Here we show that FTO as an N6-methyladenosine (m6A) RNA demethylase is degraded by selective autophagy, which is impaired by low-level arsenic exposure to promote tumorigenesis. We found that in arsenic-associated human skin lesions, FTO is upregulated, while m6A RNA methylation is downregulated. In keratinocytes, chronic relevant low-level arsenic exposure upregulated FTO, downregulated m6A RNA methylation, and induced malignant transformation and tumorigenesis. FTO deletion inhibited arsenic-induced tumorigenesis. Moreover, in mice, epidermis-specific FTO deletion prevented skin tumorigenesis induced by arsenic and UVB irradiation. Targeting FTO genetically or pharmacologically inhibits the tumorigenicity of arsenic-transformed tumor cells. We identified NEDD4L as the m6A-modified gene target of FTO. Finally, arsenic stabilizes FTO protein through inhibiting p62-mediated selective autophagy. FTO upregulation can in turn inhibit autophagy, leading to a positive feedback loop to maintain FTO accumulation. Our study reveals FTO-mediated dysregulation of mRNA m6A methylation as an epitranscriptomic mechanism to promote arsenic tumorigenicity.
A
ologous to epigenetic chemical modifications of DNA and histones, RNA modifications regulate RNA fate and gene expression. The most abundant RNA modification is N\(^\text{6}\)-methyladenosine (m\(^\text{6}\)A)\(^\text{1-4}\). m\(^\text{6}\)A methylation regulates several aspects of RNA metabolism such as mRNA decay, nuclear processing, translation, transcription, and RNA-protein interactions\(^\text{1-3}\). m\(^\text{6}\)A modification plays an important role in development, stem cell homeostasis, and diseases such as cancer\(^\text{4,5}\), as well as stress response to UV radiation or heat shock\(^\text{6}\). At the molecular level, RNA m\(^\text{6}\)A methylation is installed by writers (methyltransferases), removed by erasers (demethylases), and recognized by readers, a piece of well-coordinated machinery to regulate m\(^\text{6}\)A’s dynamics\(^\text{1-4}\). Among these m\(^\text{6}\)A effectors, FTO (fat mass and obesity-associated protein) was the first RNA m\(^\text{6}\)A demethylase discovered\(^\text{6}\) and has been shown to play oncogenic roles in leukemia\(^\text{11}\), glioblastoma\(^\text{12}\), and melanoma\(^\text{13}\). However, the role of RNA methylation in arsenic-induced skin tumorigenesis remains unclear.

Exposure to inorganic arsenic in contaminated drinking water poses an environmental public health threat for hundreds of millions of people in the US and in the world\(^\text{14-16}\). Arsenic is a natural component of the earth’s crust and is widely distributed throughout the environment. In nature, sources of arsenic exposure include food, air, and water, with the main one being arsenic-contaminated drinking water\(^\text{17}\). Arsenic is a human carcinogen. Chronic exposure to arsenic can cause skin cancer and several internal cancers such as lung, bladder, and kidney cancer\(^\text{17}\). A major target organ of arsenic is the skin. Arsenic-induced skin lesions are an early manifestation of arsenic exposure and toxicity\(^\text{18}\) and are a risk factor for subsequent cancers\(^\text{19}\). Arsenic seems to act in concert with sunlight exposure, smoking, and occupational exposures in increasing the risk of skin lesions\(^\text{20}\). At the molecular level, arsenic has been shown to cause oxidative stress, DNA damage, chromosomal aberration, and epigenetic modifications, including DNA methylation and histone modification\(^\text{17,21,22}\). However, the mechanism by which arsenic causes tumorigenesis is still poorly understood.

In this study, we discovered that chronic low-level arsenic exposure upregulates FTO and downregulates m\(^\text{6}\)A RNA methylation in keratinocytes, and induces malignant transformation and tumorigenesis through FTO and m\(^\text{6}\)A mRNA methylation. Our study reveals that arsenic inhibits autophagy and autophagic degradation of FTO through suppressing p62 transcription, leading to FTO stabilization. Furthermore, FTO forms a positive feedback loop with autophagy inhibition to maintain FTO accumulation in arsenic tumorigenesis. Taken together, these results demonstrate that m\(^\text{6}\)A RNA methylation acts as an epitranscriptomic mechanism for arsenic damage response and tumorigenesis.

Results
FTO is upregulated, while m\(^\text{6}\)A is downregulated, by arsenic in human keratinocytes and in epidermal keratinocytes in human skin. To determine whether m\(^\text{6}\)A RNA methylation and its regulators play a role in arsenic-induced tumorigenesis, we first treated HaCaT cells, a human keratinocyte cell line, continuously with a relevant low level (100 nM) of inorganic arsenite for 28 weeks to generate HaCaT cells with chronic arsenic damage (As cells)\(^\text{23,24}\) (Supplementary Fig. 1a). Arsenic substantially upregulated FTO and downregulated m\(^\text{6}\)A, while it had moderate or no effect on other m\(^\text{6}\)A regulators, including the m\(^\text{6}\)A methyltransferase proteins METTL14 and METTL3 and the m\(^\text{6}\)A demethylase ALKBH5 (Supplementary Fig. 1b). We found that treatment with a low level of arsenic for up to 72 h upregulated FTO protein and downregulated m\(^\text{6}\)A in HaCaT cells (Fig. 1a and Supplementary Fig. 1c). The effect of arsenic on FTO seemed to be dose-dependent, since only low-level arsenic upregulated FTO, whereas high-level arsenic downregulated FTO (Supplementary Fig. 1d). Similarly, FTO upregulation was also detected in NHEK cells (normal human epidermal keratinocytes) (Fig. 1b).

