using a previously described scheme (ref. 32 and *SI Appendix*, Fig. S2*A*). Knockout of Pum1 was confirmed by genotyping (*SI Appendix*, Fig. S2*B*), quantitative RT-PCR, and Western blot analysis, which did not detect either the mutant *Pum1* mRNA nor a truncated protein. $Pum1^{-/-}$ mice were much less viable than the expected Mendelian ratio (13 vs. 25%, $n = 244$, *SI Appendix*, Fig. S2*C*), indicating that loss of *Pum1* compromised prenatal development. In addition, both male and female $Pum1^{-/-}$ mice were smaller than their heterozygous and wild-type littermates at all time points observed (Fig. 1 *C*–*F* and *I* and *SI Appendix*, *Supplementary Results*, and Fig. S2), with an average weight 35% less than wild-type siblings at postnatal day (P) 28. Moreover, aged $Pum1^{-/-}$ mice had very little body fat, developed a prominent hunchback, and weighed 43% less than wild-type littermates by 11 mo (Fig. 1*D*) as previously reported (28, 33). Liver, lung, stomach, intestine, uterus, and brain weights were proportionately smaller (*SI Appendix*, Fig. S2*D*), while the testis, kidney, spleen, and heart were disproportionately smaller than in $Pum1^{+/-}$ or $Pum1^{+/+}$ littermates (*SI Appendix*, Fig. S2*E*). Notably, almost 80% of $Pum1^{-/-}$ mice were afflicted with ulcerative dermatitis at age 24 wk, in contrast to 5% of $Pum1^{-/+}$ littermates ($P < 0.05$; *SI Appendix*, Fig. S2*F*). Histological analysis revealed that the most obvious defects are in the testis as reported (27) and in the intestine in which $Pum1^{-/-}$ mice had blunted, disorganized small intestinal villi (*SI Appendix*, Fig. S2*G*, arrows), but no significant defect in cell proliferation (Ki67 staining, *SI Appendix*, Fig. S2 *G*, *Middle*), villus length, crypt length, or villus-to-crypt ratio (*SI Appendix*, Fig. S2 *G*, *Lower*). These findings are consistent with the broad expression of *Pum1* in adult tissues (34) and indicate an important role of Pum1 in postnatal growth.

**The Function of Pum1 and Pum2 Is Partially Redundant and Dose-Sensitive.** The relatively mild phenotype of $Pum1^{-/-}$ mice could be due to the high degree of homology between Pum1 and Pum2 (91% identity and 97% similarity in the Pum-HD domain) (34), which renders them functionally redundant. To address this possibility, we generated a $Pum2^{\Delta E3}$ allele in which exon 3 was deleted (ref. 33 and *SI Appendix*, Fig. S3 *A* and *B*, and *Supplementary Results*). This deletion eliminated isoform 1 (UniProt nomenclature, the 1,066-aa full-length Pum2 protein of 114.3 kD starting in exon 2) and isoform 2 (the full-length Pum2 protein missing 574 to 652 aa and 829 to 830 aa, resulting in 985 aa, 106.1 kD), but not isoform 3 (a 929-aa isoform of 99.7 kD starting in exon 4 and missing 574 to 652 aa and 829 to 830 aa) or an isoform that comigrates with isoform 2 (herein named isoform 2′; *SI Appendix*, Fig. S3*C*). These mice showed normal viability and body weight at all time points assessed (*SI Appendix*, Fig. S3 *D*–*G*) with reported neural defects (32). For simplicity, we herein denote the $Pum2^{\Delta E3}$ allele as $Pum2^{-}$.

To determine whether Pum1 and Pum2 could have redundant or dose-dependent functions, $Pum1^{+/-}$; $Pum2^{+/-}$ mice were crossed to obtain nine possible genotypes ranging from zero to four functional *Pum* alleles. At P1, all genotypes were recovered except for $Pum1^{-/-}$; $Pum2^{-/-}$ mice, indicating that these embryos do not survive to birth (Fig. 1*G*; $n = 127$, $P$ value $< 0.01$). In addition, $Pum1^{-/-}$; $Pum2^{+/-}$ pups were recovered at significantly lower numbers than expected (Fig. 1*G*; $P < 0.01$), were smaller than their littermates at P1, had no milk in their stomach, and mostly died within 24 h of birth (Fig. 1*H*) with only one surviving to P6. Histological analysis of $Pum1^{-/-}$; $Pum2^{+/-}$ pups showed thymic necrosis, hepatic congestion, and hepatic atrophy, but no obvious defects in heart, lung, kidney, spleen, or stomach. In contrast, $Pum1^{+/-}$; $Pum2^{-/-}$ mice were relatively normal, whereas $Pum1^{-/-}$ mice were smaller than $Pum2^{-/-}$ mice, which in turn were smaller than $Pum1^{+/-}$; $Pum2^{+/-}$ mice (Fig. 1*I*). In fact, this trend is already evident during embryogenesis at e9.5 (*SI Appendix*, Fig. S4*A*) and e12.5 (*SI Appendix*, Fig. S4*B*). These observations support a partially redundant and collectively essential role of Pum1 and Pum2 during embryogenesis, with Pum1 having greater effects than Pum2.

***Pum1*$^{-/-}$; *Pum2*$^{-/-}$ Embryos Show Developmental Delay at the Morula Stage and Are Lethal by e8.5.** To determine the embryonic defects that lead to the lethality of $Pum1^{-/-}$; $Pum2^{-/-}$ mice, given the

