Systematic Interrogation of 3q26 Identifies TLOC1 and SKIL as Cancer Drivers

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ABSTRACT

3q26 is frequently amplified in several cancer types with a common amplified region containing 20 genes. To identify cancer driver genes in this region, we interrogated the function of each of these genes by loss- and gain-of-function genetic screens. Specifically, we found that TLOCI (SEC62) was selectively required for the proliferation of cell lines with 3q26 amplification. Increased TLOCI expression induced anchorage-independent growth, and a second 3q26 gene, SKIL (SNON), facilitated cell invasion in immortalized human mammary epithelial cells. Expression of both TLOCI and SKIL induced subcutaneous tumor growth. Proteomic studies showed that TLOCI binds to DDX3X, which is essential for TLOCI-induced transformation and affected protein translation. SKIL induced invasion through upregulation of SLUG (SNAI2) expression. Together, these studies identify TLOCI and SKIL as driver genes at 3q26 and more broadly suggest that cooperating genes may be amplified in other regions with somatic copy number gain.

SIGNIFICANCE: These studies identify TLOCI and SKIL as driver genes in 3q26. These observations provide evidence that regions of somatic copy number gain may harbor cooperating genes of different but complementary functions. Cancer Discov; 3(9): 1044-57. © 2013 AACR.

INTRODUCTION

The most common alterations found in cancer genomes are recurrent somatic copy number alterations (SCNA; refs. 1, 2). Although some of these SCNAs harbor known oncogenes or tumor suppressor genes, the gene(s) targeted by most of these SCNAs remain unclear. For example, a recent study of more than 3,000 cancer samples identified 158 recurrent SCNAs in several cancer types, of which 122 did not harbor a known oncogene or tumor suppressor gene (1).

3q26 has been reported to be amplified in several cancer types including breast, prostate, ovarian, non–small cell lung, and head and neck squamous carcinomas. The distal arm of 3q also contains other oncogene candidates including PIK3CA, SOX2, and TP63 (3–5). However, the analysis of a large number of human cancers showed that the minimal common amplified region at 3q26 contains 20 genes, which frequently does not include the neighboring genes PIK3CA, SOX2, or TP63.

Here, we applied both gain- and loss-of-function approaches to interrogate the 20 genes resident in the minimal common amplified region of 3q26 for effects on proliferation, anchorage-independent growth, and invasion. We found two genes that cooperated to confer a tumorigenic phenotype: TLOCI and SKIL.

RESULTS

3q26 Is Frequently Amplified in Ovarian, Breast, and Non–Small Cell Lung Cancers

We previously used the Genomic Identification of Significant Targets in Cancer (GISTIC) analytic approach to identify recurrent regions of SCNA in a set of 3,131 tumor samples (1). Many of the most frequently amplified regions harbored known oncogenes, but the identity of specific driver genes was unknown for several recurrently amplified regions. Specifically, 3q26 was amplified in 22% of these tumor samples, and 8.4% of tumors harbored focal amplifications, as defined as a region less than half a chromosome arm long. The minimal common amplified region contained 20 protein-coding genes. When we investigated the specific cancer types in Tumorscape (6) that harbor this ampiclon, we found that 3q26 is amplified in 43.7% of ovarian, 31.7% of breast, and 31.2% of non–small cell lung carcinomas (NSCLC; ref. 1; Fig. 1A). When we interrogated the current Cancer Genome Atlas (TCGA; 5,547 samples; refs. 2, 7), we found that 3q26 is amplified in lung squamous cell (31.5%), serous ovarian (19.3%), cervical squamous cell (11.8%), head and neck (11.5%), pancreatic (7.1%), uterine corpus endometrial (7.1%), and stomach adenocarcinoma cancers (6.1%).

Amplification of 3q26 often extends to include larger regions of 3q. Because PIK3CA, SOX2, and TP63 also reside on 3q, we investigated at what frequency these genes are amplified in conjunction with 3q26. Of more than 3,000 cancer samples in Tumorscape, 718 (22%) displayed amplification of 3q26. Of these 718 samples, 59 did not include PIK3CA, SOX2, or TP63 (Fig. 1B). To investigate whether PIK3CA is mutated at a higher frequency in samples that lack amplification of the minimal common amplified region, we analyzed data from 3,953 samples from the TCGA (2) for which both copy number and mutation information were available. We found no significant enrichment of PIK3CA mutations in samples that either harbor or lack amplification of 3q26 ($P = 0.37, \chi^2$ test). Together these observations confirm that...
3q26 is frequently amplified in several cancer types in a manner that is independent of copy number alterations of PIK3CA, SOX2, or TP63.

**Systematic Interrogation of 3q26 Identifies TLOC1 and SKIL as Transforming Genes**

To identify genes resident in 3q26 that contribute to malignant transformation, we interrogated the function of the genes in the region by conducting systematic gain- and loss-of-function studies (Fig. 1C). Specifically, we suppressed or overexpressed each of the 20 genes present in the minimal common amplified region and assessed the effects on proliferation, anchorage-independent growth, and invasion.

