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Article

The RNA Methyltransferase Misu (NSun2) Mediates Myc-Induced Proliferation and Is Upregulated in Tumors

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Summary

Background: Myc is a well-known proto-oncogene, but its functions in normal tissue remain enigmatic. In adult epidermis, Myc stimulates exit from the stem cell compartment, decreasing cell adhesion and, by an unknown mechanism, triggering proliferation of transit-amplifying cells.
Results: We describe a novel direct target gene of Myc, Misu, that is expressed at low levels in normal epidermis but is upregulated on Myc activation. Misu encodes a previously uncharacterized RNA methyltransferase with high sequence homology to NSun2 and defines a new family of mammalian SUN-domain-containing proteins. The nucleolar localization of Misu is dependent on RNA polymerase III transcripts, and knockdown of Misu decreases nucleolar size. In G2 phase of the cell cycle, Misu is found in cytoplasmic vesicles, and it decorates the spindle in mitosis. Misu expression is highest in S phase, and RNAi constructs block Myc-induced keratinocyte proliferation and cell-cycle progression. Misu is expressed at low levels in normal tissues, but is highly induced in a range of tumors. Growth of human squamous-cell-carcinoma xenografts is decreased by Misu RNAi.
Conclusions: Misu is a novel downstream Myc target that methylates RNA polymerase III transcripts. Misu mediates Myc-induced cell proliferation and growth and is a potential target for cancer therapies.

Introduction

Myc is a nuclear protein that heterodimerizes with the ubiquitously expressed protein Max to function as a sequence-specific transcription factor, binding to E box elements [1, 2]. Myc/Max heterodimers activate gene transcription by recruiting a protein complex containing histone-acetyltransferase activity [3]. Myc and Max repress transcription by interfering with the transcriptional activator Miz-1 or recruiting a corepressor containing the histone deacetylase complex [4, 5].

Overexpression of Myc promotes tumorigenesis, but its functions in normal cells are still enigmatic. Recent studies have revealed a role in regulating adult stem cell homeostasis in the epidermis [6–8], intestinal epithelium, and blood [9]. In the epidermis and hemopoietic tissue, Myc induces exit from the stem cell niche by downregulating genes encoding proteins involved in cell adhesion [6, 8–11]. In addition, Myc impairs epidermal cell migration, which may explain why sebocyte differentiation is promoted at the expense of the hair lineages [8, 10, 11].

Myc plays a direct role in controlling cell division and growth. Many Myc target genes encode components of the protein-synthesis machinery [10]. In addition, Myc directly activates RNA polymerase (pol) III transcription and enhances expression of transfer RNAs (tRNA), 5S rRNA, and a subset of other small RNAs [12]. Myc also activates RNA pol I and thereby induces ribosomal RNA synthesis [13, 14]. As a result, Myc overexpression leads to an increase in nuclear, nucleolar, and cell size [15]. Nucleolar activity plays an important role in cell-cycle control [16].

In this report, we describe a novel gene that is activated by Myc and encodes a previously uncharacterized RNA methyltransferase named Misu. We show that Misu regulates nucleolar size, is a component of the mitotic spindle, and mediates the effects of Myc on epidermal cell growth and proliferation. Misu is expressed at low levels in most normal tissues and is overexpressed in different types of tumor. Because inhibition of Misu reduces tumor formation in vivo, it may be a good target for anticancer drugs.

Results

Misu Expression Correlates with Activation of Myc In Vitro and In Vivo

In order to analyze how Myc exerts its effect in the epidermis, we performed microarray analysis with RNA extracted from whole skin of K14MycER transgenic mice and wild-type littermates [10]. In K14MycER mice, Myc is activated by topical application of 4-hydroxy-tamoxifen (4OHT).

When Myc was activated for 1 or 4 days in K14MycER epidermis, there was strong upregulation of a mRNA with accession number BC013625 (Figure 1A). In wild-type animals, expression of BC013625 mRNA was low, whether or not the skin was treated with 4OHT (Figure 1A). The microarray results were validated by northern blotting total RNA from wild-type and transgenic back skin (Figure 1B).

BLAST searches revealed BC013625 to encode a novel protein with a conserved SUN domain. SUN, also known as Fmu, was first described in *E. coli* and catalyzes the formation of 5-methylcytidine (m^5C) in 16S rRNA [17, 18]. We named the new protein Misu, for *M*yc-*i*nduced *SU*N-domain-containing protein. The protein with the highest overall homology to Misu (36%) is yeast NCL1 (or Trm4) [19], which is a tRNA m^5C methyltransferase (MTase) [20]. Misu showed weak similarity (23%) to a human protein called p120 or nucleolar-associated antigen. p120 is expressed in rapidly dividing