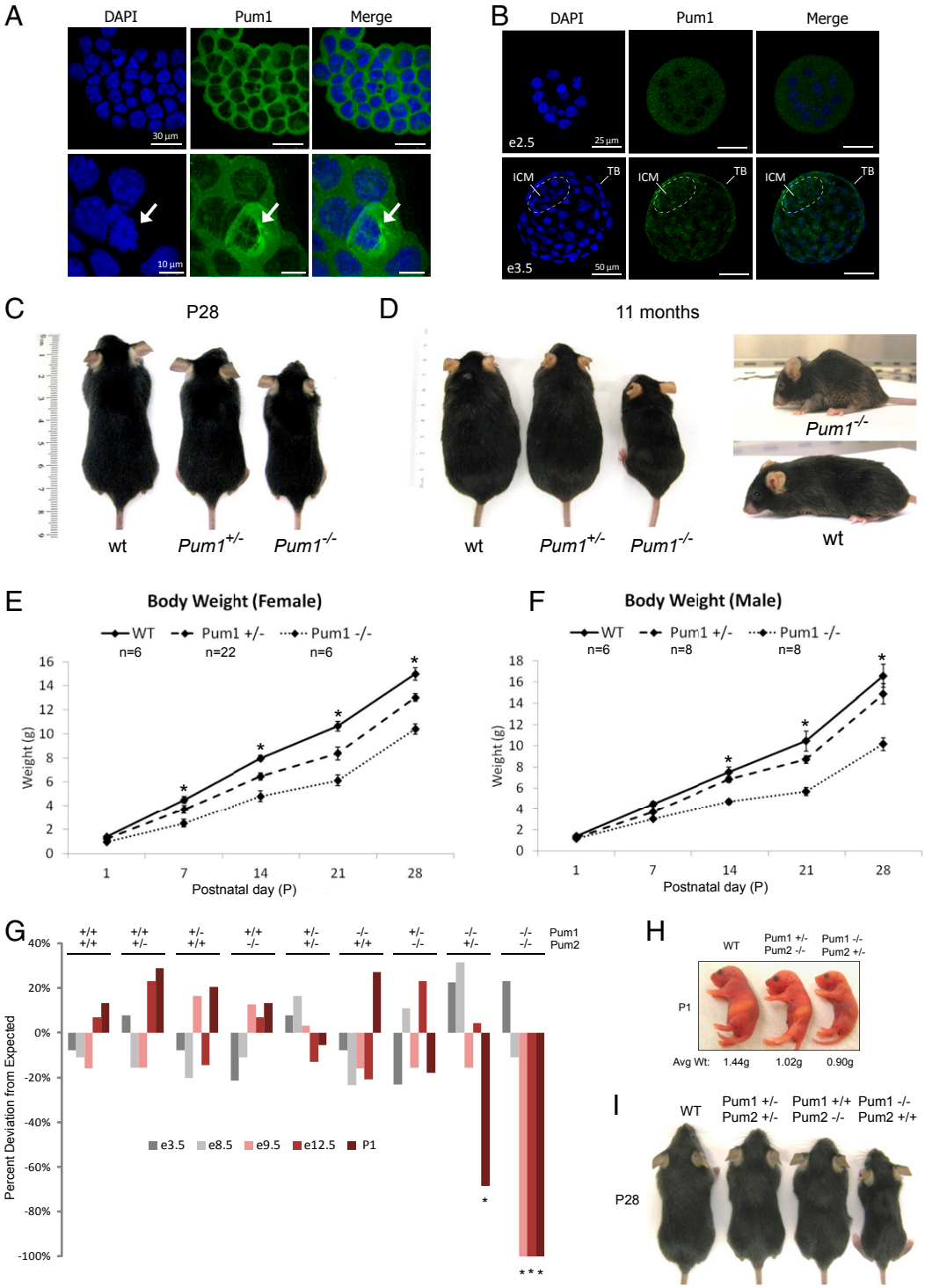


**Fig. 1.** Pumilio proteins are required for embryogenesis and normal growth. (*A*) Immunofluorescence staining of Pum1 in wild-type ESCs. Pum1 is diffusely cytoplasmic and is enriched in the nuclear periphery of some mitotic cells (arrows). Nuclei were counterstained with DAPI (blue). (*B*) Immunofluorescence staining of e2.5 morulae (*Upper*) and e3.5 blastocysts (*Lower*). Pum1 is expressed in both inner cell mass (ICM) and trophoblast (TB) cells, with greater cytoplasmic expression levels in the ICM. (*C* and *D*) Phenotype of P28 (*C*) and 11-mo-old (*D*) wild-type (wt), $Pum1^{+/-}$, and $Pum1^{-/-}$ littermates. $Pum1^{-/-}$ mice have a hunched appearance that becomes more prominent with age and weigh 43% less than wild-type mice (*D*, *Upper Right*). (*E* and *F*) Body weight (in grams) of wild-type (solid line), $Pum1^{+/-}$ (dashed line), and $Pum1^{-/-}$ (dotted line) littermates at P1, P7, P14, P21, and P28. P28 $Pum1^{-/-}$ mice weigh 35% less than wild-type mice. Error bars indicate SEM. *$P$ value < 0.01. (*G*) Deviation of the ratio of observed from expected genotypes of a $Pum1^{+/-}$; $Pum2^{+/-}$ x $Pum1^{+/-}$; $Pum2^{+/-}$ mating at e3.5 (dark gray, $n = 52$), e8.5 (light gray, $n = 36$), e9.5 (pink, $n = 21$), e12.5 (red, $n = 23$), and P1 (dark red, $n = 127$). The observed ratio of $Pum1^{-/-}$; $Pum2^{+/-}$ pups is 65% less than expected at P1. No $Pum1^{-/-}$; $Pum2^{-/-}$ pups were recovered at e9.5, e12.5, and 1dpp. * and ***indicates $P < 0.01$ and <0.001, respectively. (*H*) Morphology and average weight (in grams) of P1 wild-type, $Pum1^{+/-}$; $Pum2^{-/-}$, and $Pum1^{-/-}$; $Pum2^{+/-}$ littermates. $Pum1^{+/-}$; $Pum2^{-/-}$ pups weigh 29% less than WT littermates; $Pum1^{-/-}$; $Pum2^{+/-}$ pups weigh 38% less than WT littermates, have no milk in their stomachs, and die within 24 h of birth. (*I*) Phenotype of P28 Pum-deficient mice; $Pum1^{-/-}$ mice are smaller than $Pum2^{-/-}$ mice and $Pum1^{+/-}$; $Pum2^{+/-}$ mice, which are both indistinguishable from wild type at this age.

known stem-cell function of Pum proteins in lower organisms, we first examined *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ and sibling *Pum1*$^{+/-}$; *Pum2*$^{+/-}$ e3.5 blastocysts in which the inner cell mass had just been established. These embryos were isolated from uteri and genotyped individually by DNA extraction followed by nested PCR. Four *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos were recovered from 52 blastocysts (*SI Appendix*, Fig. S5*A*) at the expected Mendelian ratio, indicating that double-knockout embryos are viable up to e3.5. However, two of the four *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos still appeared morula-like without a defined blastocoel cavity (*SI Appendix*, Fig. S5*A*, asterisks), whereas all 48 of the remaining e3.5 embryos that carried at least one wild-type Pum1 or Pum2 allele had progressed to the blastocyst stage. To obtain a better quantification of the penetrance of the *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ phenotype, we isolated another 17 double-mutant e3.5 embryos from doxycycline-treated *Pum1*$^{Flox/Flox}$; *Pum2*$^{Flox/Flox}$; *rtTA[ROSA]26-Cre* mice (*Materials and Methods*), of which eight appeared as morulae and only nine developed to blastocysts (Fig. 2*B*). In contrast, among 28 *Pum1*$^{+/-}$; *Pum2*$^{+/-}$ e3.5 embryos, only one appeared morula-like, but all 27 others were blastocysts (Fig. 2*A*). Hence, 10 of 21 (47%) *Pum1*$^{-/-}$; *Pum2*$^{-/-}$e3.5 embryos were still at the morula stage, in contrast to 1 of 75 (1.33%) control e3.5 embryos (*SI Appendix*, Table S1). To determine if the 47% *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ morulae were due to developmental delay or arrest, we cultured them overnight and all developed into blastocysts (*SI Appendix*, Fig. S5*B*), indicating a developmental delay. The incomplete penetrance of the delayed phenotype further indicates that the defects of *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos might have just begun to manifest at this stage.

To further investigate the nature of the lethality of *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos, we examined the morphology and expression of key cell-fate markers in doxycycline-induced *Pum1*$^{-/-}$; *Pum2*$^{-/-}$embryos at e3.5, e4.5, e5.5, e6.5, e7.5, e9.5, and e12.5 stages and *Pum1*$^{+/-}$; *Pum2*$^{+/-}$ control embryos at the same stages (*Materials and Methods*). At e3.5, the *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ morulae express the pluripotency marker Oct4 normally but do not express the endoderm marker Gata4 (Fig. 2*B*). This indicates that *Pum1*$^{-/-}$; *Pum2*$^{-/-}$embryos already show a defect in endoderm lineage differentiation at e3.5.

During subsequent embryogenesis, the *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos further display defects in differentiation. By e4.5, 56% of the mutant embryos showed developmental delay or arrest (*SI Appendix*, Table S1). In these embryos, the Gata4-positive primitive endoderm cells are randomly positioned in the inner cell mass, equivalent to e3.5 wild-type embryos and indicating a delay in this lineage (Fig. 2*D* and *SI Appendix*, Fig. S5 *C–F*). By e5.5, 62% of the mutant embryos show incomplete epiblast cell transformation, with Gata4-positive cells still positioned next to the blastocoel cavity (cf. Fig. 2 *E* and *F*). This mislocalization continues in most of the e6.5 *Pum* mutant embryos (cf. *SI Appendix*, Fig. S7 *A* and *B*). The trophectoderm lineage, as indicated by Cdx2 expression, developed normally from e3.5 to e5.5 in the mutant embryos (*SI Appendix*, Fig. S6), but by e6.5, Cdx2-positve cells were no longer detectable (*SI Appendix*, Fig. S7*B*). By e7.5, all cells in the mutant embryos became disorganized, and no obvious structure was observable (cf. *SI Appendix*, Fig. S7 *C* and *D*). By e8.5, only two *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos were recovered from 36 embryos derived from a *Pum1*$^{+/-}$; *Pum2*$^{+/-}$ self-cross; both were significantly smaller and ill-developed (Fig. 2*G*, asterisk). They have a primitive head fold but an overall lack of tissue with thin neural tissue (cf. Fig. 2 *H–K*). No *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos were recovered at e9.5 or e12.5 (Fig. 1*G*). Therefore, the major defects observed at e7.5 likely reflect the terminal phenotype immediately before the e8.5 lethal phase of the mutant embryos.

***Pum1* and *Pum2* Deficiency Does Not Significantly Affect the Proliferation or Viability of ESCs.** Because *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ embryos display developmental delay at the morula-to-blastocyst transition at which ESCs form, we further investigated the function and mechanisms of action of Pum1 and Pum2 in ESCs. ESCs also allowed us to bypass the challenge of studying such mechanisms in scarcely available *Pum* double-mutant embryos at this stage. We first derived *Pum1*$^{Flox/+}$ and *Pum1*$^{Flox/Flox}$ ESC lines from e3.5 blastocysts of *Pum1*$^{Flox/+}$ mice. The ESC lines were transfected with a *pBabe-Puro-Cre* plasmid expressing Cre recombinase and selected in media containing puromycin to generate Pum1$^{Flox/Flox}$ (wild type), *Pum1*$^{+/-}$, and *Pum1*$^{-/-}$ cell lines, respectively. Knockout of *Pum1* was confirmed by genotyping, quantitative RT-PCR, and Western blot analysis (*SI Appendix*, Fig. S8 *A–C*), which indicates that neither the mutant mRNA nor a truncated protein was detectably expressed.