To identify genes whose expression was necessary for the proliferation of cell lines that harbored the 3q26 amplicon, we conducted an arrayed short hairpin RNA (shRNA) screen in 11 cell lines that do or do not harbor amplification of 3q26 (Fig. 1D). We used RNA interference (RNAi) Gene Enrichment Ranking (RIGER) analysis (8) to identify genes that were selectively essential for proliferation of cell lines with 3q26 amplification. This method takes the effect of all shRNAs for one gene into account and compares the score for each gene to other genes. Specifically, we summarized the effects of multiple shRNAs (five on average) targeting each gene into a single final score called normalized enrichment score (NES), which considers the relative ranking of and the magnitude of gene-specific suppression effects of the shRNAs (Supplementary Table S1). The enrichment score reflects the degree to which the shRNAs targeting each gene are overrepresented at the top or bottom of the ranked list. The scores are further normalized to account for the size of each set of shRNAs against each gene to yield a NES. Using
this analysis, we found that suppression of GPR160, TLOC1, TNIK, and PHC3 selectively inhibited the proliferation of cells harboring the 3q26 amplification, with a false discovery rate (FDR) of less than 0.25 (Fig. 2A). We confirmed that the degree of gene suppression induced by TLOC1-specific shRNAs correlated with the effects on cell proliferation in cells that harbor 3q26 copy number gain (Supplementary Fig. S1).

In parallel to these studies, we overexpressed each of the genes in immortalized human mammary epithelial cells (HMLE) expressing an activated allele of MAP2K1 (MEKDD). In prior studies, we showed that these cells (HMLE–MEKDD) do not grow in an anchorage-independent manner nor do they form tumors in animals, but the expression of oncogenes such as HRAS, AKT1, and IKBKE rendered these cells tumorigenic (9). As such, these cells serve as an experimental model for mammary epithelial transformation. We then assessed the consequences of expressing these genes in two assays, anchorage-independent growth and Matrigel invasion, that measure phenotypes associated with malignant transformation.

In the anchorage-independent growth assay, TLOC1 was the only gene that induced increased anchorage-independent growth in HMLE–MEKDD cells in a manner comparable with that observed when we expressed a myristoylated version of AKT1 (>2 SD above the median; Fig. 2B). We note that three genes, PLD1, PRKCI, and MYNN, scored >1 SD over the median.

In addition, we carried out proliferation experiments on a set of cell lines that do or do not harbor the 3q26 amplification. By stably expressing shRNAs, we found that cell lines with 3q26 amplification required the expression of TLOC1 for proliferation as compared with cells that lack this amplification (Fig. 2C). We concluded that TLOC1 expression was required for the proliferation of cell lines that harbor the 3q26 amplification.

In contrast, when we assessed whether expression of each of these genes affected the capacity to induce invasion in a
Transwell Matrigel invasion assay, we found that SKIL induced significant invasion (Fig. 2D and E; P = 0.02; Supplementary Fig. S2), more than what we observed when we suppressed CDH1 (E-cadherin). These observations identify SKIL as a gene resident in the 3q26 amplicon involved in increased cell invasion.

These observations implicated TLOC1 and SKIL as genes that contribute to distinct cancer phenotypes. To determine whether overexpression of TLOC1 or SKIL sufficed to confer tumorigenic growth to immortalized cells, we overexpressed each of these genes in murine embryonic fibroblasts and assessed tumorigenicity in a subcutaneous tumor assay. Similar to what we observed when we expressed the breast cancer oncogene INKBPE1 in these cells (12 tumors per 12 sites; ref. 9), we found that TLOC1 (six tumors per 12 sites) and SKIL (six tumors per 12 sites) induced tumor formation (Fig. 2F). These observations show that TLOC1 and SKIL contribute to tumor initiation and implicate both TLOC1 and SKIL as potential targets of this amplified region.

**Expression of TLOC1 and SKIL Correlates with 3q26 Copy Number Gain**

When we assessed TLOC1 protein expression in the HMLE-MEKDD cells by immunoblotting, we found that TLOC1 migrated faster than the predicted size of 46 kD (Fig. 2G). We sequenced the construct used in the screen and determined that it encoded a novel 220-amino acid version of TLOC1, which represents a splice variant lacking amino acids 182 to 360 compared with the reported TLOC1 isoform in Ensembl (ENSP00000337688). This splice variant excludes exons 6 and 7 and includes a fusion of exon 5 into the middle of the last exon 8 by intraexonal splicing (GenBank accession number KC005990). To determine whether this smaller mRNA isoform is expressed in cancer cell lines, we cloned
TLOCI and SKIL as Cancer Drivers in 3q26

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Figure 2. (Continued) E, immunoblots for Flag-epitope–tagged SKIL immune complexes (IP) from cells expressing SKIL used in D, tumor formation of NIH3T3 cells stably expressing control vector (hcRED), TLOCI, SKIL, or IKBKE as assessed at 8 weeks. G, immunoblots for Flag-epitope–tagged TLOCI in Flag-bead immune complexes (IP) from TLOCI overexpressing cells used in D. Arrows indicate specific bands. H, V5-immunoblots for V5–tagged 399- or 220-amino acids splice variants of TLOCI and hcRED in HMLE–MEKDD cells. Arrows indicate specific bands. I, the 220- and 399-amino acids splice variants of TLOCI significantly induced anchorage-independent growth in HMLE–MEKDD as compared with hcRED control (P < 0.01, Student t test). Bars indicate average fold colony number. J, expression levels of endogenous 399- and 220-amino acid forms of TLOCI (top) and SKIL (bottom) in a panel of cell lines. Cell lines with 3q26 amplification are indicated with red text and a + sign. Cell lines that were determined by qRT-PCR on genomic DNA to harbor moderately increased copy number of 3q26 are indicated with a − sign and cells with normal 3q26 status with black text and a − sign. Cell lines that were determined by qRT-PCR to harbor moderately increased copy number of SKIL are indicated with white text and a + sign. Cell lines with normal or increased 3q26 copy number, respectively (Supplementary Table S2). These observations confirmed that expression of SKIL increases or normal 3q26 copy number, respectively (Supplementary Table S2). These observations confirmed that increased or normal 3q26 copy number, respectively (Supplementary Table S2). These observations confirmed that increased or normal 3q26 copy number, respectively. These observations confirmed that increased or normal 3q26 copy number, respectively.