As-treated cells developed tumors in all four mice, while control cells did not (Fig. 1c and Supplementary Fig. 1e). We isolated the tumorigenic cells (As-T, Arsenic-Tumorigenic cells) from one tumor in mice (#1) as well as other tumors formed by the As-treated cells (As cells) in mice (Fig. 1c and Supplementary Fig. 1a). In addition to As cells, FTO was also upregulated in the four As-T cells, while the m\(^\text{6}\)A level was downregulated in As and four As-T cells as compared with control cells (Fig. 1d). In addition, as compared with control cells, As and As-T cells showed increased proliferation (Fig. 1e) and survival (Fig. 1f, and Supplementary Fig. 1f). However, in contrast to arsenic, cadmium, another carcinogenic heavy metal present in the air, water, soil, sediment\(^\text{25}\), had no effect on FTO in HaCaT cells (Supplementary Fig. 1g), suggesting FTO as a target unique for arsenic exposure. Future investigation is needed to determine whether cadmium and other heavy metals affect FTO or other m\(^\text{6}\)A regulators in keratinocytes and other cell types.

To determine the role of FTO and m\(^\text{6}\)A in arsenic-induced tumorigenesis, we next assessed whether the levels of m\(^\text{6}\)A and FTO are altered in arsenic-induced premalignant human skin lesions, arsenical keratoses, as compared with normal human skin. In humans, it is known that chronic arsenic exposure causes arsenical keratoses, which can persist indefinitely and may develop into invasive skin cancers such as squamous cell carcinoma\(^\text{26}\). Normal human skin samples were obtained from the archives in the tissue bank of the Section of Dermatology (Department of Medicine, University of Chicago). The arsenical keratoses were obtained from the clinical follow-up for arsenic-exposed cohorts that evaluate the health effects of exposure, including skin cancer, in Bangladesh. To remove the signal from DNA, we treated all samples with DNase I\(^\text{18}\) (Fig. 1g). As compared with normal human skin, we found that FTO is upregulated and the m\(^\text{6}\)A level is downregulated in arsenical keratoses (Fig. 1h–l). In addition, we found that the m\(^\text{6}\)A level is negatively correlated with the FTO level (Fig. 1m). These findings suggest that FTO acts as a protumorigenic factor in arsenic-induced skin lesions.

FTO is required for arsenic-induced tumorigenicity. To determine the functional significance of FTO upregulation in arsenic-induced tumorigenesis, we assessed the consequence of FTO knockout (KO) in As-T cells using CRISPR (Fig. 2a). FTO deletion decreased cell growth (Fig. 2b), anchorage-independent growth (Fig. 2c), clonogenic growth (Supplementary Fig. 2a), 3D cell growth (Supplementary Fig. 2b), and survival when cells were placed in suspension (Supplementary Fig. 2c). Notably, FTO deletion partially reversed the effect of arsenic on cell survival (Supplementary Fig. 2c). It is possible that FTO and other pathways affected by arsenic also promote cell survival. Nevertheless, FTO deletion drastically inhibited tumor growth of As-T cells in mice (Fig. 2d and Supplementary Fig. 2d). Furthermore, knockdown of FTO using siRNA also decreased cell proliferation in As cells (Supplementary Fig. 2e). While overexpression of FTO slightly increased cell proliferation in HaCaT cells (Supplementary Fig. 2f), it did not induce tumor formation in NSG mice (Supplementary Fig. 2g), indicating that FTO overexpression alone is not sufficient to induce tumorigenesis. It is likely that FTO upregulation is critical for arsenic tumorigenesis, while it seems that both FTO upregulation and other molecular alterations induced by arsenic are required for malignant transformation and tumorigenesis.
Next, we used the arsenic-UVB skin co-carcinogenesis mouse model to determine the role of FTO in arsenic-induced skin tumorigenesis. We first created mice with epidermis-specific FTO deletion (K14Cre;Fto fl/fl). Then we treated the mice with arsenic, UVB irradiation, or both, as reported previously. We found that arsenic accelerates skin tumorigenesis, consistent with the previous report. Epidermis-specific FTO deletion inhibited skin tumorigenesis induced by UVB irradiation alone or the combination of UVB irradiation and arsenic, with a greater effect on the latter (Fig. 2e, f). In addition, skin-specific FTO deletion reduced epidermal hyperplasia induced by UVB irradiation alone or the combination of UVB irradiation and arsenic (Fig. 2g, h).

Histological analysis showed that the tumors developed in these groups are either papillomas or squamous cell carcinomas (SCC) (representative images shown in Supplementary Fig. 2h).

To determine the therapeutic potential by targeting FTO in As-T cells, we first tested the effect of inducible FTO knockdown using a doxycycline-inducible shRNA knockdown system. Inducible FTO knockdown (Fig. 2i) decreased cell growth (Supplementary Fig. 2i), tumor growth (Fig. 2j), and tumor weight (Supplementary Fig. 2j). To determine the translational potential of targeting FTO, we tested the effect of the FTO inhibitors CS1 and CS2 identified recently. CS1 and CS2 increased the m^6A level (Supplementary Fig. 2k), while it...
decreased cell growth (Supplementary Fig. 2l), tumor growth (Fig. 2k), and tumor weight (Fig. 2l), supporting the anti-tumor effects of these FTO inhibitors in As-T cells. Overall, these findings demonstrate that FTO is required for arsenic-induced malignant transformation and tumorigenesis and that targeting FTO genetically or pharmacologically inhibits the tumorigenicity of arsenic-treated cells.

m6A seq and RNA seq analyses identify NEDD4L as the critical target for arsenic-induced FTO upregulation. To determine the potential m6A targets of arsenic exposure, we used m6A-seq coupled with RNA-seq to determine the potential m6A demethylation gene targets across the whole transcriptome for arsenic. As compared with control cells, As-T cells showed decreased m6A enrichment in 5'UTR and 3'UTR (Fig. 3a) and altered total peak
distribution (Supplementary Fig. 3a). As-T cells also showed a decreased number of m6A genes compared with control cells (Supplementary Fig. 3b). Sequence analysis of m6A peaks showed the previously identified m6A target sites (GGACU)29,30 (Fig. 3b).