However, repeated attempts to derive a *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ ESC line were unsuccessful, either from blastocysts of *Pum1*$^{+/-}$; *Pum2*$^{+/-}$ offspring or from Cre-mediated excision of *Pum1*$^{Flox/Flox}$; *Pum2*$^{Flox/Flox}$ ESCs. Hence, we transfected *Pum1*$^{Flox/Flox}$; *Pum2*$^{Flox/Flox}$ cell lines carrying an inducible doxycycline-responsive *Cre* gene (*rtTA[ROSA]26*) with a doxycycline-inducible *Pum1* complementary DNA (cDNA) construct. Addition of doxycycline-induced deletions in both *Pum1* and *Pum2* genes while concurrently inducing the expression of the exogenous *Pum1* cDNA rescued the *Pum1-and-Pum2* deficiency (*Materials and Methods*). The genotype of the resulting *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ ESCs was confirmed by Western blotting (*SI Appendix*, Fig. S8*D*). These ESCs were maintainable after the withdrawal of doxycycline to stop *Pum1* expression. Their proliferation was then compared to that of WT, *Pum1*$^{-/-}$, and *Pum2*$^{-/-}$ ESCs when cultured with or without feeders. The cell doubling times of all of the ESCs cell lines were ∼15 h (*SI Appendix*, Fig. S8 *E–H*). More double-mutant ESCs were positive for Annexin V (an early apoptotic marker) and for propidium iodide (a marker for pyknotic nuclei) than wild-type ESCs, but this difference is not statistically significant (*SI Appendix*, Fig. S8 *I* and *J*), indicating that Pum1 and Pum2 are not essential for the viability of ESCs.

**Pum1 Promotes ESC Differentiation Whereas Pum2 Promotes ESC Self-Renewal; Together They Are Required for ESC Pluripotency.** To pinpoint the defects of *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ ESCs, we cultured each of the cell-type and wild-type ESCs under a spectrum of differentiation-promoting conditions. Under normal culture conditions with feeder cells in the presence of leukemia inhibitory factor (LIF), the loss of either *Pum1* or *Pum2* did not affect the percentage of alkaline phosphatase (AP)-positive colonies, but the loss of both *Pum1* and *Pum2* caused a more significant decrease in AP-positive colonies (Fig. 3 *A–C*), indicating the decreased self-renewal capacity of *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ ESCs. However, under a mild differentiation-promoting culture condition without LIF, *Pum1*$^{-/-}$ cells generated more AP-positive colonies whereas *Pum2*$^{-/-}$ and double-knockout ESCs generated fewer AP-positive colonies than wild-type ESCs (Fig. 3 *A*, *B*, and *D*). These results indicate that, under mild differentiation-promoting conditions, Pum1 functions to promote differentiation as previously reported for haploid ESCs (35) whereas Pum2 functions to promote self-renewal.

To confirm this, we cultured ESCs under a moderate differentiation-promoting condition with LIF but without feeder cells. We again observed an increased number of AP-positive colonies from *Pum1*$^{-/-}$ ESCs and a decreased number of AP-positive colonies from *Pum2*$^{-/-}$ ESCs, and double-knockout ESCs generated an intermediate number of AP-positive colonies (Fig. 3 *A*, *B*, and *E*), which supports the above conclusion. We then removed both LIF and feeder cells. Under this highly differentiation-promoting condition, *Pum1*$^{-/-}$ ESCs again generated more AP-positive colonies whereas *Pum2*$^{-/-}$ ESCs generated fewer AP-positive colonies than wild-type ESCs. The double-mutant ESCs again generated an intermediate number of AP-positive colonies (Fig. 3 *A*, *B*, and *F*). Overall, these data support a model in which Pum1 promotes ESC differentiation and Pum2 promotes ESC self-renewal.

The above conclusion is also supported by expression levels of key pluripotency genes in *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and double-mutant ESCs. When cultured with feeder cells and LIF, *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and double-mutant ESCs all expressed similar levels of Sox2 and Oct4 as wild-type ESCs, but Nanog increased in *Pum1*$^{-/-}$ ESCs and decreased in *Pum2*$^{-/-}$ and double-mutant ESCs (Fig. 3*G*). However, without feeder cells and LIF, *Pum1*$^{-/-}$ ESCs

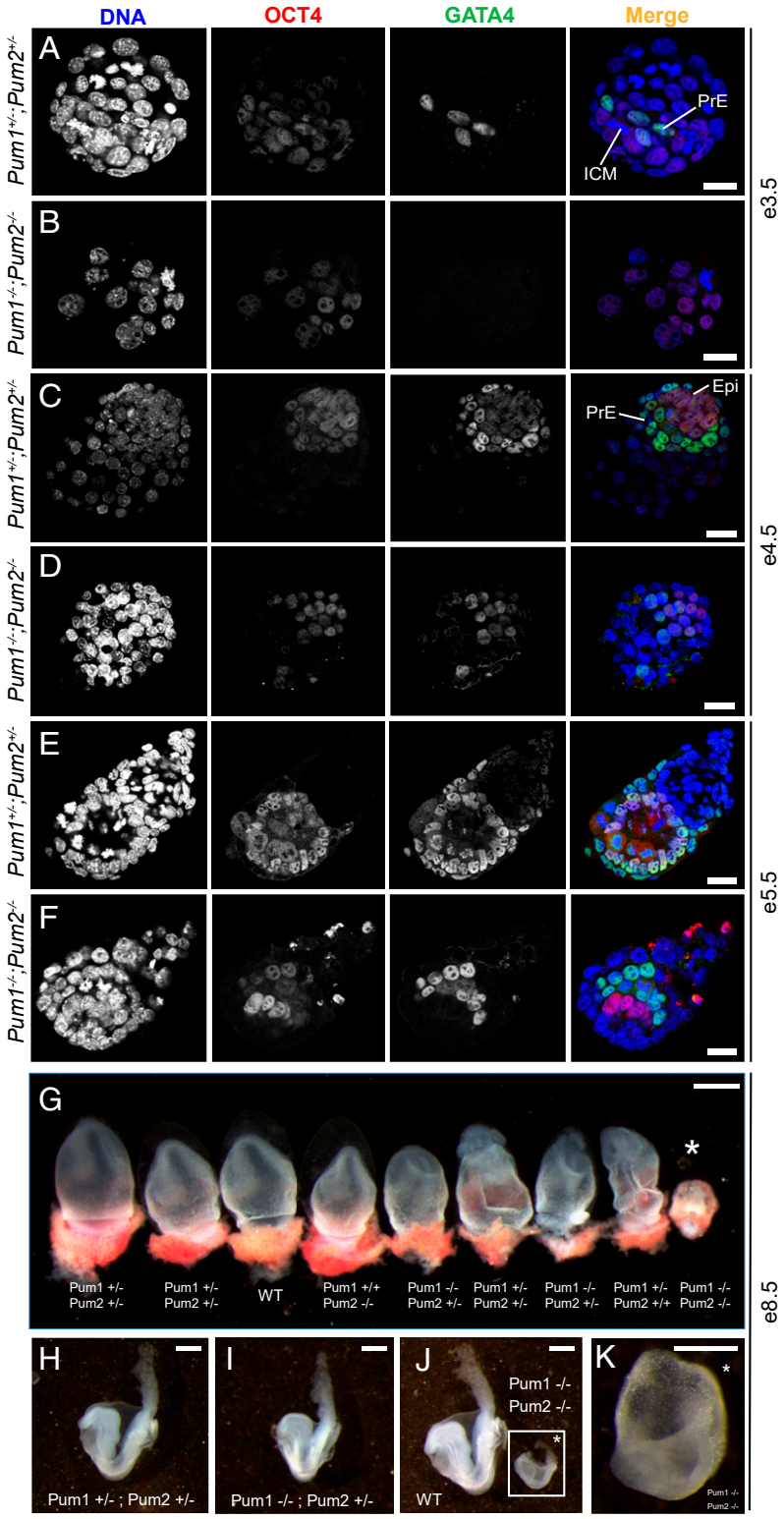


**Fig. 2.** *Pum1*$^{-/-}$*; Pum2*$^{-/-}$ mice are developmentally delayed and embryonic lethal by e8.5. Immunofluorescence staining of wild-type and mutant embryos for Oct4 (red) and endoderm marker Gata4 (green). (*A* and *B*) The e3.5 mutant embryos have no (or a smaller) blastocoel cavity and lack Gata4-positive primitive endoderm (PrE) cells. (*C* and *D*) The e4.5 mutant embryos have randomly positioned PrE cells in the ICM. (*E* and *F*) The e5.5 wild-type embryos develop an inner layer of Oct4-positive epiblast cells and an outer layer of visceral Gata4-positive endoderm cells. In mutant embryos, Gata4-positive PrE cells remain next to the blastocoel cavity. Nuclei were stained with DAPI (blue). (Scale bars, *A*–*F*, 25 μm.) (*G*) The e8.5 embryonic and extraembryonic tissues from *Pum1*$^{+/-}$*; Pum2*$^{+/-}$ mated mice. Yolk sacs were used for genotyping. A *Pum1*$^{-/-}$*; Pum2*$^{-/-}$ embryo (far right) is smaller than all littermates. (Scale bar, 1 mm.) (*H*–*K*) Higher magnification of dissected e8.5 embryos; the *Pum1*$^{-/-}$*; Pum2*$^{-/-}$ double-knockout embryo (*Inset* in *J*, magnified in *K*) shows developmental delay, a primitive head fold, and overall lack of tissue with especially thin neural tissue. (Scale bars, *H*–*K*, 500 μm.)

expressed higher levels of Nanog, Sox2, and Oct4 than wild-type ESCs, whereas *Pum2*$^{-/-}$ ESCs expressed a lower level of Nanog than wild-type ESCs (Fig. 3*H*, cf. blue, red, and green boxes). Double-knockout ESCs showed significantly reduced expression of Nanog and Sox2 and somewhat reduced expression of Oct4 (Fig. 3*H*). These results confirm that Pum1 promotes differentiation whereas Pum2 promotes self-renewal, while losing both genes severely compromises ESC pluripotency.