To investigate whether gene expression or protein levels correlated with amplification status, we conducted two analyses. To assess whether cell lines that harbor 3q26 amplifications exhibit increased gene expression levels of TLOCI or SKIL, we conducted comparative marker selection test, based on a t test metric, on the gene expression data available for these samples to identify genes that significantly differed between these two classes. We found that TLOCI and SKIL transcript levels, out of a total of 20,500 transcripts, were the third and 64th highest differentially expressed between cell lines with increased or normal 3q26 copy number. The longer parallel bar represents the mean expression and the whiskers SD. Quantification of TLOCI and SKIL expression levels relative to actin are from the previous panel. The values are standardized to expression levels in the HMEC cells. n.s., not significant. Error bars, 1 SD. hcRED, Heteractis crispa Red; IP, immunoprecipitation; IB, immunoblot.
our screen, we investigated the TLOC1 and SKIL protein expression in 13 cell lines. We found significantly higher TLOC1 protein expression levels (both TLOC1 399 amino acids and TLOC1 220 amino acids) in amplified cell lines as compared with cell lines that harbor normal copy number ($P = 0.02$ and 0.04; Fig. 2J and K). We did not find a strict correlation between SKIL protein levels and SKIL copy number ($P = 0.41$; Fig. 2J and K). Specifically, we found that two cell lines (H28 and COLO320) that harbor moderately increased copy number at 3q26 by quantitative real-time PCR (qRT-PCR) on genomic DNA did not exhibit higher expression of SKIL protein (Supplementary Fig. S3). Together, these observations confirmed that the transcript levels of TLOC1 and SKIL correlated with 3q26 amplification.

**Identification of TLOC1-Interacting Proteins That Are Essential for Transformation**

TLOC1 is part of the translocon complex (11), a channel complex in the endoplasmic reticulum (ER) through which newly synthesized proteins are transported into the ER lumen. This complex includes SEC61, TLOC1, and SEC63. TLOC1 and SEC63 have been suggested to recruit newly synthesized proteins into the ER lumen. These proteins, msk (IPO7), and CGI0077 (DDX5; Supplementary Table S4), which has been previously described to associate with TLOC1 (14).

Supplementary Table S3). Eighteen of these proteins were identified by at least four unique peptides (Fig. 3B and Supplementary Table S3). The peptide coverage of these 18 proteins ranged from 3% to 33% (Fig. 3B). Of these 18 proteins, four have been previously found as interacting proteins with the *Drosophila* homolog of TLOC1, Trp1: rb (AP3B1), Trp1 (TLOC1), msk (IP07), and CGI0077 (DDX5; Supplementary Table S4). We also identified HSPA5 (Bip, Kar2p), which has previously been described to associate with TLOC1 (14).

When we examined the top 18 identified proteins, we found enrichment in proteins implicated in translation (Fig. 3C and Supplementary Table S3). Specifically, four of 18 proteins have been reported to be involved in translation: HNRNPM, DDX3X, PABPC1, and EIF3CL (15–18). To identify proteins essential for TLOC1-induced cell transformation, we suppressed each of the associated proteins with two independent shRNAs and tested for

![Figure 3](image-url)
their effect on anchorage-independent growth in TLOC1-overexpressing HMLE-MEKDD cells. Of these candidates, DDX3X and SRRM2 significantly reduced anchorage-independent growth (Fig. 3D). Because DDX3X previously has been reported to be involved in translation (17) and gave the strongest phenotype, we focused on DDX3X for subsequent experiments.

To confirm that DDX3X interacted with TLOC1, we reisolated V5-tagged immune complexes and detected endogenous DDX3X by immunoblotting (Fig. 3E). To identify domains of TLOC1 that were responsible for the association with DDX3X, we generated a set of TLOC1 truncation mutants. These truncation mutants were generated from the 220-amino acid–long splice variant of TLOC1 (Fig. 3F). We found that the 81 N-terminal amino acids were necessary for the interaction of TLOC1 with DDX3X. In parallel, we tested the transforming ability of each of these truncation mutants and found that the 81 N-terminal amino acids also were necessary for TLOC1 to induce anchorage-independent growth (Fig. 3G). These observations showed that the 81 N-terminal amino acids are required for the association of TLOC1 with DDX3X and for TLOC1-induced cell transformation.