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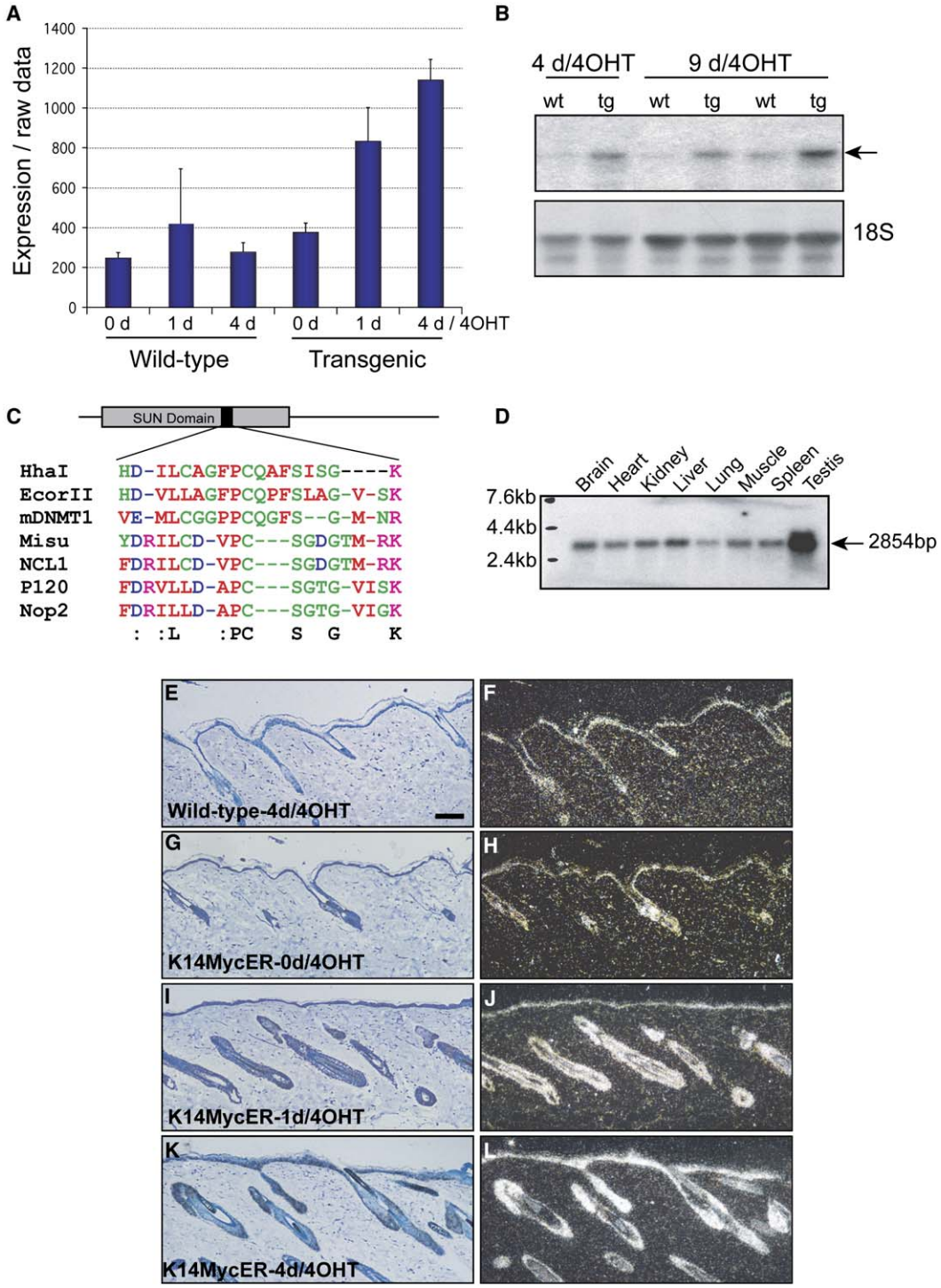

Figure 1. Expression of Misu RNA Correlates with Myc Activation

(A) RNA expression profile of Misu in skin of K14MycER-transgenic and wild-type mice that were untreated (0 days) or treated with 4OHT (1 day and 4 days). Error bars represent standard deviations.

(B) Northern blot of total RNA from skin of wild-type (wt) and transgenic (tg) mice, treated for 4 days or 9 days with 4OHT, probed for Misu (arrow) or, as a loading control, 18S RNA.

(C) Sequence surrounding the first conserved cysteine within the SUN domain of Misu, DNA MTases (HhaI, EcoRI, mDnmt1), and RNA MTases (NCL1, p120, Nop2).

(D) Tissue northern blot probed for Misu (arrow).

(E–L) In situ hybridization of Misu in back skin of wild-type mouse treated with 4OHT for 4 days (E and F) or K14MycER transgenic untreated (G and H) or treated with 4OHT for 1 day (I and J) or 4 days (K and L). Bright- and dark-field views of the same sections are shown. Scale bar represents 100 μm.

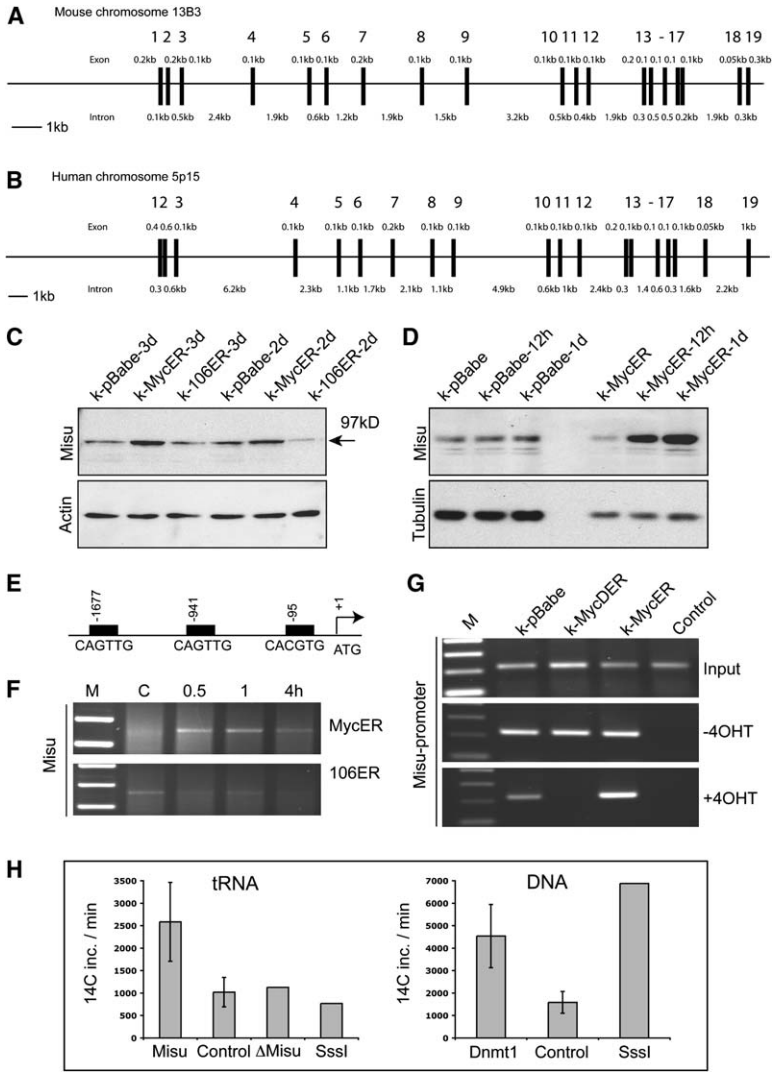

Figure 2. Misu Is a Direct Target Gene of Myc, with RNA Methyl Transferase Activity and Conserved Genomic Organization

(A) Mouse and (B) human Misu genes consist of 19 exons.

(C and D) Western blots showing upregulation of Misu by activation of MycER in human keratinocytes (k) compared to controls (k-pBabe; k-106ER). Actin and tubulin served as loading controls.

(E) Three putative Myc binding sites in the Misu promoter.

(F) RT-PCR showing amplification of Misu in keratinocytes infected with MycER but not 106ER after 30 min pretreatment with cycloheximide. RNA was isolated 0.5, 1, and 4 hr after treatment with 4OHT.