Next, we used the following criteria and analyses and identified NEDD4L (neural precursor cell-expressed developmentally downregulated 4) like as a potential m6A target of arsenic, m6A modification, and FTO: (1) decreased m6A enrichment, (2) altered mRNA level, (3) potential role in cancer, and (4) altered expression in both human and mouse SCC. We compared the genes with altered expression and m6A enrichment in our m6A seq and RNA seq datasets from control and As-T cells, with genes with altered expression from human and mouse skin cancers (squamous cell carcinoma, SCC) (GSE84292 and GSE84293) generated from the previous studies31. There were 40 genes altered among the four groups, including 10 downregulated genes (GSI, LPIN1, ME1, NCOA7, NEDD4L, PDLIM4, TRPV4, CLDN1, CRIP2, GAS6) and 3 upregulated genes (GINS4, RAD54L, SLC16A1) that shared changes in As-T, mouse and human cSCCs (Fig. 3c, and Supplementary Fig. 3c). Among all these genes, we elected to initially focus on NEDD4L, as it is found to be downregulated in several cancers32–35, suggesting a tumor-suppressive role for NEDD4L.

As and four As-T cells showed decreased mRNA and protein levels of NEDD4L as compared with control cells (Fig. 3d, e). m6A-seq analysis showed that As-T cells exhibited decreased m6A levels in the exon 1 (NC_000018.10:58221610-58221760) region of the NEDD4L isoform (id = NM_001144966.3) (Fig. 3f). Consistently, m6A IP qPCR analysis showed that in As-T cells the m6A level for the NEDD4L full-length transcript was reduced as compared with control cells (Fig. 3g), consistent with elevated FTO in these cells (Fig. 1d). To determine the m6A site at the NEDD4L transcript regulated by FTO, we performed the m6A IP qPCR analysis of m6A enrichment in the differentially methylated region of the NEDD4L transcript in control and As-T cells. As-T cells showed decreased m6A enrichment in the exon 1 (NC_000018.10:58221610-58221760) region of NEDD4L as compared with control cells (Fig. 3h). Furthermore, NEDD4L mRNA stability was decreased in As-T compared with control cells (Fig. 3i), suggesting that arsenic regulates NEDD4L RNA metabolism and decay. FTO deletion increased the m6A level and abundance of the NEDD4L transcript (Fig. 3j). m6A IP qPCR analysis of m6A enrichment showed that FTO deletion increased the m6A level in the exon 1 region of NEDD4L in As-T cells (Fig. 3k). Future investigation is needed to determine the importance of other potential m6A sites in the NEDD4L genes in arsenic tumorigenesis. Moreover, we found that FTO deletion increased the NEDD4L mRNA and protein levels and mRNA stability (Fig. 3l, m, and Supplementary Fig. 3d), suggesting that increased m6A enrichment on the NEDD4L transcript elevates its stability instead of leading to transcript decay36,37.

To determine whether FTO regulates NEDD4L mRNA m6A modification and expression through its demethylase activity, we tested the effect of the FTO demethylase-inactive mutants. In cells with FTO deletion, wild-type (WT) FTO decreased NEDD4L mRNA and protein levels, while FTO demethylase-inactive mutants had no effect (Fig. 3n, o). We also showed that the FTO inhibitors CS1 and CS2 increased the protein and mRNA levels of NEDD4L (Supplementary Fig. 3e, f), while it had no effect on the protein or mRNA levels of FTO, consistent with the previous studies38. These data strongly indicate that FTO upregulation by arsenic decreases the NEDD4L mRNA stability through its RNA demethylase activity.

Identification of NEDD4L as the functional target of FTO. Next, we investigated whether NEDD4L is responsible for FTO’s function in arsenic tumorigenicity. First, we assessed whether NEDD4L is negatively associated with FTO in arsenical keratoses, as compared with normal human skin. We found that FTO is upregulated, while NEDD4L is downregulated in arsenical keratoses as compared with normal skin (Fig. 4a, b). NEDD4L levels were negatively correlated with FTO levels (Fig. 4c).

Second, we analyzed the alteration of NEDD4L in arsenic-UVB-treated skin and tumors in mice and the effect of FTO deletion. We found FTO is upregulated by UVB alone, arsenic alone, or the combination of both (Fig. 4d and Supplementary Fig. 4a). Moreover, while NEDD4L was upregulated by UVB irradiation, it was downregulated by arsenic (Fig. 4e). Other mechanisms may mediate UVB-induced NEDD4L upregulation, which remains to be investigated in detail. Nevertheless, NEDD4L levels were negatively correlated with FTO levels (Fig. 4f). Furthermore, FTO deletion increased NEDD4L levels in skin and tumors from mice treated with both UVB irradiation and arsenic (Fig. 4g, h, and Supplementary Fig. 4b).

Third, we knocked down NEDD4L and observed that it partially reversed the effect of FTO deletion on cell proliferation, anchorage-independent growth, and cell survival when placed in suspension (Fig. 4i-l). Consistently, NEDD4L knockdown also partially reversed the effect of FTO deletion on tumor growth in vivo (Fig. 4m, n). These data indicate that NEDD4L is an important target of FTO, and other FTO targets may also play an important role in FTO’s function.

Next, we investigated the potential target of NEDD4L. NEDD4L is shown to act as a ubiquitin ligase controlling several pathways, including the WNT signaling by targeting Dvl238. As WNT signaling is one critical pathway in skin cancer39, we elected to test the role of the Wnt/β-catenin pathway in NEDD4L’s function. Using TOP Flash luciferase assay, we found that WNT signaling is increased in As-T cells as compared with control cells (Supplementary Fig. 4d). We observed that Dvl2, as well as β-catenin, a downstream target of WNT/Dvl240, is upregulated in As-T cells as compared with control cells.
FTO deletion decreased the levels of Dvl2 and β-catenin and WNT activity, which was prevented by NEDD4L knockdown (Supplementary Fig. 4e-f). Future investigation is needed to elucidate the role of other pathways, including the TGF-β and PI3K/AKT pathways, in the function of NEDD4L in skin cancer. These results demonstrate that NEDD4L is a critical downstream target of FTO responsible for its function in arsenic-induced malignant transformation.