**Figure 4.** TLOC1 increased the cap- versus IRES-dependent translation ratio by decreasing IRES-dependent translation. **A,** illustration of the bicistronic translation reporter used to measure translation levels. The Renilla luciferase reporter is driven by cap-dependent translation and the firefly luciferase expression and an internal ribosome entry site (IRES) regulates Firefly luciferase expression (ref. 19; Fig. 4A). We found that TLOC1 overexpression significantly reduced IRES-dependent translation (P = 0.002), which resulted in a significant net increase of the ratio of cap-dependent to IRES-dependent translation (P = 0.01; Fig. 4B). Furthermore, we tested this reporter system with the TLOC1 truncation mutants and found that the full-length form and the shorter C-terminal truncation version significantly inhibited the IRES-dependent translation reporter, whereas the 149-amino acid N-terminal truncation mutant, which does not interact with DDX3X, failed to affect this reporter (Fig. 4C). We also tested whether suppression of DDX3X affected translation in cells expressing TLOC1. We found that the increased cap/IRES-dependent

**TLOC1 Affects Cap-Dependent versus IRES-Dependent Translation**

To assess whether TLOC1 and DDX3X affected protein translation, we manipulated TLOC1 and DDX3X expression and measured the effects on translation using a bicistronic translation reporter system. In this system, cap-dependent translation controls Renilla luciferase expression and an internal ribosome entry site (IRES) regulates Firefly luciferase expression (ref. 19; Fig. 4A). We found that TLOC1 overexpression significantly reduced IRES-dependent translation (P = 0.002), which resulted in a significant net increase of the ratio of cap-dependent to IRES-dependent translation (P = 0.01; Fig. 4B). Furthermore, we tested this reporter system with the TLOC1 truncation mutants and found that the full-length form and the shorter C-terminal truncation version significantly inhibited the IRES-dependent translation reporter, whereas the 149-amino acid N-terminal truncation mutant, which does not interact with DDX3X, failed to affect this reporter (Fig. 4C). We also tested whether suppression of DDX3X affected translation in cells expressing TLOC1. We found that the increased cap/IRES-dependent

**Figure 4.** TLOC1 increased the cap- versus IRES-dependent translation ratio by decreasing IRES-dependent translation. **A,** illustration of the bicistronic translation reporter used to measure translation levels. The Renilla luciferase reporter is driven by cap-dependent translation and the firefly luciferase by IRES-dependent translation. **B,** TLOC1 overexpression significantly increased the ratio of cap- versus IRES-dependent translation by inhibition of IRES-dependent translation (P = 0.01). Student t test. The ratio of cap/IRES-dependent translation was calculated and is illustrated in the right. **C,** overexpression of the transforming TLOC1 truncation mutant ΔC27 significantly (P = 0.03, Student t test) inhibited IRES-dependent translation as compared with the non-transforming variant ΔN149. **D,** suppression of DDX3X in TLOC1 overexpressing cells significantly reversed the TLOC1-induced change in translation ratio (P = 0.03). Student t test). **E,** TLOC1 and DDX3X binds to 7-methylated GTP beads, and TLOC1. **F,** TLOC1 overexpression increased EIF4G protein expression. **G,** TLOC1 overexpression in HMLE-MEKDD cells decreased EIF4EBP1 phosphorylation on Threonines 37 and 45 and Serine 65. Lysates were prepared from TLOC1- or BFP-overexpressing HMLE-MEKDD cells, which had been grown with (+GFs) or without (−GFs) growth factors for 24 hours. Error bars, 1 SD. BFP, blue fluorescent protein; RLU, relative light unit; FLU, fluorescent light unit; IB, immunoblot.
translation induced by TLOC1 expression was significantly reversed upon DDXX3 suppression (Fig. 4D). Together, these observations show that TLOC1 levels modulate cap/IREs-dependent translation, which is reversed by suppression of DDXX3.

Eukaryotic initiation factor (EIF) 4G binds to the mRNA cap structure to initiate translation. EIF4G is a scaffold protein that interacts with EIF4E and promotes the assembly of EIF4E and EIF4A (DDXX2A) to create the initiation complex EIF4F. EIF4A, like DDXX3, is also an RNA helicase. Several other proteins, for example, EIF4EBP1, bind to or modify this complex to alter its activity and specificity. Hypophosphorylated EIF4EBP1 is known to inhibit translation by sequestering EIF4E. Phosphorylation of EIF4EBP1 releases EIF4E and allows EIF4E to assemble the EIF4F complex and induce translation (20). EIF4EBP1 phosphorylation levels are regulated by the mTOR pathway.

To determine whether TLOC1 and DDXX3 bind translation-related complexes, we isolated 7-methylated GTP-binding complexes and assessed the associated levels of EIF4E, EIF4G, TLOC1, and DDXX3 in HMLE-MEKDD cells expressing TLOC1. We found that all of these proteins bound 7-methylated GTP but not actin or blue fluorescent protein (BFP), which served as controls for nonspecific binding (Fig. 4E). We also found that TLOC1 overexpression increased EIF4G levels as well as the amount of EIF4G1 associated with the 7-methylated GTP beads (Fig. 4F). These observations show that TLOC1 and DDXX3 interact with a translation protein complex through 7-methylated GTP and that TLOC1 overexpression increases EIF4G protein levels as well as the levels of EIF4G that associate with 7-methylated GTP beads.