***Pum2*$^{-/-}$ ESCs Show Precocious Expression of Differentiation Genes and *Pum1*$^{-/-}$*; Pum2*$^{-/-}$ ESCs Are Severely Defective in Differentiating into Three Germ-Layer Lineages.** To further investigate the role of Pum1 and Pum2 in ESC self-renewal and differentiation, embryoid bodies (EBs) were generated from *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and double-mutant ESCs and assayed for markers of pluripotency as well as endodermal, mesodermal, and ectodermal lineages (Fig. 4 *A*–*J*). *Pum1*$^{-/-}$ and *Pum2*$^{-/-}$ EBs showed a similar rate of growth and

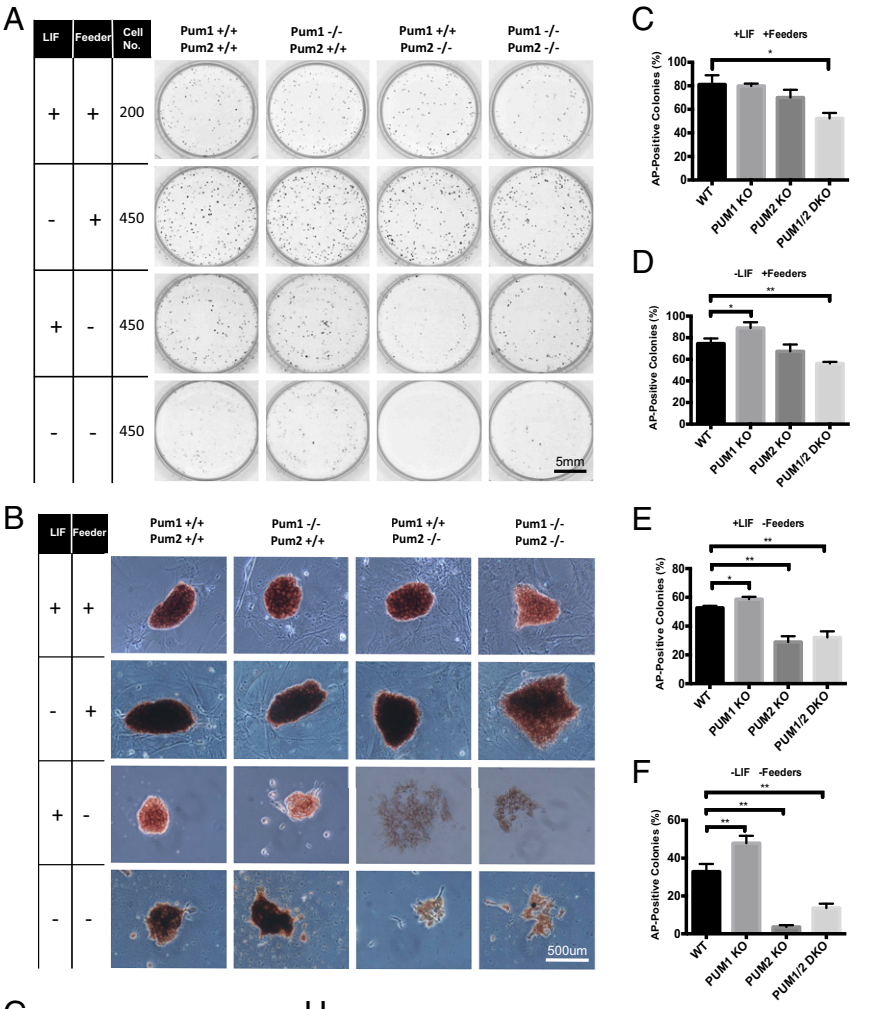


**Fig. 3.** *Pum1; Pum2* double knockout (DKO) ESCs have decreased self-renewal capacity. (*A* and *B*) Alkaline phosphatase (AP) staining of ESCs cultured under self-renewal and differentiation conditions as viewed by low (*A*) and high (*B*) magnifications. (*C–F*) Quantification of AP staining in different culture conditions. Percentage of cell-colony numbers are shown as mean ± SD. t-test: $*P < 0.05$, $**P < 0.01$. (*G* and *H*) Western blot analysis of pluripotency markers in wild-type, $Pum1^{-/-}$, $Pum2^{-/-}$, and $Pum1^{-/-}$; $Pum2^{-/-}$ ESCs cultured under self-renewal (*G*) and differentiation (*H*) conditions. Boxes highlight lower protein levels of Nanog (blue), Sox2 (red), and Oct4 (green) in $Pum1^{-/-}$; $Pum2^{-/-}$ ESCs compared to wild type, lower levels of Nanog (blue) in $Pum1^{-/-}$ cells compared to wild type, and higher levels of all three pluripotency markers in $Pum1^{-/-}$ ESCs compared to wild type.

morphology as wild-type EBs up to 20 d. However, beating cardiomyocytes appeared earlier in $Pum2^{-/-}$ EBs (16 d) compared to wild-type and $Pum1^{-/-}$ EBs (20 d), indicating an accelerated differentiation of $Pum2^{-/-}$ ESCs along the mesodermal lineage. In support of this notion, $Pum2^{-/-}$ EBs showed precocious expression of the mesodermal markers Brachyury and Goosecoid, both peaking at day 4 (Fig. 4 *C* and *D*). Moreover, $Pum2^{-/-}$ EBs had accelerated expression of ectodermal marker FGF5 and endodermal markers Foxa2 and Gata6 (Fig. 4 *E*, *H*, and *J*). Together, the above observations indicate role of Pum2 in promoting self-renewal and pluripotency.

In contrast, Pum1/2 double-mutant EBs initially grew much slower but eventually reached wild-type size by days 8 to 10 and became much larger than wild-type EBs by day 12 (Fig. 4*A*). They formed prominent cysts indicative of primitive ectoderm differentiation (Fig. 4*A*), but had no beating cardiomyocytes even beyond day 20, consistent with a defect in mesodermal differentiation. The expression of the pluripotency marker Nanog expectedly decreased over the 14-d course of differentiation (Fig. 4*B*), but the expression of the mesodermal markers Brachyury and Goosecoid, ectodermal markers FGF5, Sox1, and Mash1, and endodermal marker Gata6 all failed to increase even after 14 d in culture (Fig. 4 *C–G* and *J*). The expression of two other endodermal markers, Foxa2 and Gata4, was also severely delayed up to day 10 (Fig. 4 *H* and *I*). These observations indicate that $Pum1^{-/-}$; $Pum2^{-/-}$ ESCs are severely defective in differentiation, in addition to impaired self-renewal as described above.

***Pum1*$^{-/-}$; *Pum2*$^{-/-}$ ESCs Do Not Properly Differentiate into Three Germ-Layer Lineages in Teratomas.** To confirm the in vitro differentiation defects of Pum-deficient ESCs, four cell lines were injected subcutaneously for teratoma assays (Fig. 4*K* and *SI Appendix*, Fig. S9). The growth rate of $Pum1^{-/-}$; $Pum2^{-/-}$ teratomas was transiently higher than that of $Pum1^{-/-}$, $Pum2^{+/+}$, and $Pum1^{+/+}$, $Pum2^{-/-}$ but eventually showed no significant difference (*SI Appendix*, Fig. S9 *A–C*). However, hematoxylin-eosin staining (H&E) revealed that the double-mutant teratomas contained many more undifferentiated cells and lacked mesoderm-like structures (Fig. 4*K*, for typical mesodermal structures; *SI Appendix*, Fig. S9*D*). In addition, the double-mutant teratomas contained fewer Sox1-positive ectoderm structures and Foxa2-positive endodermal cells (*SI Appendix*, Fig. S9 *E* and *F*). Together, these data confirm that double-mutant ESCs are defective in in vivo differentiation into the three germ lineages.

**Pum1 and Pum2 Form An Interregulatory Feedback Loop in ESCs.** To further investigate the functional relationship between Pum1 and Pum2, we found multiple putative PRE sequences as defined by Morris et al. (36) in the 3′ UTR of both Pum1 and Pum2, among which one in Pum1 and two in Pum 2 fit the canonical TGTA(T/A/C)ATA consensus (Fig. 5 *A*, *Upper*). Therefore, we reasoned that Pum1 and Pum2 likely repress their own and each other's expression. To investigate this possibility, we examined the expression of Pum1 in $Pum2^{-/-}$ ESCs and vice versa. Indeed, the levels of Pum1 and Pum2 are significantly increased in $Pum2^{-/-}$ and $Pum1^{-/-}$ backgrounds, respectively, illustrating that Pum1 and Pum2 repress each other's expression (Fig. 5*A*). Such a negative inter- (and possibly auto-) regulatory feedback loop would maintain steady-state levels of Pum1 and Pum2, meanwhile allowing one Pum to be overexpressed in the absence of the other, which may partially compensate for defects caused by the loss of either individual protein.