FTO regulates NEDD4L mRNA stability through m6A and IGF2BPs. To determine whether FTO regulates NEDD4L through m6A, we assessed the effect of manipulating the m6A RNA methyltransferase factors METTL14 and METTL3. METTL14 knockdown at least partially reversed the effect of FTO deletion on NEDD4L mRNA stability, mRNA level, and protein level (Fig. 5a, b, and Supplementary Fig. 5a). Furthermore, METTL14 knockdown partially reversed the effect of FTO deletion on tumor growth (Fig. 5c and Supplementary Fig. 5b). Similarly, METTL3 knockdown also partially reversed the effect of FTO deletion on cell growth (Fig. 5d and Supplementary Fig. 5c), and anchorage-independent growth (Fig. 5e). Over-expression of wild-type METTL3, but not the catalytically dead
Fig. 3 Identification of the potential target of arsenic-induced FTO upregulation via transcriptome-wide m6A-seq and RNA-seq analyses. a Distribution of m6A peaks across the length of mRNA in control and As-T cells. b Venn diagram showing the overlap between genes with altered expression or m6A enrichment in four datasets: m6A-seq (unique m6A peak in control/As-T), RNA seq (As-T/control), RNA seq [Mouse cuSCC/chronically irradiated skin (CHI)], and RNA seq [Human cuSCC/normal skin (NS)]. NEDD4L is found in all groups. c Immuno blot analysis of FTO and GAPDH in control, As, and four As-T cells. d qPCR analysis of NEDD4L mRNA levels in d (n = 3). e Distribution of m6A peaks in ‘Exon 1 (NC_000018.10:58221610-58221760)’ of the NEDD4L transcript in control and As-T cells. g m6A IP qPCR analysis of m6A enrichment across the NEDD4L transcript in control and As-T cells (n = 3). h m6A IP qPCR analysis of m6A enrichment in the differentially methylated region of the NEDD4L transcript (NC_000018.10:58221610-58221760) in control and As-T cells (n = 3). i qPCR analysis of NEDD4L mRNA stability following treatment with actinomycin. D (ActD, 2 μM) in control and As-T cells (n = 3). j m6A IP qPCR analysis of m6A enrichment across the NEDD4L transcript in As-T cells with or without FTO deletion (n = 3). k m6A IP qPCR analysis of m6A enrichment in the differentially methylated region of the NEDD4L transcript (NC_000018.10:58221610-58221760) in wild-type (WT) and FTO KO cells (n = 3). l qPCR analysis of NEDD4L mRNA level in cells with or without FTO deletion (n = 3). m qPCR analysis of NEDD4L mRNA stability following treatment with ActD in As-T cells with or without FTO deletion (n = 3). n Immunoblot analysis of NEDD4L and FTO in FTO-KO cells transfected with wild-type (WT) or demethylase-inactive mutant FTO (Mutant 1, H231A/D233A; Mutant 2, R316Q/R322Q). o qPCR analysis of NEDD4L mRNA levels in cells as in n (n = 3). All data were performed on n = 3 biologically independent samples. Error bars are shown as mean ± S.D. e, g, m, o p-values by two-tailed unpaired t-tests are indicated (e, g, m, o).

As increases the FTO stability through impairing autophagy. To determine the mechanism by which arsenic upregulates FTO, we first assessed whether arsenic affects FTO mRNA levels. As compared with control cells, FTO mRNA levels remained unchanged in As and As-T cells (Fig. 6a), indicating that arsenic did not alter FTO mRNA abundance. Next, we assessed the effect of arsenic on FTO protein stability by treating the cells with cycloheximide (CHX) over a time course. As compared with control cells, FTO protein stability was increased in As-T cells (Fig. 6b, c), indicating that arsenic increased FTO protein stability. Next, we assessed the role of FTO protein degradation through either the proteasome or the autophagic-lysosomal pathways, two critical protein degradation pathways. When the proteasome was inhibited by MG132, control cells showed FTO protein accumulation, similar to As-T cells (Supplementary Fig. 6a, b). In contrast, when the lysosome was inhibited by the lysosome inhibitor bafilomycin A1 (BfnA1), control cells showed significantly more FTO protein accumulation than As-T cells did (Fig. 6d, e). In addition, As-T cells showed reduced autophagy, as indicated by decreased accumulation of LC3-II, the surrogate marker for autophagosome accumulation, when lysosome was inhibited (Fig. 6d). To further determine the role of autophagy in FTO stability, we assessed the effect of genetic inhibition of autophagy. Deletion of the essential autophagy genes ATG5 or ATG7 increased FTO protein stability as indicated by the increased half-life (Fig. 6f, g, and Supplementary Fig. 6c). These findings suggest that arsenic mimics the effect of autophagy inhibition in stabilizing FTO.

Next, we used the PLA assay to assess the plausible interaction between FTO and autophagosomes. We first validated the specificity of the PLA assay for FTO and LC3 (Supplementary Fig. 6d-f). Treatment with BfnA1 increased the interaction between FTO and LC3 (Fig. 6h, i). This was further confirmed by the co-localization of FTO and LC3 by immunofluorescence staining (Fig. 6j). It is noteworthy that FTO was localized mainly in the nucleus, consistent with the previous studies; lysosome inhibition seemed to increase the FTO level in both the nucleus and the cytosol (Fig. 6j). Gene set enrichment analysis (GSEA) detected downregulation of both ‘Lysosome’ and ‘Phagosome’ pathway-related genes in mouse cuSCCs as compared with non-tumor skin (Fig. 6k, l). The downregulated genes were shown in the heatmap (Supplementary Fig. 6g, h). Moreover, we also found that ‘Autophagy-animal’, ‘Lysosome’, and ‘Phagosome’ pathway-related genes are downregulated in As-T cells compared to control cells (Supplementary Fig. 10a, b). Electron microscopy analysis showed that the number of autophagic vacuoles is decreased in As-T cells as compared with control cells (Fig. 6m, n), further supporting the suppression of autophagy by arsenic.