To investigate whether TLOC1 expression had any effects on known regulators of translation, we investigated the phosphorylation status of EIF4EBP1. We found that TLOC1 overexpression led to decreased EIF4EBP1 phosphorylation at threonines 37 and 45 and at serine 65 (Fig. 4G). In aggregate, these observations provide evidence that overexpression of TLOC1 affects the ratio of cap-dependent translation through interactions with proteins involved in regulating protein translation, including DDXX3.

**SLUG Is Required for SKIL-Induced Cell Invasion**

SKIL has been reported to inhibit the TGF-β signaling axis by binding to SMAD4 and SMAD2, recruiting the transcriptional corepressor N-CoR and suppressing SMAD-induced transcription (21). SMAD4 is a tumor suppressor gene frequently inactivated in pancreatic cancer, and has been correlated with an invasive phenotype (22). To determine whether overexpression of SKIL or suppression of SMAD4 induced similar cell states, we analyzed existing gene expression data from the CCLE (10). After downloading the data from the CCLE, we calculated the average expression and SD for SKIL and SMAD4 on a set of 634 samples (all CCLE cell lines excluding samples derived from hematologic malignancies). High SKIL expression was defined as one SD above average SKIL expression and low SMAD4 expression one SD below average SMAD4 expression. Ninety-three cell lines were identified to have high SKIL expression, and 59 cell lines exhibited low SMAD4 levels. Of these cell lines, 15 cell lines possessed both high SKIL expression and low SMAD4 expression. By comparative marker selection analysis (23), we identified the top 50 genes that were correlated with either high SKIL expression or low SMAD4. Among these genes, 13 were present in both lists, which is significantly higher than what is expected by chance ($P < 0.001$, binomial distribution test; Fig. S4; Supplementary Table S5). At least six of the intersecting genes have been reported to be involved in regulation of invasion, including TCF8 (ZEB1; ref. 24), EHF (25), FOXL1 (26), MARVELD3 (27), ST14 (28), and TWIST1 (29). These observations suggest that high SKIL or low SMAD4 gene expression affected signaling pathways implicated in invasion.

Because we found an overlap between the expression of genes related to invasion and epithelial-to-mesenchymal transition (EMT) in cells having high SKIL or low SMAD4 expression, we tested the role of EMT master regulators in invasion. TWIST1, SNAIL (SNAI1), and SLUG (SNAI2) are well-established transcriptional regulators of invasion and EMT (30) and have been reported to be components of the TGF-β signaling pathway (31). To test whether invasion induced by expression of SKIL was dependent on the expression of any of these genes, we suppressed the expression of these genes using specific shRNAs in SKIL-expressing HMLE-MEKDD cells. We found that suppression of SLUG significantly inhibited invasion ($P = 0.03$; Fig. 5B) but did not affect cell proliferation (Fig. 5C). We also found that SLUG protein levels were upregulated in SKIL-expressing cells (Fig. 5D). To investigate whether SLUG had any effect on SKIL-induced gene expression, we investigated how SLUG suppression affected a set of TGF-β- and invasion-related genes. We found that several genes were upregulated by SKIL and reversed upon SLUG suppression, including SNAI1, TWIST1, PLAUR, and VIM (Fig. 5E). Furthermore, upon overexpression of SLUG, the same subset of genes was upregulated (Fig. 5F).

Because SKIL has been suggested to inhibit SMAD4 function, we tested the effect of suppression of SMAD4 in our system. We found that suppression of SMAD4 induced invasion and that suppression of SLUG significantly reversed this phenotype ($P = 0.02$ and 0.05; Fig. 5G). These observations implicate SKIL as a regulator of SMAD4-mediated invasion, which required SLUG.

**TLOC1 and SKIL Cooperate to Induce Transformation**

Because TLOC1 and SKIL frequently are coamplified, we investigated whether manipulating the expression of these genes induced cell transformation. We found that overexpression of TLOC1 and SKIL together induced a significant increase in anchorage-independent growth ($P = 0.003$; Fig. 6A), which failed to be explained by a general increase in cell proliferation (Fig. 6B).

Having investigated cooperative effects of SKIL and TLOC1, we tested the effects of coexpressing TLOC1 and SKIL with PIK3CA or SOX2 from neighboring amplification peaks. We found that both PIK3CA and SOX2 overexpression induced anchorage-independent growth in HMLE-MEKDD cells but exhibited no cooperative effect with TLOC1 or SKIL (Fig. 6C). These observations suggest that TLOC1 and SKIL induce transformation independently of PIK3CA or SOX2.
TLOC1 and SKIL as Cancer Drivers in 3q26

**DISCUSSION**

3q26 is amplified in several types of cancer, including lung, ovarian, and breast, and is correlated with poor prognosis and an invasive phenotype (32). Here, we have systematically interrogated the function of the genes harbored in the minimal common amplified region by suppressing or overexpressing each of these in human cancer cell lines. We found that TLOC1 expression induced anchorage-independent growth, whereas SKIL induced invasion, and both of these genes induced tumor formation. Coexpression of TLOC1 and SKIL induced cooperative cell transformation. Together, these observations identify TLOC1 and SKIL as transcription factors that regulate translation of terminal oligopyrimidine-containing mRNAs (34). Taken together, these observations suggest that TLOC1 and DDXX3X are components of a macromolecular complex involved in translational regulation.