(G) ChIP of Misu promoter with antibody to Myc. PCR of input control amplifies Misu promoter in keratinocytes infected with empty vector (k-pBabe), a dominant-negative mutant of Myc (k-MycDER), and MycER (k-MycER). One control was isotype-matched antibody (upper panel). Without 4OHT, the Misu promoter is detected equally in all samples except the control (middle panel). After 2 days of 4OHT treatment, Misu can be detected in k-MycER, to a lower extent in k-pBabe, but not in k-MycDER (lower panel). M denotes molecular weight markers.

(H) Incorporation of radioactive (^{14}C)-labeled SAM into tRNA (left-hand panel) or hemimethylated DNA (right-hand panel). As negative controls, Misu and Dnmt1 were transcribed in the anti-sense direction (control). ΔMisu is the S3 splice variant. Error bars represent standard deviations.

cells [21] and shows substantial homology (67%) to the yeast rRNA m^{5}C MTase Nop2 [22].

Within the SUN domain, a pair of conserved cysteines, approximately 50 amino acids apart, represent the potential active site [22]. Figure 1C shows the highly conserved core sequence surrounding the first cysteine that is found in Misu, NCL1, p120, and Nop2 (Figure 1C) [19, 20]. This part of the SUN domain has sequence and structural similarities with motif IV of DNA:m^{5}C MTases such as bacterial HhaI and EcorII and mammalian Dnmt1 (Figure 1C) [20, 23]. The conserved sequence motifs and structural homologies among S-adenosyl-L-methionine-dependent DNA and RNA MTases strongly suggest an evolutionary relationship [23, 24].

Misu mRNA was present in all tissues analyzed (Figure 1D). In wild-type mouse skin treated for 4 days with 4OHT (Figures 1E and 1F) or in untreated K14MycER skin (Figures 1G and 1H), Misu expression was very weak. Following 4OHT treatment of transgenic skin (Figures 1I–1L), Misu expression was strongly upregulated in all cells that express the K14 promoter: the basal layer of interfollicular epidermis, the periphery of the sebaceous glands, and along the hair-follicle outer-root sheath [10].

Misu Defines a New Family of Mammalian SUN-Domain-Containing Genes and Is Alternatively Spliced

BLAST searches revealed five previously uncharacterized mouse SUN-domain-containing proteins, in addition to Misu and p120 (Figure S1A in the Supplemental Data available online). One, NSun2 (accession number NM_145354), has regions of sequence identity with Misu, but the predicted protein is smaller (691 versus 757 amino acids; Figure S1A). However, GenBank entry NP_060225 for human NSun2 corresponds to the predicted full-length sequence of human Misu. Misu localized to chromosome 13B3 in mouse (Figure 2A) and 5p15 in human (Figure 2B).

Human and mouse Misu are each encoded by 19 exons (Figures 2A and 2B). We obtained evidence for the existence and expression of three splice variants (Figures S1 and S2), two of which lack an intact SUN domain and can therefore function as dominant-negative inhibitors of Misu (Figure 2H, ΔMisu).

To analyze whether Myc activation increased Misu protein expression, we performed western blotting of 4OHT-treated cultured human keratinocytes that had

been transduced with a MycER retroviral vector (k-MycER) (Figures 2C and 2D). As controls, keratinocytes expressing the empty retroviral vector (k-pBabe) or a mutant form of MycER with a deletion within the transactivation domain (k-106ER) were also examined (Figures 2C and 2D). Misu protein expression was increased in k-MycER within 12 hr of 4OHT treatment, but not in control cells (Figure 2D).

There are three putative binding sites for Myc upstream from the predicted start methionine of human Misu (Figure 2E). To analyze whether Misu RNA synthesis was independent of de novo protein synthesis, we performed RT-PCR on MycER expressing keratinocytes that had been treated with cycloheximide for 30 min prior to addition of 4OHT (Figure 2F). Misu expression was detected in keratinocytes transduced with MycER, but not in control cells transduced with Myc106ER (Figure 2F).

We also performed chromatin immunoprecipitation (ChIP) analysis in primary human keratinocytes (Figure 2G). The Misu promoter was amplified by PCR in all samples prior to immunoprecipitation with an antibody to Myc (Input, Figure 2G, upper panel). As controls we used an isotype-matched antibody (control) or lysates from cells transduced with a Myc mutant (MycDER) that is unable to bind to E boxes (MycDER, Figure 2G) [25]. Without 4OHT treatment, the intensity of the PCR fragment was comparable in all samples, indicating that the promoter was precipitated by endogenous Myc (Figure 2G, middle panel). On treatment with 4OHT, the interaction of Myc with the Misu promoter was increased in k-MycER, but not in k-pBabe, and was blocked in k-MycDER (Figure 2G, lower panel). We concluded that Misu is a direct downstream target of Myc.

Misu Methylates RNA

To determine whether Misu had methyltransferase activity, we performed methylation assays, measuring the incorporation of ^{14}C-labeled S-adenosyl-L-methionine (SAM) into RNA (Figure 2H). Given that yeast RNA:m^{5}C MTases can methylate a wide range of RNAs in vitro [26], and Misu showed the highest homology to NCL1, a tRNA methyltransferase, we used tRNA as a representative RNA target substrate. Full-length Misu cDNA was in vitro translated and incubated with tRNA (Figure 2H, left panel). As a negative control we used Misu cDNA transcribed in the antisense direction (Figure 2H, left panel, control).

We detected a 2.5-fold increase in ^{14}C incorporation into tRNA in the presence of Misu compared to controls (Figure 2H, left panel). Misu also catalyzed ^{14}C incorporation into rRNA (Figure S3E). DNase treatment did not abolish the methylation activity of Misu toward RNA (data not shown). There was no significant ^{14}C-SAM incorporation into tRNA in the presence of the 60 kDa Misu splice variant (ΔMisu, corresponding to the S3 splice variant initiated in exon 7; Figure S2B) or SssI, a DNA MTase isolated from *Spiroplasma sp.* (Figure 2H). In contrast, incorporation of ^{14}C-SAM into hemimethylated DNA was stimulated by SssI and the DNA MTase Dnmt1 (Figure 2H, right panel). In vitro, Misu and Dnmt1 showed comparable methylation activities toward their target substrates (Figure 2H).