To determine the mechanism by which arsenic inhibits autophagy, we first assessed the role of Metal-activated transcription factor 1 (MTF1), a multipotent regulator of transcription often involved in the adaptation to stress, including arsenic treatment.53,54 When the proteasome was inhibited by MG132, control cells showed FTO protein accumulation, similar to As-T cells (Supplementary Fig. 6a, b). In contrast, when the lysosome was inhibited by the lysosome inhibitor bafilomycin A1 (BfnA1), control cells showed significantly more FTO protein accumulation than As-T cells did (Fig. 6d, e). In addition, As-T cells showed reduced autophagy, as indicated by decreased accumulation of LC3-II, the surrogate marker for autophagosome accumulation, when lysosome was inhibited (Fig. 6d). To further determine the role of autophagy in FTO stability, we assessed the effect of genetic inhibition of autophagy. Deletion of the essential autophagy genes ATG5 or ATG7 increased FTO protein stability as indicated by the increased half-life (Fig. 6f, g, and Supplementary Fig. 6c). These findings suggest that arsenic mimics the effect of autophagy inhibition in stabilizing FTO.

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To determine the mechanism by which arsenic inhibits autophagy, we first assessed the role of Metal-activated transcription factor 1 (MTF1), a multipotent regulator of transcription often involved in the adaptation to stress, including arsenic treatment.53,54 While low-dose acute or chronic arsenic treatment did not induce MTF1, acute high-dose arsenic treatment did increase the MTF1 level (Supplementary Fig. 6i-l). However, MTF1 knockdown had no effect on the FTO level (Supplementary Fig. 6m), suggesting that MTF1 is not a major player in chronic low-level arsenic damage. Future studies are required to assess the role of MTF1 in arsenic tumorigenicity in detail. Taken together, these findings demonstrate that arsenic stabilizes FTO protein by inhibiting autophagic degradation.
Next, we assessed whether p62 regulates FTO protein stability. Indeed, overexpression of p62 reduced the FTO protein level, whereas p62 knockdown increased it (Fig. 7d and Supplementary Fig. 7a). Moreover, p62 inhibition increased FTO protein stability in both HaCaT and MEF cells (Supplementary Fig. 7b–e), whereas overexpression of p62 deceased it in As-T cells that expressed low levels of p62 (Fig. 7e, f). Correspondingly, overexpression of p62 increased NEDD4L mRNA levels in WT cells but not in cells with FTO deletion (Fig. 7g and Supplementary Fig. 7f), indicating that p62 regulates NEDD4L expression through FTO.

Next, we assessed whether FTO interacts with p62. First, we observed that the co-localization of FTO and p62 is increased by...
deletion of ATG7 or by the lysosome inhibitor BfnA1 in MEF cells (Supplementary Fig. 7g). PLA assays supported the co-localization of p62 and FTO in HaCaT cells (Supplementary Fig. 7h, i). Co-immunoprecipitation analysis showed that FTO binds with p62 (Fig. 7h). As an autophagy receptor, p62 can interact with polyubiquitin chains or its ubiquitinated protein substrates through its UBA domain and with LC3/GABARAP proteins noncovalently via a short linear sequence known as LIR to deliver its substrates to autophagosomes and then lysosomes for degradation. Indeed, we found that FTO is ubiquitinated in As-T cells (Fig. 7i). Moreover, deletion of the UBA domain of p62 abolished the FTO-p62 interaction (Fig. 7j, k), indicating that FTO-p62 interaction requires p62’s UBA domain. Furthermore, overexpression of wild-type (WT) p62, but not the LIR-binding deficient mutant (W338A), reduced the FTO-p62 interaction (Fig. 7j, l). 

Immuine electron microscopy analysis showed that FTO colocalizes with the p62 cluster in the cytoplasm (Fig. 7m). These p62-positive structures are shown to be liquid droplets formed by liquid-liquid phase separation and degraded by autophagy, rather than monomeric or oligomerized p62. It is possible that FTO is translocated into the p62 bodies depending on the context and degraded by autophagy. Lastly, as compared with normal human skin, human arsenical keratosis showed decreased p62 levels (Fig. 7n and Supplementary Fig. 7j). In addition, we found that p62 is negatively correlated with FTO (Fig. 7o).

Next, we assessed the role of p62 in FTO localization in the autophagosomes. Overexpression of p62 increased autophagy in p62-low As-T cells (Supplementary Fig. 8a). In addition, it also increased the co-localization of FTO with LC3 (Supplementary Fig. 8b, c) in As-T cells, whereas p62 deletion decreased it (Supplementary Fig. 8d-h) in MEF cells. Notably, p62 deletion markedly decreased but did not completely abolish, the FTO-LC3 PLA signal, suggesting that while p62 is a crucial autophagy receptor for FTO colocalization with LC3, other autophagy factors may also play a role. Immunofluorescence analysis further confirmed that p62 deletion reduced the co-localization of FTO with LC3 (Supplementary Fig. 8i). Our data indicate that p62 is necessary and sufficient for FTO localization in the autophagosomes.

Furthermore, we showed that FTO also regulates autophagy in response to arsenic as a positive feedback signaling system. We found that FTO deletion in As-T cells increases autophagy, as shown by p62 accumulation and formation of LC3-II upon BfnA1 treatment (Supplementary Fig. 9a). FTO deletion decreased p62 mRNA levels, consistent with decreased p62 protein levels in FTO-depleted cells under homeostatic conditions (Supplementary Fig. 9a, b). In addition, we found that FTO deletion increased the phosphorylation of AMPK, decreased phosphorylation of the mTOR substrate p70S6K, and increased protein levels of ATG5 and ATG7, as well as increasing the LC3-II level, under serum starvation conditions that can induce autophagy (Supplementary Fig. 9c).