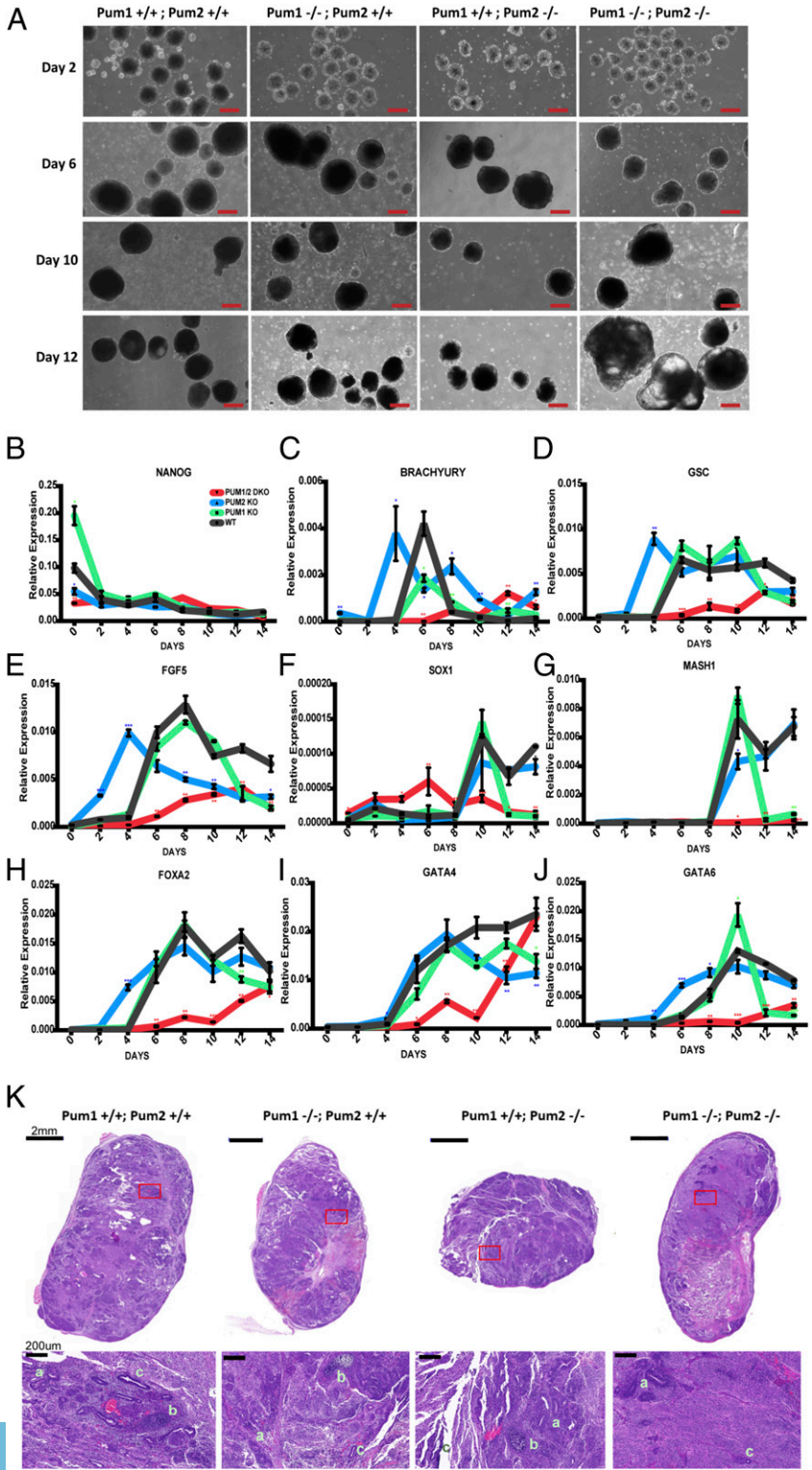


**Fig. 4.** *Pum1; Pum2* DKO ESCs have abnormal expression of key germ-layer differentiation markers in EBs and teratomas. (*A*) Morphological comparison of wild-type and mutant EBs on day 2, day 6, day 10, and day 12. (Scale bars, 250 μm.) (*B–J*) Relative expression of markers for pluripotency and three germ layers as quantified by qRT-PCR and normalized over *GAPDH* mRNA. Results are presented as mean ± SD. t-test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*K*) H&E staining of teratoma histological sections: (a) ectoderm-like pattern; (b) mesoderm-like pattern; (c) endoderm-like pattern.

**Pum1 Binds to the mRNAs of 1,461 Genes in ESCs.** Given the well-established role of Pumilio proteins as translational repressors, we next sought to identify mRNA targets of Pum1 and Pum2 in ESCs to explore potential mechanisms of the observed defects in ESC self-renewal and differentiation. Endogenous Pum1 RNA immunoprecipitation and microarray (RIP-Chip) experiments were conducted as previously described (37) on total ESC lysates to identify the associated mRNAs (*SI Appendix*, Fig. S10 *A* and *B*). Data were normalized using loess regression and quantile normalization; the *P* values and fold enrichment for each probe were analyzed by Volcano, scatter, and MA plots (Fig. 5*B* and *SI Appendix*, Fig. S10 *C*–*E*).

A total of 1,461 unique mRNAs were significantly enriched in the Pum1 immunoprecipitation (IP) as compared to the negative control (>1.5-fold, *P* value < 0.001). Of these mRNAs, the top 500 by *P* value and fold enrichment are shown in Dataset S1 and the top 47 gene probes in Fig. 5*C*. Several individual targets including Cyclin B1 and Suppressor of Zeste 12 homolog (Suz12) were verified by RIP and quantitative RT-PCR (Fig. 5*D*). The top 47 gene probes were assessed by Gene Set Enrichment Analysis (GSEA) to assign the enriched targets to functionally related gene sets. GSEA revealed that Pum1 mRNA targets are particularly enriched for genes involved as nuclear components and in transcriptional regulation, protein phosphorylation, cell-cycle regulation, and chromatin regulation (Fig. 5*E*). To examine whether the targets identified from our RIP-Chip data display the canonical Pum-binding motif PRE (TGTA[ATC]ATA), we searched for this motif at their 3′ UTR sequences (27, 38) by PERL regular expression search. We found that 52% of Pum1-targeted genes and 65% of Pum2-targeted genes have at least one motif, similar to 46.5% of human Pum1 as reported by Morris et al. (36), indicating that either the canonical PRE is important but not essential for Pum targeting, or some targets are not due to direct Pum binding, or both.

**Pum2 Binds to the mRNAs of 379 Genes in ESCs.** The high homology between Pum1 and Pum2 (39) implies that they may have similar or overlapping target sets. To investigate this possibility, we performed a Pum2 RIP-Chip to identify its in vitro mRNA targets, using the same experimental conditions and data normalization as in Pum1 RIP-Chip. GSEA of the top mRNA targets revealed that, like Pum1, Pum2 binds to mRNAs in ESCs that are functionally enriched for proteins involved in transcriptional control, cell-cycle regulation, and metabolism. Further analysis revealed that, of the 379 most enriched mRNA targets of Pum2 (>1.5-fold, *P* value < 0.001) (Dataset S2), 354 were also Pum1 targets that were enriched for proteins involved in transcriptional regulation, cell-cycle regulation, and nuclear proteins, while 25 were Pum2-specific (Fig. 5 *F* and *G* and Dataset S3), and Pum1-specific target mRNAs are highly enriched in transcriptional factors (*SI Appendix*, Fig. S10*F*), reflecting Pum1 and -2 as key regulators of regulators.

**Pum1 and Pum2 Impact the Stability of Hundreds of mRNAs in Diverse Cellular Processes.** To systematically analyze the effect of Pum1 and Pum2 on mRNAs and their translation in ESCs, we first investigated the changes of the transcriptome in *Pum1*$^{-/-}$; *Pum2*$^{-/-}$, and double-mutant ESCs by RNA deep sequencing. In *Pum1*$^{-/-}$ ESCs, 45 genes are up-regulated, yet 8 genes are down-regulated (*SI Appendix*, Fig. S11 *A*, *B*, and *G*). In *Pum2*$^{-/-}$ ESCs, however, 880 genes are up-regulated, yet 481 genes are down-regulated (*SI Appendix*, Fig. S11 *C* and *D*). The up-regulated genes are enriched in development, cell cycle, cell proliferation, cell differentiation, and apoptotic pathways, whereas the down-regulated genes are enriched in nucleosome assembly and chromatin-related activities (*SI Appendix*, Fig. S11 *H* and *I*). In *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ ESCs, 773 genes are up-regulated, yet 475 genes are down-regulated (*SI Appendix*, Fig. S11 *E* and *F*). The up-regulated genes are also enriched in development, cell cycle, and apoptotic pathways, whereas the down-regulated genes are also enriched in development, nucleosome assembly, and neuron development activities (*SI Appendix*, Fig. S11 *J* and *K*).