Subcellular Distribution of Misu Changes during the Cell Cycle

Misu methytransferase activity in vivo will depend on its nuclear location. rRNA:m^{5}C MTases are found in the nucleoli [27]. tRNA MTases are found both in the nucleoli and close to the nuclear envelope, where the splicing and processing of tRNA takes place [28, 29]. In keratinocytes and SZ95 sebocytes, the level of Misu varied throughout the cell cycle, with lowest expression in early G1 (Figures 3A–3C). In G1, Misu was predominantly found in the nucleoli, where it colocalized with nucleolin (arrows, Figures 3D–3F). In S phase cells, the number and size of nucleoli increase, and nucleolin is found in both nucleoli and nucleoplasm (Figure 3D, asterisks). S phase cells had the highest levels of Misu, which was distributed more uniformly throughout the nucleus (asterisks, Figures 3E and 3F) than in G1. We confirmed that Misu was upregulated during S phase by western blotting SZ95 cells that had been treated for 4 hr with trichostatin A (TSA) or Actinomycin D (ActD) to trigger accumulation of S phase cells (Figures 3M and 3N). Misu was also upregulated in FACS-sorted populations of S phase SZ95 cells that had not been treated with drugs (Figure 3O).

In a small percentage of cells in G2, Misu was detected in cytoplasmic vesicles that did not correspond with mitochondria [30] (arrows, Figures 3G–3I). In M phase, Misu was found along the spindle and colocalized with γ-tubulin at centrioles (Figures 3J–3L). This distribution has never been described for RNA MTases, but it has been reported for ATRX [31]. ARTX is a centromeric heterochromatin binding protein, involved in methylating repetitive DNA sequences, that is required for meiotic spindle organization.

Endogenous Nucleolar Localization of Misu Is Dependent on RNA Species Transcribed by RNA Pol III

In order to examine whether the localization of Misu was dependent on the presence of nucleolar-RNA species, we treated cells with RNaseA prior to immunostaining (Figures 4A–4H). RNaseA treatment abolished the localization of Misu in nucleoli (Figure 4B), but not in centrioles and cytoplasmic vesicles (Figure 4C, arrow and data not shown). Like Misu, the localization of nucleolin was sensitive to RNaseA but not to DNase treatment (Figures 4D–4H).

After treatment with 0.05 μg/ml, Actinomycin D, which selectively inhibits RNA pol I, Misu localized to the perinucleolar compartment, a region highly enriched in RNA pol III transcripts (Figure 4J, arrow) [32]. Ten micrograms per milliliter α-amanitin, which selectively inhibits RNA pol II, had no effect on the localization of Misu (Figure 4K, arrow). In contrast, 100 μg/ml α-amanitin inhibits RNA pol II and III and depleted Misu from the nucleoli (Figure 4L, arrow). We conclude that the nucleolar localization of Misu depended on the presence of RNA pol III transcripts.

Misu Is Required for Myc-Induced Proliferation

To examine whether Misu was required for Myc-induced proliferation, we generated three different Misu RNAi constructs (RNAi1,3,4). RNAi1 and 3 inhibited Misu expression by a maximum of 50%, whereas RNAi4

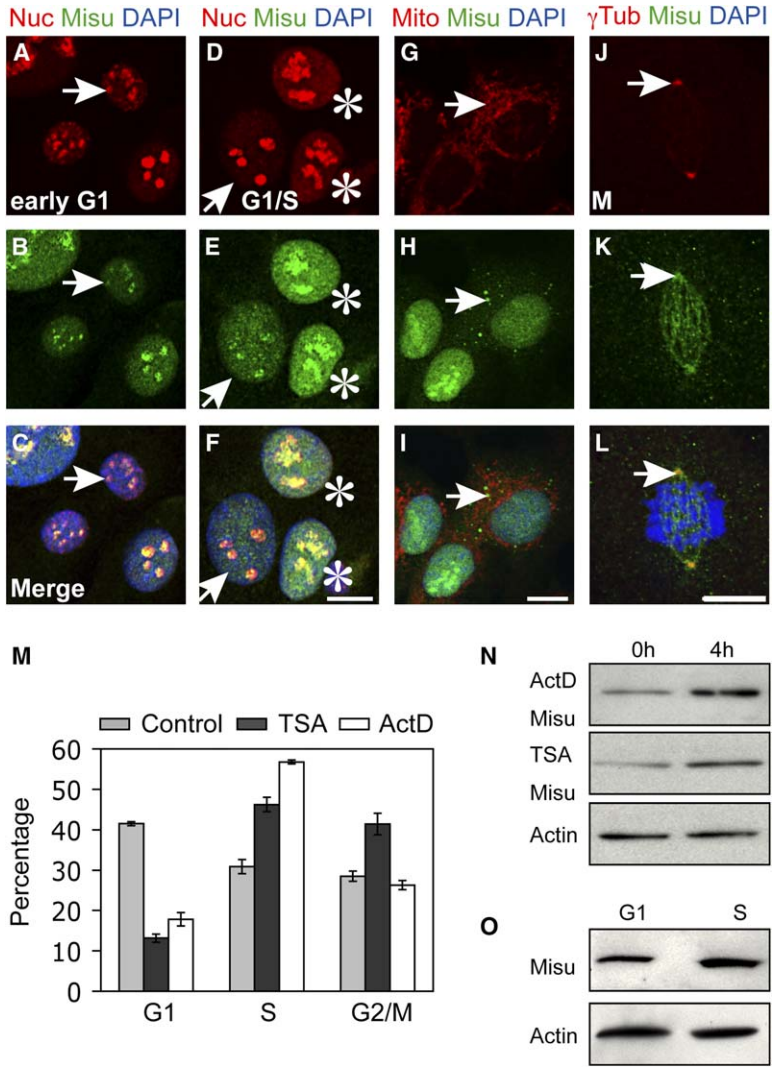

Figure 3. Subcellular Localization of Misu and Regulation during the Cell Cycle

(A–L) Immunofluorescence staining for Misu (green) and nucleolin (red in [A]–[F]) and γ-tubulin (γTub, red in [J]–[L]) or mitochondrial dye (Mito, red in [G]–[I]) with DAPI (blue) counterstain. (A–C) Arrows show colocalization of Misu with nucleolin at nucleoli. (D–F) Cells in G1 (arrow) and S phase (asterisks) are shown. (G–I) Cytoplasmic Misu does not colocalize with mitochondria (arrow). (J–L) Colocalization of Misu with γ-tubulin at spindle poles.

(M) Cells arrested in S phase or G2/M phase of the cell cycle after treatment with trichostatin A (TSA) or Actinomycin D (ActD). Error bars represent standard deviations.