Next, we assessed the role of TFEB, a transcription factor that controls transcription of CLEAR and autophagy genes and is regulated by phosphorylation. We found that, as compared with control cells, As-T cells showed reduced levels of TFEB protein and phosphorylation levels (S211), but not the TFEB mRNA level (Supplementary Fig. 9d, e). It is possible that chronic arsenic treatment decreases either translation or protein stability of TFEB and that the decreased TFEB phosphorylation is due to the decreased TFEB abundance, which requires further investigation. FTO deletion decreased phosphorylation of TFEB at S122 and the expression of ATG5 and ATG7 but not p62 mRNA (Supplementary Fig. 9f-h), possibly due to the reduced mTOR signaling (Supplementary Fig. 9c). These findings suggest that TFEB has a complex role in arsenic damage response and FTO function, which requires further investigation. Our results suggest a positive feedback loop between FTO and autophagy dysregulation, leading to self-sustained maintenance of FTO upregulation in arsenic-induced malignant transformation and tumorigenesis. Taken together, these findings demonstrate that p62 binds with FTO to mediates FTO degradation, and that arsenic inhibits FTO degradation by suppressing p62 expression.

Arsenic downregulates p62 expression by inhibiting the TNF/NF-xB pathway. To determine how arsenic downregulates p62 expression, we performed pathway analysis to determine which shared signaling pathways were downregulated in As and As-T cells as compared with control cells using Metascape. Among many pathways downregulated by arsenic, the cytokine production and cytokine signaling pathways, in particular, were downregulated in As and As-T cells as compared with control cells (Fig. 8a). Moreover, KEGG network analysis showed that ‘Autophagy-animal’, ‘Phagosome’, ‘Lysosome’, and ‘NF-xB and TNF signaling pathway’ was downregulated in As-T cells as compared with control cells (Supplementary Fig. 10a, b). GSEA analysis showed that TNF-signaling genes were downregulated in As-T cells as compared with control cells, and in mouse SCC as compared with non-tumor skin (Fig. 8b and Supplementary Fig. 10c-g). qPCR analysis showed that several TNF-related genes (NFKB1, TNF, TNFRSF1A, TNFAIP3) and autophagy-related genes (ATG5, ATG7, and p62) were decreased in arsenic-treated cells as compared with control cells (Fig. 8c).

It is known that p62 expression can be induced by NF-xB activation. Previous studies have shown that in myometrial cells the known NF-xB-inducing cytokine TNFα induces p62 expression. Therefore, we hypothesized that arsenic downregulates p62 expression through downregulating signaling of cytokines such as TNFα. Indeed, NF-xB activity was decreased in...
arsenic-treated cells (As and As-T cells) as compared with control cells (Fig. 8d), while it was increased after TNFα treatment (Fig. 8e). TNFα treatment increased the protein and mRNA levels of p62 and NEDD4L, and the protein levels of ATG5 and ATG7, while it decreased FTO protein level (Fig. 8f, g). Knocking down p62 or RELA, also known as p65 as part of the NF-κB heterodimer, prevented the effect of TNFα (Fig. 8h, i). A PLA assay showed that TNFα increased the colocalization of FTO with either p62 or LC3, which was prevented by knocking down either p62 or RELA (Fig. 8j, k, and Supplementary Fig. 10h, i).
Previous studies showed that Nrf2 and TFEB also regulate p62 expression. However, we found that in HaCaT cells knockdown of either Nrf2 or TFEB had no effect on FTO levels, although it had a small to moderate effect on the p62 levels (Supplementary Fig. 10j–l). Future studies are needed to assess the role of Nrf2 and TEFB in arsenic-regulation of p62 in greater detail. Taken together, our findings demonstrate that arsenic suppresses p62 expression by downregulating the NF-κB pathway to upregulate FTO.

**Discussion**

RNA m6A modifications are shown to play important roles in cancer development and therapeutic response. However, the role...
of m6A in tumorigenesis caused by environmental carcinogens such as arsenic remains unknown. Here we discovered that chronic low-level arsenic exposure upregulates FTO stability and downregulates m6A enrichment in keratinocytes, leading to malignant transformation and tumorigenesis. At the molecular level, arsenic inhibits FTO protein degradation via impairing p62-dependent selective autophagy. FTO interacts with p62 in the autophagosomes, and p62 is required for autophagic degradation of FTO. Furthermore, arsenic inhibits p62 expression by suppressing the TNFα/NF-κB signaling pathway. We identified NEDD4L as a critical downstream target of FTO in arsenic tumorigenicity. NEDD4L RNA stability is regulated by m6A modification, through the m6A readers IGF2BPs and FTO. Furthermore, FTO and autophagy dysfunction form a positive feedback loop to maintain the upregulation of FTO. Taken together, these results establish RNA modification and FTO as an epitranscriptomic mechanism in arsenic-induced tumorigenesis (Supplementary Fig. 11).

We found that FTO is upregulated and m6A enrichment is downregulated by chronic exposure to relevant low levels of arsenic. The downregulation of FTO induced by arsenic seems to depend on the dose of arsenic since only low-level arsenic upregulates FTO (Supplementary Fig. 1d). However, high-level arsenic downregulates FTO, similar to previous reports.2,7,22 Such FTO upregulation is required for arsenic-induced tumorigenesis, as FTO deletion diminished arsenic-induced tumorigenesis in both a xenograft tumor model and an arsenic-UVB co-carcinogenesis model. FTO’s pro-tumorigenic role in arsenic damage response is mediated through m6A RNA methylation, as knockdown of METTL14/METTL3 reversed the effect of FTO deletion in vivo and in vitro. Furthermore, the FTO level is higher, while the m6A level is lower in human arsenical keratosis, a premalignant lesion induced by arsenic than in normal human skin. These findings provide an epitranscriptomic mechanism by which arsenic upregulates the RNA demethylase FTO to promote tumorigenesis.

Emerging evidence suggests that FTO and m6A RNA methylation regulate their functions in cancer and other diseases through different downstream gene targets depending on the context and cell lineage.6,7 For example, in leukemia, Li and colleagues have shown that FTO promotes tumorigenesis by suppressing the expression of ASB2 and RARA genes.10 In melanoma, we have recently shown that FTO promotes the mRNA stability of the melanoma-promoting genes PDCD1, CXCR4, and SOX10.11 Using m6A IP seq followed by qPCR validation, we found that FTO reduces the m6A levels of the NEDD4L transcript, leading to its increased decay.