**Pum1 and Pum2 Impact the Translation of Hundreds of mRNAs in Diverse Cellular Processes.** We then systematically analyzed the effect of Pum1 and Pum2 on the translation of all mRNAs in ESCs by ribosome protection assays of *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and double-mutant ESCs.

We first assessed the quality of our ribosomal protection assay by analyzing the distribution of ribosome footprints on mRNAs at single-nucleotide resolution. The ribosome footprints are predominantly 28 nucleotides long (the width of the ribosome; *SI Appendix*, Fig. S12*A*). The footprint shows clear trinucleotide periodicity in all samples (Fig. 6*A* and *SI Appendix*, Fig. S13 *A*–*I*), and most footprint reads are in the protein-coding region, but not 5′ or 3′ UTRs; *SI Appendix*, Fig. S12*B*). Together, the above findings validate the high quality of our ribosome protection data.

We next analyzed changes in mRNA translation in *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and the double-mutant ESCs. All replicates show high correlation (*SI Appendix*, Fig. S13*J*), indicating the high quality of our data. The ribosome distribution pattern in mutant ESCs was similar to that of wild-type cells; ribosomal stalling was not observed in wild-type or mutant ESCs (Fig. 6*B* and *SI Appendix*, Fig. S13 *K* and *L*), indicating that Pum1 and/or Pum2 deficiencies do not affect the dynamics of translation. In *Pum1*$^{-/-}$ and *Pum2*$^{-/-}$ ESCs, 182 and 820 genes, respectively, show increased translation, whereas 109 and 427 genes, respectively, show decreased translation (*SI Appendix*, Fig. S12 *C* and *D*). In the double-mutant ESCs, 828 genes display increased translation, whereas 448 genes show decreased translation (*SI Appendix*, Fig. S12*E*). The up- and down-regulated mRNAs in the three types of mutant ESCs partially overlap (Fig. 6*C*), consistent with the overlapping function of Pum1 and Pum2 (*SI Appendix*, *Supplementary Results*). Gene ontology analysis revealed that the up-regulated genes in mutant ESCs are enriched in nucleosome assembly, DNA methylation, and epigenetic regulation (*SI Appendix*, Fig. S14 *A*–*C*). The down-regulated genes in mutant ESCs are enriched in ubiquitination, response to hypoxia, long-term memory, and transcriptional pathways *SI Appendix*, Fig. S14 *D*–*F*. These findings reflect the diversity of genes regulated by Pum1 and Pum2.

**Pum1 Represses the Translation of Pum2 and Cyclin B1 mRNAs.** To validate our ribosomal protection assay data, we compared the steady-state mRNA and protein levels of several targets in wild-type, *Pum1*$^{+/-}$, and *Pum1*$^{-/-}$ ESC lines. There was no significant difference in the mRNA levels of Pum1-target *Pum2* and *Cyclin B1* mRNAs (*SI Appendix*, Fig. S15*A*). However, their protein levels were significantly increased by approximately twofold in Pum1-deficient ESCs (*SI Appendix*, Fig. S15*B*), indicating that Pum1 translationally represses these targets in vivo.

To further assess whether the translational repression is mediated by PREs, we utilized a luciferase reporter construct in which the 3′ UTRs of *Cyclin B1* mRNA, which contains three PREs, was cloned directly downstream of the protein-coding sequence of firefly luciferase (*SI Appendix*, Fig. S15*C*). A plasmid encoding renilla luciferase was cotransfected into cells as a transfection control, and the ratio of firefly to renilla luciferase was used to indicate the effects of Pum proteins on the translation of target mRNAs in vivo. The full-length Cyclin B1 3′ UTR resulted in an 80% reduction of firefly luciferase expression levels (*SI Appendix*, Fig. S15*C*). We then generated mutations within the eight-nucleotide core of each of the three PREs within the 3′ UTR of *Cyclin B1*, as previously reported (40). Mutating each PRE resulted in a 10 to 40% release of the suppression of luciferase expression levels, and mutating all three PREs allowed for a 50% increase of firefly expression (*SI Appendix*, Fig. S15*C*). This indicates that Pum proteins suppress Cyclin B1 translation mainly by binding to PREs.

**Pum Proteins Repress or Activate Translation and Turnover of Different Subsets of Target mRNAs.** To investigate how Pum proteins regulate the translation of their direct target genes, we examined the change in translational efficiency of Pum1- and/or Pum2-bound

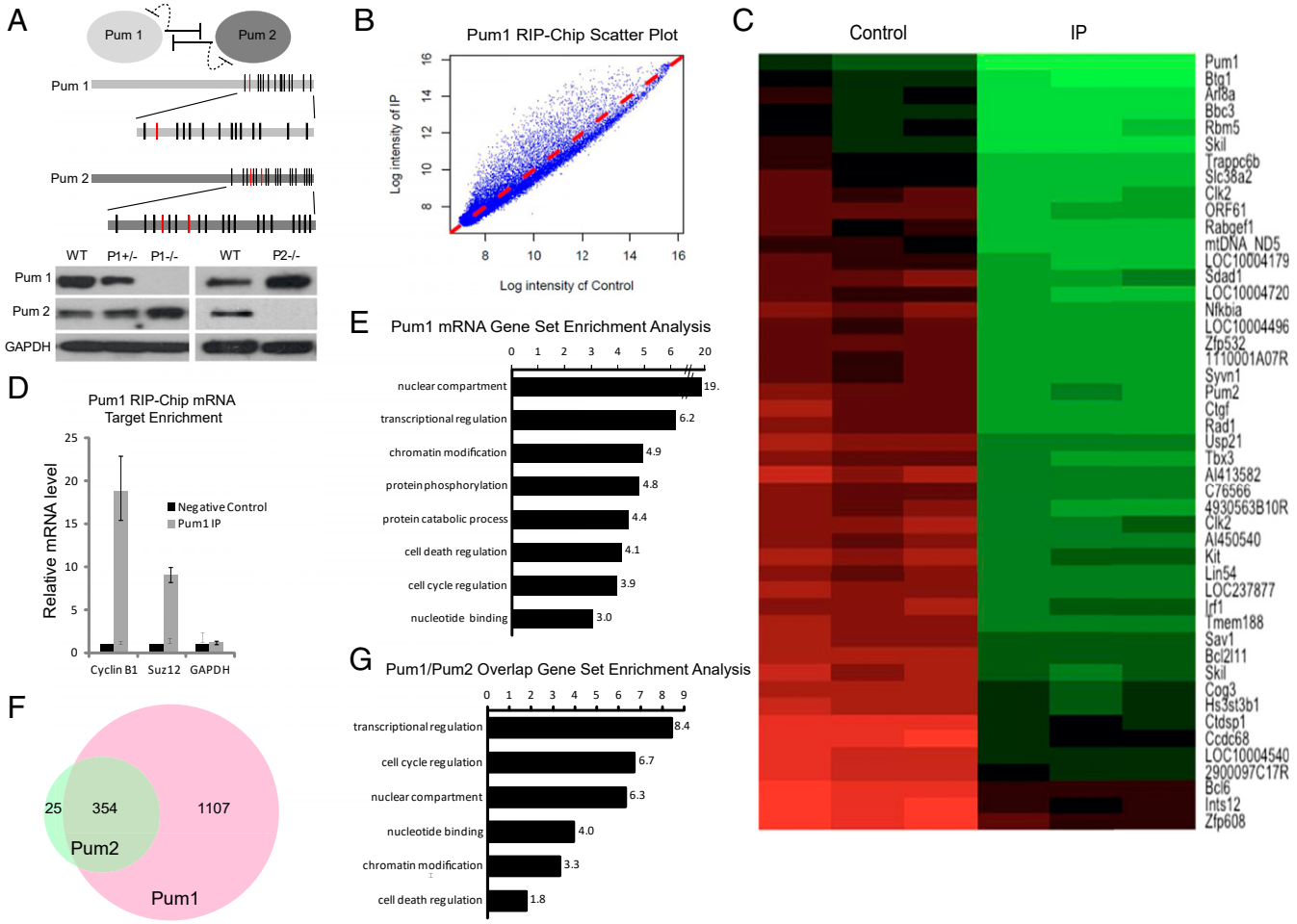


**Fig. 5.** Pum1 and Pum2 autoregulate each other and bind overlapping sets of functionally related mRNAs in ESCs. (*A*) Pum1 and Pum2 participate in an interregulatory loop (*Upper*); *Pum1* and *Pum2* mRNA have multiple putative PRE sites in their 3′ UTR (*Middle*: red lines, strict canonical TGTANATA motif; black lines, degenerate TDTANAWH motif). Western blotting analysis reveals increased Pum2 levels in $Pum1^{-/-}$ ESCs (*Lower Left*) and increased Pum1 levels in $Pum2^{-/-}$ ESCs (*Lower Right*). (*B*) Scatter plot of the log intensity of IP samples (*y* axis) and of negative controls (incubated with blocking peptide against which the antibody was raised [*x* axis]). (*C*) Heat map of enrichment in IP and control samples for the top 47 gene probes. (*D*) qRT-PCR of known Pum1 mRNA targets confirming enrichment in IP samples; error bars indicate SEM of three replicates. (*E*) GSEA of top 500 Pum1 mRNA targets by fold enrichment and *P* value. (*F*) Venn diagram indicating total number of significantly enriched ($P < 0.001$, fold enrichment >1.5) mRNA targets of Pum1 (pink) and Pum2 (green). (*G*) GSEA of the 354 overlapping mRNA targets of Pum1 and Pum2.

mRNAs in their corresponding mutant and double-mutant ESCs. In $Pum1^{-/-}$ and $Pum2^{-/-}$ ESCs, 10 (0.7%) and 10 (2.6%) of 1,461 Pum1 and 379 Pum2 targets, respectively, are translationally up-regulated (Fig. 6*D*). In $Pum1^{-/-}$; $Pum2^{-/-}$ ESCs, only 44 (3%) of the 1,486 direct Pum1- and/or Pum2-bound mRNAs are translationally up-regulated (Fig. 6*D*). These results indicate that Pum1 and Pum2 only suppress small subsets of their target mRNAs, in contrast to the common belief that Pum proteins regulate all of their target genes.