(N) Western blot of Misu before (0 hr) and after treatment with TSA and ActD for 4 hr.

(O) Western blot of Misu in FACS-sorted G1-phase and S phase SZ95 cells.

decreased Misu expression by up to 90% (Figure 4M, upper panel). These results were confirmed by Misu immunofluorescence staining of keratinocytes (Figures 4N–4Q) and SZ95 cells (Figures S4A–S4H).

MycER activation in SZ95 cells or keratinocytes resulted in an increase in nucleolar size, accompanied by increased deposition of Misu, nucleolin, and Ki67 (Figures 4R–4T; data not shown). Introduction of Misu RNAi into MycER-expressing cells significantly reduced nucleolar size, to a level comparable to control cells (Figures 4R–4U).

When primary human keratinocytes were transduced with a Misu RNAi4 retroviral vector, proliferation was markedly decreased compared to empty-vector-infected controls or keratinocytes infected with RNAi1 or 3 (Figures 5A and 5B). Proliferation of human keratinocytes was not affected by a scrambled RNAi sequence (Figures S4I and S4J).

Misu RNAi4 abolished the MycER-activation-induced increase in growth rate of keratinocytes (Figures 5B and 5C) and prevented Myc-induced accumulation of keratinocytes in G2/M of the cell cycle (Figure 5D). Misu RNAi did not trigger keratinocyte apoptosis or terminal differentiation. On the contrary, α6β4 integrin expression increased and transglutaminase-1 expression decreased (Figures S5A–S5D).

Myc activation in the epidermis not only stimulates proliferation but also promotes terminal differentiation [6, 7]. To test whether this was also dependent on Misu, we reconstituted human epidermis by growing keratinocytes at the air-liquid interface on dead, de-epidermized dermis (DED) [6] (Figures 5E–5P). Epidermis formed by keratinocytes transduced with an empty retroviral vector (k-eV) was well differentiated (Figures 5E and 5I), with low numbers of proliferating cells in the basal layer (Figure 5M). Epidermis reconstituted with Misu-deficient keratinocytes (k-MisuRNAi) underwent normal proliferation (Figure 5N) and terminal differentiation, although the number of differentiated layers was slightly reduced (Figures 5F and 5J).

As reported previously, keratinocytes infected with MycER formed a multilayered epidermis with disorganized suprabasal layers, more differentiated layers (Figure 5G), and increased proliferation (Figure 5O) [6]. In the presence of Misu RNAi, these effects were abolished (Figures 5H, 5L, and 5P). We conclude that Misu RNAi prevents not only Myc-induced proliferation but also Myc-induced terminal differentiation.

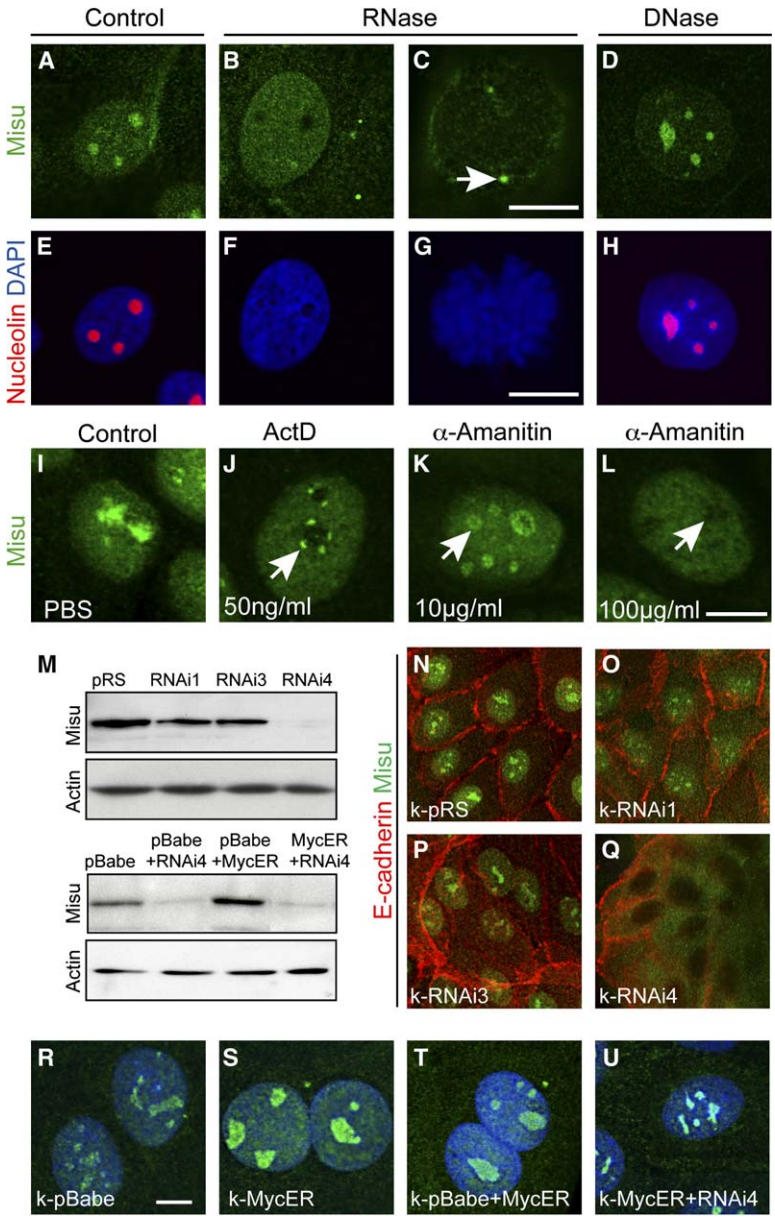

Figure 4. RNA-Dependent Nucleolar Localization of Misu and Effects of Misu RNAi on Nucleolar Size

(A–H) RNA-dependent colocalization of Misu with nucleolin in nucleoli. Immunofluorescence staining of Misu (A–D) and nucleolin (E–H). SZ95 cells were treated with PBS (A, E, and I), RNase (B, C, F, and G), or DNase (D and H).

(I–L) Localization of Misu in nucleoli (arrows) after treatment with PBS (I), 50 ng/ml actinomycin D (ActD, [J]), 10 μg/ml α-amanitin (K), or 100 μg/ml α-amanitin (L).