Several lines of evidence now implicate translation as a key process perturbed in cancer cells (35). For example, MYC-induced transformation has been shown to drive the shift from cap- to IRES-dependent translation during the cell cycle and lead to genomic instability (36). The finding that TLOC1 and DDXX3X induced translation through inhibition of IRES-dependent translation is consistent with this model. We note that mutations involving DDXX3X have recently been described in several cancer types, including head and neck cancer (37).

It remains unclear whether perturbation of translation contributes to transformation through specific transcripts or global dysregulation of translation. Several recent reports suggest that subsets of mRNAs are specifically regulated. For example, oncogenic mTOR signaling has been shown to control the translation of a set of proinvasive transcripts in prostate cancer (38), and has also been reported to control translation of terminal oligopyrimidine (TOP)-motif-containing mRNAs (39). These observations suggest that perturbation of translation control likely affects specific transcripts that contribute to transformation.
We found that TLOC1 overexpression decreased EIF4EBP1 phosphorylation, which is predicted to induce decreased translation. Overexpression of EIF4A2 also induces tumorigenic growth of NIH3T3 cells (40), and an inhibitor targeting EIF4A2, 4EGI-1, was shown to inhibit growth of human breast and melanoma cancer xenografts (41). The finding that TLOC1 is not only amplified in a significant fraction of cancers but also contributes directly to cell transformation suggests that dysregulation of protein translation by perturbation of TLOC1 or DDX3X contributes to tumorigenicity.

We identified a shorter splice variant of TLOC1 that also had transforming capacity, an inhibitory effect on translation, and an association with DDX3X. Because this form lacks the two transmembrane domains of TLOC1, we predicted that this form does not bind to the ER. However, despite these differences, we found that both isoforms induced cell transformation. Two mRNA forms of the TLOC1 ortholog in Drosophila melanogaster, Trip1, have been shown to be selectively expressed: a 1.6-kb form, which is expressed in the male reproductive system, and a 2.2-kb form confined to other tissues (42). The difference between the 2.2 and 1.6 kb transcripts is close to the difference of the 537 bases of the two TLOC1 forms. These observations suggest that these two isoforms may have tissue-specific effects but that both are transforming when inappropriately expressed.

We also identified SKIL in 3q26 as an inducer of invasion. SKIL has been reported to inhibit SMAD4 function by repressing its transcriptional activity by recruitment of the transcriptional corepressor N-CoR (21). Although SKIL protein levels did not strictly correlate with copy number, we showed that both overexpression of SKIL and suppression of SMAD4 induced an invasive phenotype through upregulation of SLUG, thus illustrating two common genomic events in cancer that lead to inactivation of the TGF-β pathway and subsequently induction of an invasive phenotype.

PIK3CA and SOX2 are other oncogenes that also reside on the 3q arm and are often co-amplified with 3q26. To investigate whether TLOC1 or SKIL acted in a cooperative manner with PIK3CA or SOX2, we expressed combinations of genes but did not observe any cooperation between PIK3CA or SOX2 and TLOC1 and SKIL. These observations suggest that PIK3CA or SOX2 act independently of TLOC1 and SKIL; however, we note that our experiments do not eliminate the possibility that these genes cooperate in other assays in vivo. Moreover, we confirmed that PIK3CA mutations occur at equal frequency in cells that harbor or lack increased copy number at 3q26.

We found that TLOC1 and SKIL cooperated to induce anchorage-independent growth. Cooperating cancer drivers in amplification peaks have previously been described. YAP1 and BIRC2 (cIAP1), which reside in the same amplicon in liver cancer, have been shown to have a cooperative effect on tumorigenesis (43). In addition, EGFR is frequently co-amplified with the neighboring gene SEC61G in glioma, and both have been shown to be required for tumor cell survival (44). Of note, SEC61G and TLOC1 interact in the translocation channel. In a similar manner, 8p22 has been reported to include a cluster of cooperating tumor suppressors (33). Although we showed that overexpression of TLOC1 affects protein translation and anchorage-independent growth, and SKIL affects cell invasion, it remains unclear whether these functions cooperate or act in parallel in vivo.

Moreover, further studies are necessary to determine whether targeting these pathways will lead to clinical responses. Indeed, 4EG1-1 has been shown to inhibit growth of human breast and melanoma cancer xenografts (41). In addition, inhibitors of DDX3X, which has also been reported to be important for HIV propagation, have been developed (45). Further work will be necessary to determine whether 3q26 amplification predicts the response of cancer cells to these inhibitors.

In aggregate, these studies extend work suggesting that regions of recurrent SCNAs may harbor more than one driver gene that participate in different aspects of tumor initiation or progression. Future efforts to interrogate such regions will not only require systematic interrogation of resident genes but also the use of multiple assays to assess potentially complementary phenotypes.
**METHODS**

**Copy Number Analysis**

GISTIC profiles were created from Tumorscape with 3,131 tumor samples as previously described (1, 6). To create amplification diagrams of tumor samples and the screened cell lines, copy number illustrations were made with the Integrative Genomics Viewer (46). The TCGA was also queried for amplification of genes in the minimal amplified region of 3q26 (2, 7).