Similarly, only 7 (0.5%) and 8 (2.1%) of 1,461 Pum1 and 379 Pum2 target mRNAs are translationally down-regulated in $Pum1^{-/-}$ and $Pum2^{-/-}$ ESCs, respectively (Fig. 6*E*). In $Pum1^{-/-}$; $Pum2^{-/-}$ ESCs, only 49 (3.3%) of 1486 Pum1- and/or Pum2-bound mRNAs are translationally down-regulated (Fig. 6*E*). These results indicate that Pum1 and Pum2 can promote the translation of a similar number of their target mRNAs, in contrast to the well-known function of Pum proteins in translational repression.

We then investigated whether Pum1 and Pum2 regulate the stability of their direct targets. In $Pum1^{-/-}$ and $Pum2^{-/-}$ ESCs, 1 (0.07%) and 5 (1.32%) of 1,461 Pum1 and 379 Pum2 targets, respectively, become less stable, whereas in $Pum1^{-/-}$; $Pum2^{-/-}$ ESCs, only 40 (2.70%) of the 1,486 direct Pum1- and/or Pum2-bound mRNAs become less stable. In $Pum1^{-/-}$ and $Pum2^{-/-}$ ESCs, 5 (0.34%) and 32 (8.44%) of 1,461 Pum1 and 379 Pum2 targets, respectively, become more stable, whereas in $Pum1^{-/-}$; $Pum2^{-/-}$ ESCs, only 110 (7.40%) of the 1,486 direct Pum1- and/or Pum2-bound mRNAs become more stable. There is little overlap between translationally regulated and stability-regulated target mRNAs (*SI Appendix*, Fig. S12*F*), indicating that Pum proteins exert a single mode of regulation toward an individual target mRNA. Furthermore, few of the target mRNAs are regulated by Pum1 and Pum2 the same way (*SI Appendix*, Fig. S12*J*), indicating that Pum1 and Pum2 do not exert the same regulatory effort toward a given common target mRNA.

## Discussion

**Pum1 and Pum2 Are Collectively Essential for Early Embryogenesis.** Pum1 mRNA is expressed more ubiquitously in fetal and adult tissues than Pum2 mRNA (34). Consistent with this, we observed that Pum1-deficient mice have a more severe phenotype than Pum2-deficient mice. $Pum1^{-/-}$ mice display significantly decreased body weight and have uniformly smaller organs, but $Pum2^{-/-}$ mice exhibit only slightly lower body weights (1.7 to 4.4 g less) than wild-type littermates, validating previous reports (24, 31). Thus, Pum1 plays a more important role than Pum2 in embryogenesis. The partial redundancy between Pum1 and

Pum2 indicate overlapping functions, which together are essential for embryogenesis, since knocking out both Pum proteins causes developmental delay starting at e3.5 (when ESCs are established) and lethality by e8.5. More importantly, our molecular analysis reveals that the embryonic lethality of the double-mutant embryos may initially be due to early defects in self-renewal and differentiation—two complementary aspects of pluripotency—followed by abnormal differentiation and hypoplasia during germ-layer formation, ultimately resulting in a disorganized and inviable embryo by ~e7.5 to 8.5.

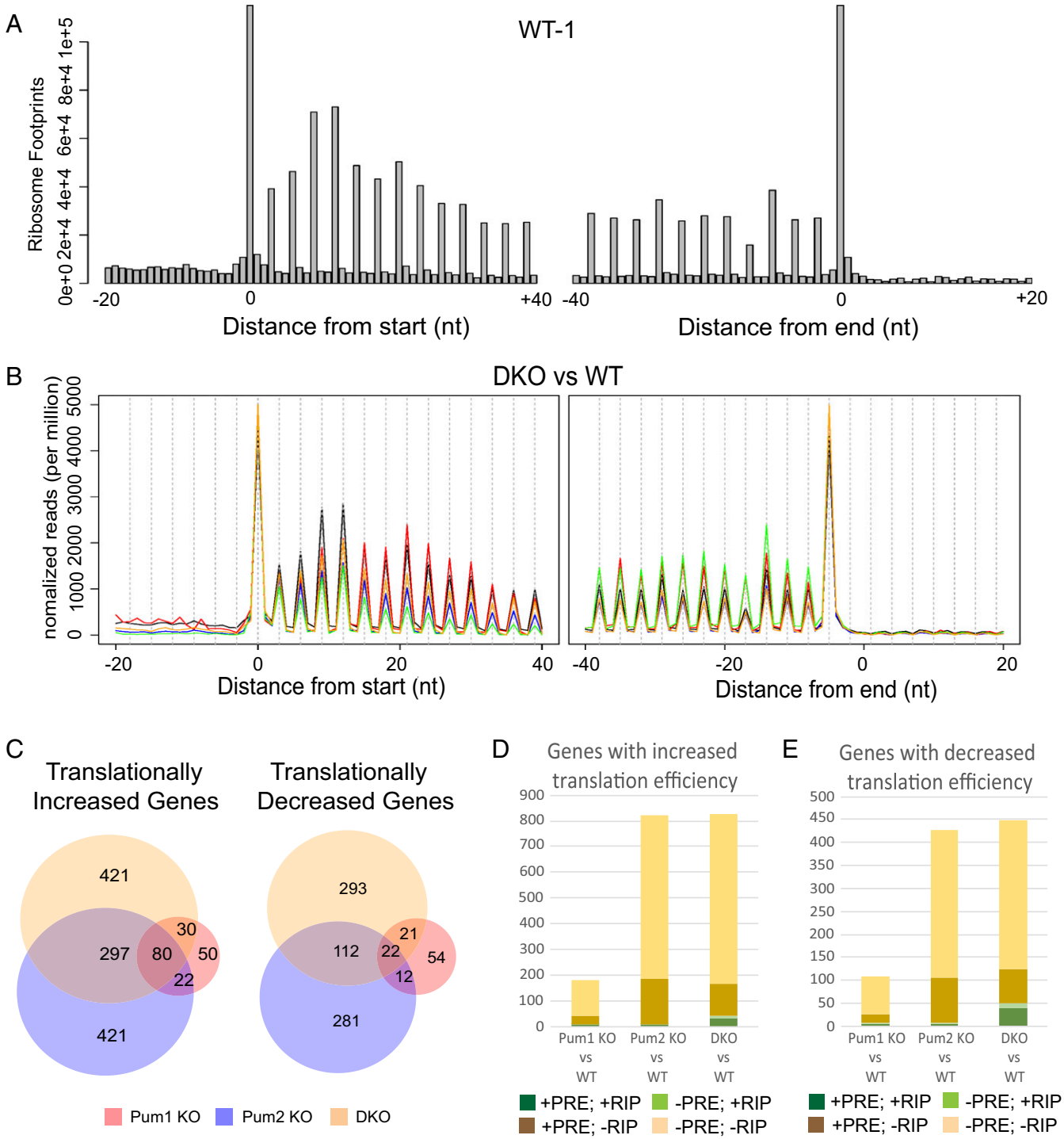


**Fig. 6.** Pum1 and Pum2 regulate the translation of many mRNAs in ESCs. (*A*) The metaplot of ribosomal P-site mapping around the start and stop codons of annotated protein-coding regions in wild-type ESCs. The trinucleotide periodicity feature of ribosomal protection assay is clearly evident. +20-bp, −40-bp region relative to start codon is displayed for translation starting region; +40-bp, −20-bp region relative to end codon is displayed for translation stopping region. (*B*) The metaplot of P-site mapping around the start and the stop codon of annotated CDs in wild-type (black and red) and *Pum* double-mutant (blue, green, and orange) ESCs. Reads were normalized to sequencing depth. (*C*) Venn diagrams of translationally increased and decreased genes in *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and *Pum* double mutants compared to wild type. (*D*–*E*) The proportion of direct target and PRE-containing mRNA isoforms that are in translationally increased (*D*) or decreased (*E*) in *Pum1*$^{-/-}$, *Pum2*$^{-/-}$, and *Pum* double mutants compared to wild type.