(M) Western blots of Misu in (upper panel) keratinocytes infected with empty vector (pRS) or Misu RNAi constructs (RNAi1,3,4). Lower panel shows keratinocytes coinfected with empty vector (pBabe), RNAi4, or MycER and treated with 4OHT.

(N–Q) Immunofluorescence staining of Misu (green) and E-cadherin (red) in keratinocytes infected with empty vector (k-pRS) or Misu RNAi constructs.

(R–U) Activation of Myc increases keratinocyte nucleolar size in the absence (S and T) but not the presence (U) of Misu RNAi. Cells were transduced with the constructs shown. Green fluorescence shows Misu. Blue fluorescence shows DAPI. Scale bars represent 10 μm.

Misu Is Expressed at Low Levels in Normal Tissues and Is Upregulated in Tumors

Misu protein expression was very weak in human and mouse skin, except in sebaceous glands and at the bulb (base) of growing (anagen) hair follicles (Figures 6A, 6B, 6E, and 6F, data not shown). In K14MycER mice treated with 4OHT for 4 days, Misu expression was strongly increased, with immunoreactivity not only in the sebaceous glands and hair-follicle bulb, but also in the basal layer of the interfollicular epidermis and along the hair-follicle outer root sheath (Figures 6C, 6D, 6G, and 6H), all the sites of transgene expression. In all Misu-expressing cells, the protein had a nuclear localization.

Misu expression was increased in benign (papillomas) and malignant (squamous cell carcinomas) mouse skin tumors (Figures 6I–6L; Table S1). In papillomas, cells expressing Misu were confined to the proliferative layers (Figures 6I and 6J, arrow). In squamous cell carcinomas, the Misu-positive cells extended throughout the tumor mass (Figures 6K and 6L). Two out of five human oral squamous cell carcinomas were strongly positive for Misu (Table S1 and data not shown).

Misu expression was very low in normal human tissues (Table S1; Figures 6M and 6N and data not shown), the only exception being the pancreas (Figure 6O). In the colon, Misu was only expressed in a small number of epithelial cells (Figure 6S, arrowheads). However, in a patient with Crohn's disease, there were areas of increased expression (Figure 4U, arrowheads). It is interesting that Myc is also upregulated in such tissue, and that Crohn's disease predisposes to gastric carcinoma [33].

Misu was upregulated in 7/7 human breast carcinomas, including primaries (Figures 6P and 6Q) and lymph-node metastases (Figure 6R), and in 3/4 colon carcinomas (Table S1). The only tumors that did not show increased Misu expression were four rectal carcinomas (Table S1).

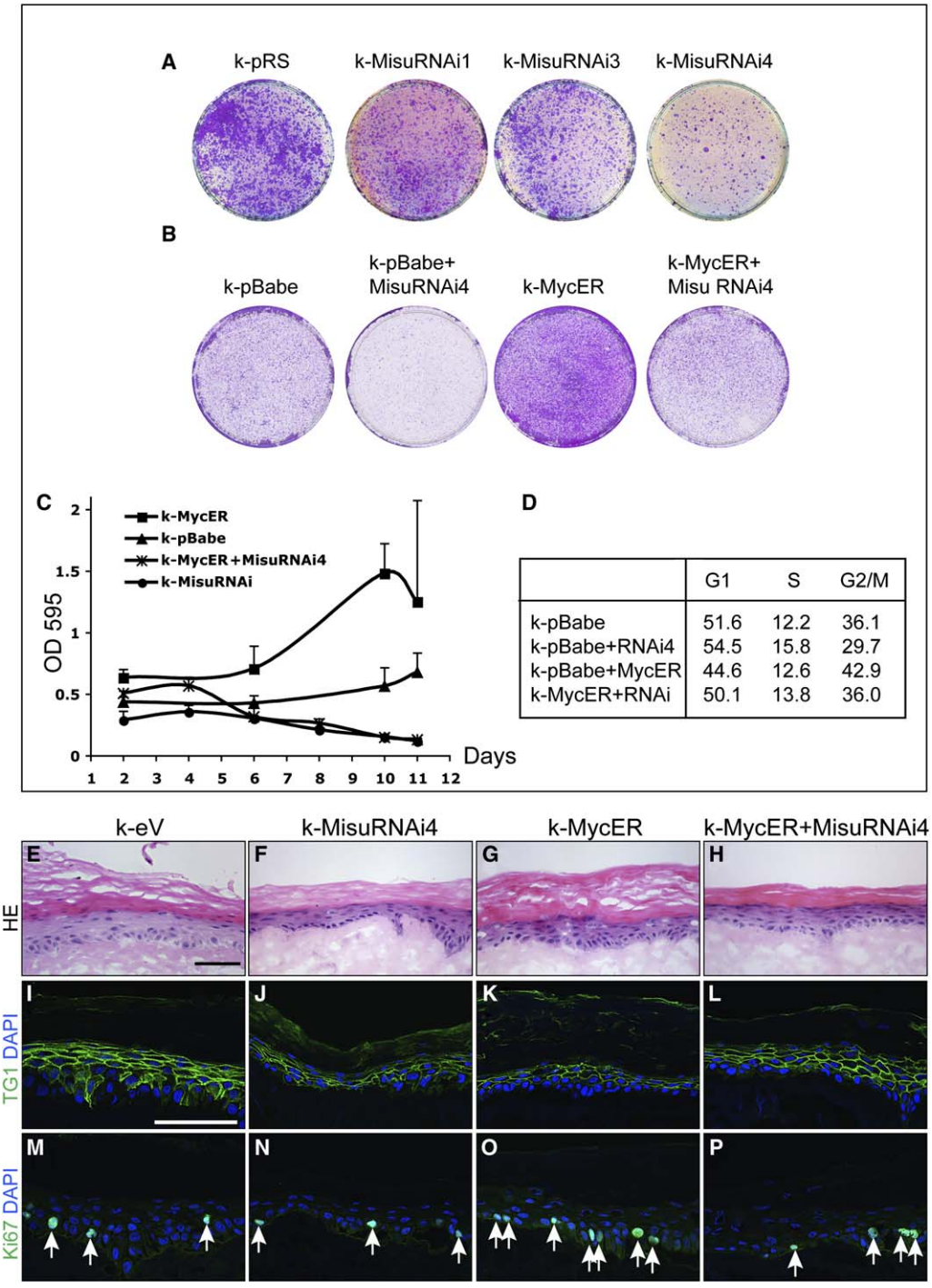

Figure 5. Misu Is Required for Myc-Induced Keratinocyte Proliferation and Differentiation

(A–C) Introduction of Misu RNAi into keratinocytes transduced with MycER (k-MycER) or empty retroviral vector (k-pRS, k-pBabe) led to inhibition of proliferation. (A) and (B) show rhodamine-blue-stained culture dishes. (C) shows growth curves, and error bars represent standard deviations.