NEDD4L belongs to the NEDD4 family of the E3 HECT domain ubiquitin ligases and has been suggested to regulate several signaling pathways16,41,42, thus acting as a tumor suppressor in several cancers.12-35 We found that knockdown of NEDD4L reversed the effect of FTO knockout on arsenic-induced tumorigenesis, establishing that NEDD4L is a critical mRNA target for FTO. However, in wild-type cells, knockdown of NEDD4L had no effect, suggesting that NEDD4L alone is dispensable for arsenic-induced tumorigenesis when FTO is intact to inhibit NEDD4L. In contrast to melanoma in which m6A decreases the RNA stability of melanoma-promoting gene transcripts,13 in arsenic-induced tumor cells, we found that m6A increases the RNA stability of NEDD4L through the readers IGF2BP1-3. Previously IGF2BP1-3 proteins have been shown to be a distinct family of m6A genes that bind to and promote the stability and translation of m6A-modified transcripts.37 We found that knockdown of IGF2BP3 increases the NEDD4L decay in FTO knockout cells and reverses the effect of FTO. Future investigations are needed to determine whether IGF2BP3 also play active roles in arsenic damage response and tumorigenicity and whether the FTO/IGF2BP3 axis also regulates NEDD4L translation.

We found that FTO regulates the Dvl2 level through inhibiting NEDD4L, resulting in increased Wnt signaling, suggesting Wnt signaling as a downstream target for the FTO/NEDD4L axis. Taken together, our findings clearly demonstrate that FTO promotes arsenic-induced tumorigenesis through m6A RNA methylation and NEDD4L.

Intriguingly we found that chronic arsenic exposure stabilizes FTO protein by compromising its autophagic degradation. From RNA-seq analysis, arsenic suppressed the expression of several autophagy genes, including the autophagy receptor p62. Arsenic inhibited autophagy by suppressing p62 expression, as adding p62 reversed the effect of arsenic. In addition, we found that FTO degradation by autophagy is mediated by p62. FTO binds with p62’s UBA domain and colocalizes in the LC3-positive autophagosomes in a p62-dependent manner. p62 knockout or knockdown increases FTO protein stability, while p62 overexpression decreases it. It appears that arsenic downregulates cytokine signaling pathways, including the TNFα pathway. p62 expression is shown to be regulated by several signaling pathways and transcription factors such as NF-κB,68 a major downstream pathway for cytokine signaling. Treatment with TNFα increases NF-κB activity, leading to increased p62 expression and decreased FTO levels. In addition, the roles of other autophagy pathways in FTO degradation will require additional investigation.

Our findings showed that chronic low-level arsenic exposure inhibits p62 expression, which at least partially leads to compromised autophagy and autophagy-mediated FTO degradation, and thus facilitates arsenic tumorigenesis. Other autophagy genes downregulated by arsenic may also play important roles in autophagy inhibition by arsenic, which needs further investigation. Our findings seem to be specific for arsenic, as previous studies have shown that multiple types of tumors exhibit elevated basal autophagy73,74, as well as p62 accumulation,79, to promote tumorigenesis and tumor progression.70,75,76 In addition, it seems that the effect of toxic metals on autophagy is also dependent on...
context, including the specific metal, cell type, concentrations of metals, and treatment duration. We found that in HaCaT cells, cadmium has no effect on FTO levels up to 72 h (Supplementary Fig. 1f). These discrepancies could be due to the specific mechanism of action for arsenic-induced tumorigenesis and suggest a context-dependent role for FTO, as well as p62 and autophagy. The regulatory and functional roles of carcinogenic metals in autophagy warrant further investigation, in particular in the context of clinically relevant low-level chronic exposure.

Moreover, we found that FTO inhibits autophagy. FTO deletion increased the expression of autophagy genes including ATG5 and ATG7, increased phosphorylation of AMPK, and decreased phosphorylation of the mTOR target p70S6K and TFEB, pathways that regulate autophagy, and enhanced autophagy. This is consistent with previous reports in which FTO increases activation of the mTORC1 pathway and thus inhibits autophagy. However, our findings are opposite to those reported in preadipocytes, in which FTO knockdown decreases the expression of ATG5 and ATG7 and reduces autophagy.

These discrepancies could be due to the cell-type specific or context-dependent role of FTO in autophagy, which warrants future investigation. Nevertheless, our findings demonstrate that...
FTO and autophagy inhibition form a positive feedback loop, leading to self-sustained FTO upregulation and malignant traits in arsenic-induced tumorigenic cells. In summary, we demonstrate that arsenic promotes FTO protein stability by impairing selective autophagy and thus induces malignant transformation and tumorigenesis. FTO decreases m^6^A modification and stability of its functional downstream target NEDD4L mRNA. Our results provide an epitranscriptomic molecular axis as the post-transcriptional mechanism in arsenic-induced tumorigenesis, and thus may help the development of new mechanism-based prevention and therapy by targeting FTO and m^6^A RNA methylation to reduce the skin cancer burden in arsenic-exposed individuals worldwide.

### Methods

#### Cell culture

HaCaT cells, MEF (mouse embryonic fibroblasts) cell line, HaCat (human keratinocyte), kindly provided by Dr. (Fusenberg), and HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco), 100U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). Normal Human Epidermal Keratinocyte (NHEK) cells were purchased from Lonza and maintained in KGM Gold keratinocyte growth basal medium (Lonza, #00192151) and KGM Gold keratinocyte growth medium supplements and growth factors (Lonza, # 00192152). WT and ATG5 KO MEF cells were kindly provided by Dr. Noboru Mizushima. ATG7 KO MEF cells were generously provided by Dr. Masaki Komatsu.