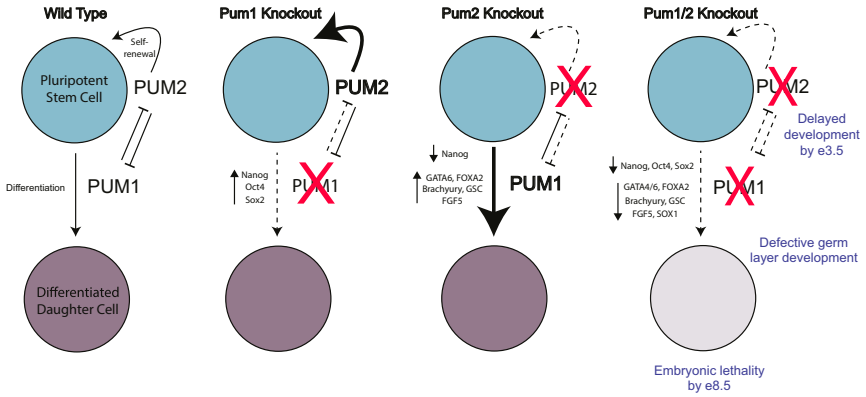


**Fig. 7.** A model of Pumilio function in ESCs and embryogenesis. During early embryogenesis, pluripotent stem cells (blue) differentiate into daughter cells (purple). Pum2 promotes ESC self-renewal while Pum1 promotes ESC differentiation; both negatively regulate each other's expression. In the Pum1 knockout, Pum2 is overexpressed, and pluripotency markers Nanog, Oct4, and Sox2 are elevated. In the Pum2 knockout, Pum1 is overexpressed; Nanog levels are decreased while there is early expression of the differentiation markers Gata6, FoxA2, Brachyury, GSC, and FGF5. In the Pum1/2 double knockout there are decreased levels of both pluripotency and differentiation markers, delayed development by e3.5, and defective germ-layer development that leads to embryonic lethality by e8.5. Overall, this model highlights the role of Pum1 in differentiation, Pum2 in self-renewal, and both collectively for normal embryogenesis.

**Pum1 and Pum2 Have Opposing but Complementary Functions in ESC Pluripotency.** In support of the above conclusion, Pum1 and Pum2 have important functions in ESCs. Remarkably, despite their extremely high homology and overlapping targets, Pum1 and Pum2 appear to have very different functions in ESC pluripotency, with Pum1 promoting differentiation and Pum2 promoting self-renewal (Fig. 7). The differentiation-promoting function of Pum1 is particularly intriguing since previous studies in invertebrate models have implicated only Pum proteins in stem-cell self-renewal. Pum1's role in differentiation is, however, consistent with the study by Leeb et al. (35) in which haploid ESCs generated an increased number of AP-positive (undifferentiated) colonies after Pum1 knockdown. In our study, Pum1 deletion in diploid ESCs not only increased the number of AP-positive colonies but also the total colony number, with the percentage of undifferentiated colonies remaining similar to that of wild-type ESCs. Therefore, Pum1 not only promotes the differentiation of ESCs, but also inhibits ESC proliferation. This function is also opposite to the known function of Pum proteins in previous studies of diverse model systems.

In contrast to Pum1-deficient ESCs, Pum2-deficient ESCs fail to maintain the expression of pluripotency genes and instead precociously express differentiation genes of the three germ layers. However, these ESC defects did not lead to corresponding defects in embryogenesis. This could be due to the much more stringent ex vivo condition that promotes differentiation, as compared to the supportive in vivo environment, since it is known that a mutation can generate a strong ESC phenotype without in vivo effects (41). In any case, the complementary functions of Pum1 and Pum2 are likely due to unique targets of the proteins that generate a combinatorial effect of regulation.

**Pum1 and Pum2 Form a Negative Interregulatory Feedback Loop.** In mouse ESCs, Pum1 and Pum 2 bind to their own mRNAs, consistent with previous reports that human PUM2 protein binds to Pum2 mRNA (42). These observations indicate that PUM proteins can autoregulate their own expression. Indeed, *Drosophila* Pum participates in a negative-feedback mechanism with Nanos to protect neurons from overactivity of Pum (43). Hence, we propose that Pum1 and Pum2 negatively regulate their own expression.

Furthermore, we show that Pum1 and Pum2 bind to each other's mRNA to repress each other's translation, forming a negative interregulatory feedback loop. Thus, the effect of knocking out one Pum might be partially compensated by the overexpression of the other, possibly through enhanced regulation of their common targets and/or ectopic binding to mRNAs that are normally bound only by the other Pum. This strategy may have a survival advantage. In addition, it allows for fine-tuning of steady-state levels of Pum1 and Pum2 in the cell, which in turn can precisely control the expression of target genes required for diverse cellular processes involved in ESC pluripotency and embryogenesis.

**Pum1 and Pum2 Are Master Regulators of Embryogenesis.** PUF proteins are described as "regulators of regulators" (36) because they control the expression of transcription factors and kinases that have many diverse regulatory effects on downstream gene expression. This study supports this notion, as transcription factors are among the most highly enriched categories of Pum target mRNAs in ESCs and early embryos. Such tiered regulation also likely explains why Pum-target mRNAs represent only a small subset of mRNAs that are affected in Pum-deficient ESCs; it is conceivable that Pum proteins repress or activate transcription factors, which then regulate many other genes. Another category of highly enriched Pum target mRNAs are involved in embryonic patterning. The impaired self-renewal of Pum-deficient ESCs and the early embryonic lethality of *Pum1*$^{-/-}$; *Pum2*$^{-/-}$ mice indicate that the dysregulation of these targets has significant consequences. The delicate and complex balance between self-renewal and differentiation, and of gene activation versus repression that must occur for embryogenesis to progress normally, require the precise control of many diverse cellular processes. It is reasonable to speculate that such coordinated regulation of large numbers of target genes is orchestrated by master regulators such as Pumilio.

The identification of 1,461 target mRNAs of Pum1 and 379 target mRNAs of Pum2 in mouse ESCs is consistent with the number of known Pum targets in human HeLa cells (36) and in the mouse testis (27). This study also reveals that Pum2 binds to an almost complete subset of Pum1 mRNA targets in ESCs, compared to previous reports of partially overlapping sets of mRNA bound to human PUM1 and PUM2 (38). The 354 common mRNA targets of Pum1 and Pum2 are particularly intriguing, since these may represent the most evolutionarily conserved targets of Pum proteins and are candidates for future study.

**Pum1 and Pum2 Can Repress or Promote Translation and Enhance or Decrease the Stability of Different Target mRNAs.** Recent studies have implicated the function of Pum proteins not only in repressing translation, but also in promoting translation and enhancing or decreasing the stability of different target mRNAs (44). Our study illustrates the importance of all four types of regulation for ESC function. Pum1 and Pum2 repress the translation of only a few of their direct target mRNAs, in contrast to the expected repression of most target mRNAs. Even more remarkably, Pum1 and Pum2 promote the translation of similar percentages of target mRNAs and stabilize or destabilize another substantial subset of

their direct target mRNAs. These findings are consistent with reports of mRNA target activation by Pum orthologs in other species (44) and suggest that mammalian Pum proteins depend on transcript-specific or developmental cues to regulate protein levels by different or even opposing mechanisms. These distinct modes of regulation might depend on protein-binding partners or post-translational modification of Pum proteins. For example, in *C. elegans*, FBF-1 and FBF-2, two nearly identical Pumilio and FBF (PUF)-domain RNA-binding proteins interact with the cytoplasmic polyadenylation element binding protein CPB-1 (45), yet the cytoplasmic polyadenylation element binding protein can switch from an activating to repressing role according to developmental cues (46). For another example, the phosphorylation of Puf3p, a PUF protein in *C. elegans*, switches its function from translational repression to translational activation (47). These modes of Pum regulation add to the already complex network of translational control and provide a rich stream of opportunities for future investigations to examine how Pum proteins have such different regulatory impacts on different target mRNAs that are essential for development.

## Materials and Methods

Methods related to *Pum1* and *Pum2* knockout mouse generation, mouse husbandry, genotyping, isolation, and analysis of *Pum* double-mutant embryos, blastocyst culture and genotyping, mouse ESC derivation, small interfering RNA knockdown, EB formation, immunofluorescence microscopy, RIP-Chip microarray, ribosome protection assay, bioinformatic analysis, and statistical analysis are all described in detail in *SI Appendix, Supplementary Methods*. Mouse care and usage strictly follow a protocol (#11087) approved by Yale University Institutional Animal Care and Use Committee.

All data in this paper are included in the manuscript and *SI Appendix* except for the ribosomal profiling data and mRNA-seq data that have been deposited in National Center for Biotechnology Information Sequence Read Archive with accession no. PRJNA602837.

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