(D) Effect of Misu RNAi4 on Myc-induced changes in cell-cycle distribution.

(E–H) Histology and (I–P) immunofluorescence staining of keratinocytes cultured on DED. Keratinocytes were infected with empty vector (k-eV; [E, I, and M]), Misu RNAi (F, J, and N), MycER (G, K, and O), or both Misu RNAi and MycER (H, L, and P) and grown in the presence of 4OHT. Reconstituted epidermis was stained with antibody to transglutaminase 1 (TG1, [I–L]) or Ki67 (M–P) with DAPI counterstain (blue). Ki67-positive cells are indicated with arrows. Scale bars represent 100 μm.

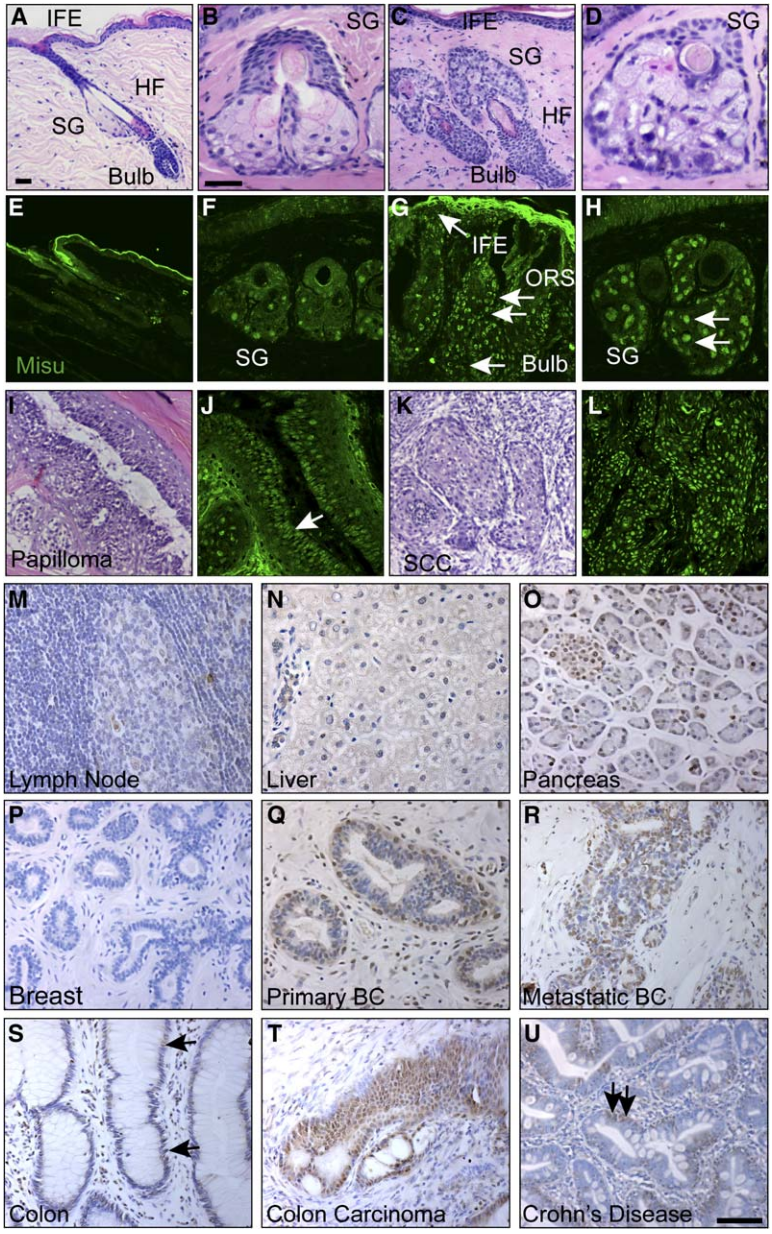

Figure 6. Expression of Misu in Normal Tissue and Tumors of Human and Mouse

(A–L) Histology (A–D) and Misu immunofluorescence staining (E–H) (MFRY5) of wild-type (A, B, E, and F) and K14MycER (C, D, G, and H) mouse back skin after 4OHT treatment. Arrows in (G) and (H) mark cells with high Misu expression. Expression of Misu was upregulated in mouse skin papilloma (I and J) and squamous cell carcinoma (K and L). Arrow in (J) shows Misu-positive cells.

(M–U) Immunohistochemistry for Misu in normal human lymph node (M), liver (N), pancreas (O), primary human breast carcinoma (BC) (Q), lymph-node metastasis of breast carcinoma (R), normal colonic epithelium (S), colon carcinoma (T), and Crohn's disease (U). (P) As a control, staining of human breast was performed with secondary antibody alone.

Scale bars represent 50 μm (A–H) and 100 μm (I–U).

Knockdown of Misu Reduces Growth of Human Squamous-Cell-Carcinoma Xenografts in Nude Mice

To investigate whether Misu RNAi could inhibit tumor growth, we transduced a human squamous-cell-carcinoma cell line, SCC15, with the RNAi constructs 1, 3, and 4 and, as a control, with the empty vector pRS (Figure 7A). As in primary keratinocytes, RNAi4 reduced Misu expression in SCC15 cells by 90%, whereas RNAi1 and 3 were less effective (data not shown). SCC15 cell proliferation in culture was not inhibited by Misu knockdown (Figure 7A). However, when SCC15 cells infected with pRS or RNAi3 and 4 were subcutaneously injected into nude mice, tumor size was substantially reduced by RNAi4 and, to a lesser extent, by RNAi3 (Figure 7B).

SCC15 transduced with the empty vector pRS formed squamous cell carcinomas with high Misu expression and a large fraction of proliferative, Ki67-positive cells (Figures 7C, 7F, and 7I). SCC15 transduced with RNAi3 or 4 instead formed cysts containing cornified material (Figures 7D and 7E). The number of Ki67- and Misu-positive cells was reduced by expression of RNAi3 or 4, the effects being most dramatic with RNAi4 (Figures 7G, 7H, 7J, and 7K). We conclude that Misu repression inhibited tumor growth in a dose-dependent manner.