**Cell Culture, Vectors, and Lentiviruses**

HMLE cells expressing STERT and SV40 Early Region have been described previously (47). To express or suppress genes, retroviral and lentiviral vectors were used (pBabe, pW2I, pLex, pLKO) as described previously (48, 49). pBabe-Puro-Flag-DEST, pLEX-Puro-V5-DEST, and pW2I-Neo-Flag-DEST constructs were generated by Gateway cloning from pDONR223 constructs acquired from either the Human ORFeome (50) or from BP cloning reactions of BP-tagged PCR products. Truncation mutants of TLOC1 were created by PCR amplification with BP-tagged primers (Supplementary Table S6) and BP ligation (Invitrogen) into pDONR223 and subsequent LR ligation (Invitrogen) into a viral destination vector. The identity of the cell lines used in this study were verified by the CCLE.

**RNAi Experiments**

shRNAs were obtained from the RNAi Consortium. The corresponding shRNAs are listed in Supplementary Table S7. Ten different GFP-specific shRNAs were used as unrelated negative controls. Lentiviral infections were conducted as described previously (49). The suppression of each shRNA was determined in a parallel experiment where MCF7 cells were infected, selected, and cultured in a similar fashion as in the screen. The shRNA suppression efficiency was determined by qRT-PCR. Primers are listed in Supplementary Table S8. The effects of suppressing genes resident in 3q26 were calculated by RIGER (8). RIGER is a method similar to Gene Set Enrichment Analysis (51) to summarize the effects of multiple shRNAs into a single per-gene score. First, we weighted each shRNA by its target suppression efficiency and computed a differential proliferation score (blue lines in Fig. 2A) for each shRNA according to the difference of mean proliferation between the cells harboring 3q26 and controls. These scores were sorted from high to low and each gene was assigned an “enrichment” score (red lines in Fig. 2A) according to how overrepresented their shRNAs were in the sorted list using a Kolmogorov-Smirnov weighted statistic. The positive (enrichment) enrichment scores are normalized using the absolute value of the mean of the positive (negative) values in a permutation-based null distribution. This null distribution was also used to generate nominal P values and FDR (see Fig. 2A). The FDR is the expected proportion of false-positives among all queries.

**Anchorage-Independent Colony Growth**

HMLE cells expressing the different genes were assayed for their colony formation capacity as described previously (9). In addiction to the 20 genes, we included myristoylated AKT1, which has been shown to collaborate with MEKDD and transform immortalized breast epithelial cells, as a positive control. As negative controls, we used corresponding vectors expressing YFP (yellow fluorescent protein), hRed (Heteractis crina Red), Lacz (β-galactosidase), or rLuc (luciferase). To test the function of TLOC1-associated proteins, each candidate was targeted with two shRNAs in TLOC1-overexpressing HMLE-MEKDD cells.

**Invasion Experiments**

Invasion capacity of cells was determined by plating 50,000 cells in the upper chamber of Matrigel (BD Biosciences)-coated Transwell invasion chambers according to the manufacturer’s instructions. The cells were seeded in media containing no growth factors, and media with a normal amount of growth factors was added to the lower chamber. An shRNA-targeting CDH1 (E-cadherin; ref. 29) or overexpression of YFP served as positive or negative control, respectively. Invasion was determined after 24 to 48 hours by staining with Giemsa or Hoechst and counting the number of invaded cells with ImageJ (W.S. Rasband, U.S. NIH, Bethesda, MD; http://imagej.nih.gov/ij/).

**Quantitative Real-Time PCR**

RNA was harvested using RNeasy (Qiagen). Complementary DNA was prepared using Advantage RT-for-PCR (Clontech). Quantitative PCR was conducted using SYBR (Applied Biosystems). Primers are listed in Supplementary Table S8. For the gene suppression experiments (Supplementary Fig. S1), MCF7 cells propagated in 24-well tissue culture plates were infected with individual shRNAs at a multiplicity of infection (MOI) < 1. After infection, the cells were selected with puromycin and passaged to eliminate dead cells. RNA was purified with RNeasy (Qiagen) and converted into cDNA by reverse transcription using Superscript II (Invitrogen, Life Technologies). Gene expression levels for each targeted gene and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined for each shRNA-infected sample by RT-PCR in a 7300 Real-Time PCR System (Applied Biosystems, Life Technologies). The relative expression level per gene was normalized against GAPDH levels and compared with a reference sample where GFP had been targeted as an unrelated shRNA control. Expression was determined by single measurements from three different biological replicates. For genomic qRT-PCR analysis, DNA was purified from cells with QIAamp (Qiagen). Genomic levels were determined by RT-PCR in a 7300 Real-Time PCR System (Applied Biosystems, Life Technologies) with primers specific for 3q26 (Supplementary Table S8). Primers specific for genomic LINE-1 were used to normalize for genomic input (Supplementary Table S8).

**Immunoblotting**

For immunoblot analyses, samples were harvested in 1% NP-40 or radioimmuno precipitation assay buffer supplemented with protease and phosphatase inhibitors. Samples were separated by SDS-PAGE and transferred to nitrocellulose filters with the iBlot system (Invitrogen, Life Technologies). Antibodies used were: anti-Flag M2 (Sigma), anti-V5 (Invitrogen, Life Technologies), TLOC1 (SEC62; #HPA014059), DDX3X (#HPA001648; Prestige Antibodies; Sigma), SNON (SKIL; #4973), SLUG (#9589), EIF4G (#9742), EIF4EBP1 (#9744), pEIF4EBP1(Thr37/45) (#9459), pEIF4EBP1(Ser65) (#9456), (Cell Signaling Technology), and SNON (SKIL; H317; sc-9141; Santa Cruz Biotechnology).