Discussion

Misu is the first SUN-domain-containing protein to be characterized in vertebrates. Although tRNAs are the class of RNA with the highest number of nucleoside modifications, only a few mammalian tRNA methylases have been characterized [24, 34–37]. The different modifications are thought to affect maintenance of tRNA structural integrity, translational efficiency, and/or fidelity [38, 39].

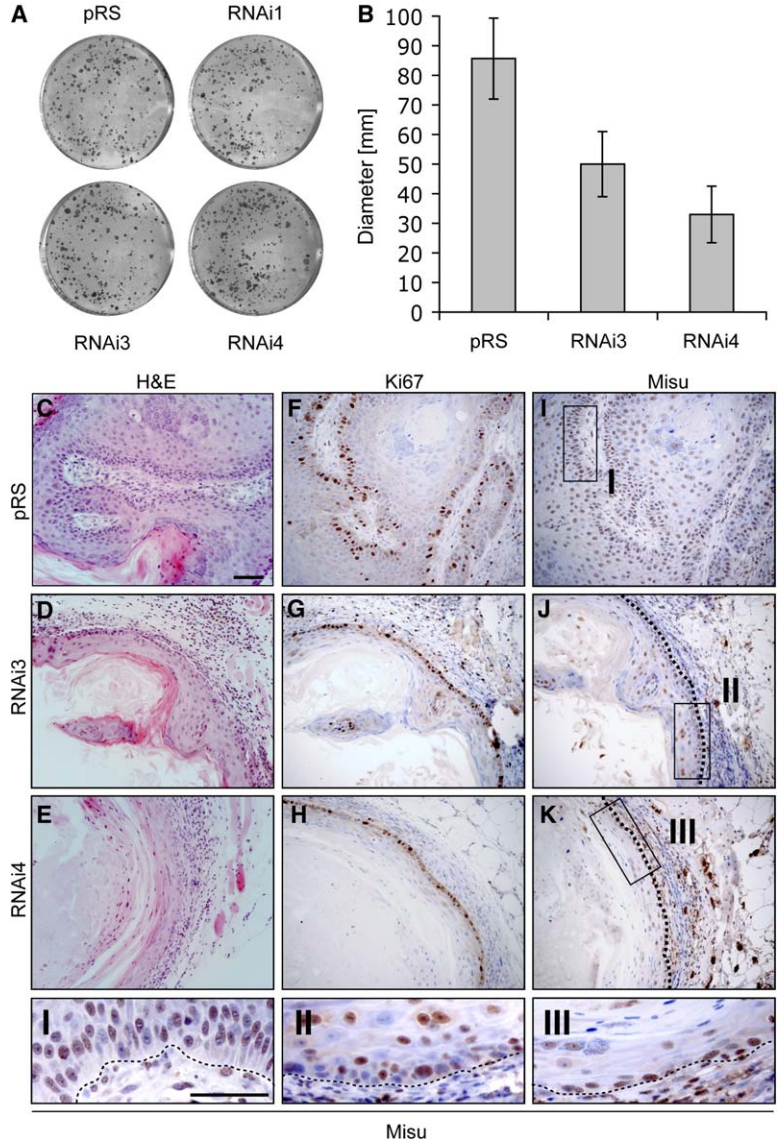

Figure 7. Inhibition of Misu Decreases Tumor Growth

(A) Introduction of Misu RNAi constructs into SCC15 cells did not affect proliferation in vitro.

(B) Tumor growth was reduced when SCC15 cells infected with Misu RNAi3 and 4 were xenografted into nude mice. pRS is control vector. Error bars represent standard deviations.

(C–E) Histology of tumors derived from SCC15 infected with pRS (C), RNAi3 (D), or RNAi4 (E).

(F–K) Immunohistochemistry of tumor sections stained with antibodies to Ki67 (F–H) and Misu (I–K). Boxed regions in (I)–(K) are shown at higher magnification in I–III. Dashed line marks interface between tumor and stroma in (J) and (K). Scale bar represents 100 μm.

The localization of Misu in nucleoli is consistent with recent evidence demonstrating that in yeast the majority of tRNA processing takes place there [28, 29]. Most nucleolar proteins are involved in ribosome biogenesis, including synthesis and processing of rRNA [40]. Thus, the nucleolus appears to be the site of most if not all RNA editing, raising the possibility that the relative locations of tRNA, rRNA, and mRNA may be spatially coordinated [41].

Nucleolar structure is dependent on RNA pol I and III transcripts, rather than the activity of the RNA polymerases themselves (Figures 4I–4L) [42]. Knockdown of Misu prevented Myc-induced increase in nucleolar size (Figures 4R–4U) [15]. Other factors that have the same effect are nutrient restriction and inhibition of TOR kinase, which results in a chromatin-mediated rearrangement of nucleolar architecture and RNA pol I localization [43]. Whether Misu has a similar effect on RNA pol III remains to be elucidated, but we have provided evidence that Myc regulates nucleolar morphology by directly activating a protein involved in RNA processing.

One difficulty in defining the role of the nucleolus in cellular metabolism is that most of its structural and functional proteins are continuously exchanged with the nucleoplasm [44]. This was true for Misu, which was found in the nucleolus in G1, throughout the nucleoplasm in S, dispersed in the cytoplasm in G2, and decorating the spindle at M phase of the cell cycle. Misu levels increased in S phase, and downregulation of Misu in human primary keratinocytes prevented Myc-induced accumulation of keratinocytes in G2/M [6]. These observations suggest a possible role for Misu in regulating the cell cycle.

In conventional culture conditions, knockdown of Misu decreased proliferation of human keratinocytes and abolished Myc-induced hyperproliferation. However, when keratinocytes were growing at a low rate in reconstituted epidermis, Misu RNAi did not affect proliferation. This would argue that low levels of Misu are sufficient for normal epidermal homeostasis, but Misu upregulation is required when keratinocyte proliferation is stimulated, for example when cells enter the transit-amplifying compartment.

Misu, like Myc, is overexpressed in human and animal tumors [45]. Our xenograft data suggest that Misu mediates the effects of Myc on tumor growth; Misu may also contribute to the nucleolar changes associated with malignant transformation [46].

Conclusion

Misu is a new Myc target gene that methylates RNA pol III transcripts and is required for Myc-induced growth and proliferation. We believe that Misu is not only the key to understanding Myc functions in normal cells, but is also an attractive target for anticancer therapies.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, five figures, and one table and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/10/971/DC1/.

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Accession Numbers

The GenBank accession number for the full-length mouse sequence reported in this paper is DQ490066.