**Proliferation Experiments**

HMLE–MEKDD cells expressing vector control or gene of interest or cancer cell lines expressing shRNA against control or gene of interest were plated in 6-well plates. Cells were counted after indicated time and replated at the same amounts, and this cycle was repeated as indicated. Increase in cell number was calculated and plotted as accumulated increase in cell number.

**TLOC1 Association Experiments**

One hundred-milligram protein lysates were generated from HMLE–MEKDD cells expressing V5-tagged LUC or TLOC1 by lysis in 1% NP-40 supplemented with protease and phosphatase inhibitors. Immune complexes were precipitated overnight with anti-V5-heads (Invitrogen). A fraction of the precipitated product was analyzed on PAGE for silver staining and immunoblotting to confirm that V5-tagged TLOC1 was isolated. The rest of the protein was separated on gels and stained with SYPRO Ruby protein gel stain, and lanes were isolated. The immunoglobulin G bands were removed from the lanes and the rest was submitted for mass spectrometry analysis. Excised gel bands were subjected to in-gel trypsin digestion. Gel pieces were washed and dehydrated with acetonitrile and rehydrated...
in 50 mmol/L ammonium bicarbonate containing 12.5 ng/μL modified sequencing-grade trypsin (Promega) at 4°C. After 45 minutes, trypsin solution was removed and replaced with 50 mmol/L ammonium bicarbonate solution and incubated at 37°C for 16 hours. Peptide-containing ammonium bicarbonate extract was removed, and remaining peptides were eluted with 50% acetonitrile containing 1% formic acid and dried in a speed-vac.

Samples were reconstituted in 2.5% acetonitrile containing 0.1% formic acid and separated over a nanoscale reverse-phase high-performance liquid chromatography (HPLC) capillary packed with C18 spherical silica beads (52). Sample was loaded in an equilibrated Famos auto sampler (LC Packings). A gradient was formed and peptides were eluted with increasing concentrations of solvent (97.5% acetonitrile containing 0.1% formic acid).

Eluted peptides were subjected to electrospray ionization and entered into an LTQ Velos ion-trap mass spectrometer (Thermo Fisher Scientific). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences were determined by matching protein databases with the acquired fragmentation pattern by SEQUEST (Thermo Fisher Scientific; ref. 53).

**Translation Measurements**

Cap- and IRES-dependent translation were determined by transient transfection of the bicistronic translation reporter into HMLE-MEKDD cells stably expressing BFP as unrelated control or TLOC1. Cells were plated in 96-well cell assay plates at 100,000 cells per well, transfected with pDL/N, and incubated for 48 hours. Renilla and Firefly luciferase activity were determined with Dual-Glo Luciferase Assay system (Promega).

**Cap-Binding Assay**

Cap-associated proteins were precipitated with 7-methylated GTP beads from cell lysates derived from TLOC1- or BFP-overexpressing cells. 7-Methylated GTP sepharose beads (GE Healthcare Life Sciences) were used according to the manufacturer’s instructions to precipitate cap-complexes.

**Gene Expression Analysis**

For the gene expression analysis between cell lines with or without 3q26 amplification, gene expression and copy number data were downloaded from CCLE for 967 cancer cell lines (54). Cell lines with a log2 amplification value higher than 0.3 at 7p15.1 were excluded. A comparative marker analysis was performed using a two-sided Student’s t-test to identify genes with significant up- or down-regulation in cell lines with 3q26 amplification compared to normal 3q.

**Tumor Formation Assays**

Tumor xenograft experiments were carried out as described previously (9). Tumor formation was assessed 8 weeks after injection. Tumors were scored when they reached 5 mm3.

**Disclosure of Potential Conflicts of Interest**

L.A. Garraway has received a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member of Foundation Medicine, Novartis, Millennium/Takeda, and Boehringer Ingelheim. R. Beroukhim has received a commercial research grant from Novartis, ownership interest (including patents) in Astrazeneca, and is a consultant/advisory board member of Novartis. W.C. Hahn is a consultant/advisory board member of Novartis. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.C. Hahn

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**Acknowledgments**

The authors thank members of the Hahn and Cichowski laboratories for helpful discussions and P. Hammerman, M. Pop, and H. Widalid for useful input. The authors also thank R. Tomatino at the Taplin Mass Spectrometry facility for carrying out the mass spectrometry experiments.

**Grant Support**

This work was supported in part by NIH/National Cancer Institute (NCI) grants R01 CA130988 (to W.C. Hahn), RC2 CA148268 (to W.C. Hahn), and U54 CA112962 (to W.C. Hahn); the Sweden–America Foundation (to D. Hagerstrand), and the Ernst O. Ek Fund (to D. Hagerstrand).

Received January 14, 2013; revised June 5, 2013; accepted June 6, 2013; published OnlineFirst June 13, 2013.

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TLOC1 and SKIL as Cancer Drivers in 3q26

Published OnlineFirst June 13, 2013; DOI: 10.1158/2159-8290.CD-12-0592

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Systematic Interrogation of 3q26 Identifies \textit{TLOC1} and \textit{SKIL} as Cancer Drivers

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