**Lentiviral generation and infection.** Lentivirus was produced by co-transfection into HEK-293T (human embryonic kidney) cells with lentiviral constructs together with the pCMVdeltag2.8 packaging plasmid and pSV-G envelope plasmid using X-tremeGENE 9. Virus-containing supernatants were collected at 24–48 h. Target cells were infected in the presence of Polybrene (8 μg/mL) (Sigma-Aldrich, St. Louis, MO) and selected with puromycin (Santa Cruz Biotechnology, Santa Cruz, CA) at 48 h. Target cells were transfected with lentiviral constructs together with empty vector (EV) or p62 (HA tag). Immunoblot analysis of FTO and HA (p62) in As-T cells as in treated with CHX over a time course. Quantification of FTO and p62 KO MEF cells were generously provided by Dr. Masaaki Komatsu. ATG5 KO MEF cells were kindly provided by Dr. Noboru Mizushima. ATG7 KO MEF cells were generously provided by Dr. Xiaoyang Wu (University of Chicago). The guide RNA sequences were FTO KO-1: GAAGGGACACCGGACTGCC; FTO KO-2: ACGGTGCTCGTGGCCAGTTGA. The Tet-On inducible FTO knockdown vectors were kindly provided by Dr. Hui-Lung Sun (University of Chicago), generated by cloning two independent shRNAs targeting FTO: shFTO-1 (shRNA TRCN0000256490 CGGTCCGCAACAGCCTGTGATCGT) and shFTO-2 (shRNA TRCN0000256504, TCTTGCATCTCATTGGTAAT) into a Tet-On doxycycline-inducible lentiviral expression vector (Addgene Plasmid #21915, kindly provided by Dmitrii Wiederschain).

**siRNA transfection.** Cells were transfected with siRNA targeting negative control (siNC) (Dharmacon, Lafayette, CO), or an individual gene or the combination of IGF2BP1, IGF2BP2, IGF2BP3, MTF1, TFE, Nof2 using GenMute® siRNA Transfection Reagent (Signagen, Ijamsville, MD) according to the manufacturer’s instructions.

**Quantitative real-time PCR (qPCR).** Quantitative real-time PCR assays were performed using a CFX Connect real-time system (Bio-Rad, Hercules, CA) with Bio-Rad iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The threshold cycle number (CQ) for each sample was determined in triplicate. The CQ values for FTO, NEDD4L, ATG5, OPTN, p62, LAMP1, IGF2BP1, IGF2BP2, IGF2BP3, ATG7, NFKB1, TNF, TNFRSF1A, and TNFAIP3 were normalized against GAPDH. Primers are shown in Supplementary Table 1.

**Luciferase reporter assay.** Cells were transfected with 1 μg of TOPFLASH/FOP FLASH or NF-κB-Luc constructs in combination with 0.025 μg of pRL-TK, a transfection efficiency control (Promega, Madison, WI), using GenJet™ Plus DNA in vitro transfection reagent (Signagen, Ijamsville, MD) according to the manufacturer’s instructions. Luciferase reporter assays (Promega) were carried out according to the manufacturer’s instructions.

**m^6^A dot blot assay.** Total RNA was isolated with RNeasy plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA samples were loaded onto a Hybond-N+ membrane (ECL Healthcare, Chicago, IL) and UV cross-linked twice to the membrane. After it was dry, the membrane was blocked with 5% BSA (in 1X PBS) for 1 h and incubated with a specific anti-m^6^A antibody (Synaptic Systems, Goettingen, Germany) overnight at 4°C. Next, the membrane was incubated with the HRP-conjugated anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature, and then developed with Thermo ECL Supersignal Western Blotting Detection Reagent (Thermo Fisher Scientific, Waltham, MA).

**Gene-specific m^6^A qPCR.** Total RNA was immunoprecipitated with an anti-m^6^A antibody followed by qPCR analysis. Primers for full-length mRNA qPCR are listed in the qPCR section. For region-specific m^6^A qPCR of NEDD4L, fragmented IP RNA and input RNA samples were prepared as described in the m^6^A IP Seq section and used as RT-qPCR templates for qPCR analysis. Target-specific primers for the m^6^A methylated region in NEDD4L (exon 1; NC_000018.10:521610-58221670) were designed by using Primer-BLAST® forward, 5’-ATACAGCCA GTGTTGACCGG-3’, and reverse, 5’-CAGTGGTGCCGCTTCTACCT-3’.

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Fig. 7 p62 is downregulated by arsenic and regulates FTO stability. a Heatmap showing altered expression of the genes in autophagy/lysosome pathways between As-T and control cells. b qPCR analysis of OPTN, p62, ATG5, and LAMPI levels in control, As, and As-T cells (n = 3). c Immunoblot analysis of FTO in control, As, and four As-T cells. d Immunoblot analysis of HA, NEDD4L, and FTO in As-T cells transfected with empty vector (EV) or p62 (HA tag). e Immunoblot analysis of FTO and HA (p62) in As-T cells as in treated with CHX over a time course. Quantification of FTO in control, As, and four As-T cells transfected with empty vector (EV) or p62 (HA tag). f Immunoblot analysis of FTO and HA (p62) following immunoprecipitation using species-matched control IgG and an anti-p62 antibody in HaCaT cells. The immunoblot analysis of FTO and HA (p62) in As-T cells transfected with plasmids expressing WT and ΔUBA p62. g Immunoblot analysis of Flag-FTO and HA-p62 (WT or ΔUBA) following immunoprecipitation assay of the binding of Flag-FTO with HA-p62 WT or HA-p62 ΔUBA in HaLa cells transfected with Flag-EV (empty vector), Flag-FTO, HA-p62 WT, or HA-p62 ΔUBA. h Immunoblot analysis of FTO and HA in As-T cells transfected with constructs expressing WT p62 or LIR (W338A) mutant p62 (HA). i Representative images of immune electron microscopy (IEM) analysis for p62 and FTO in control cells. The upper panel shows the negative controls without primary antibodies. The bottom panel shows IEM staining of FTO (10 nm) and p62 (15 nm). j Quantification of the p62 levels in normal human skin (n = 6) and arsenical keratones (n = 3). Related to Supplementary Fig. 7j. a.u.: arbitrary units.
Cells in a 15 cm dish at 70–80% confluence were cross-linked twice with UVC radiation (254 nm, 150 mJ/cm²) using Stratalinker on ice. Cells were harvested and lysed, followed by RNase T1 digestion. A 1:1:1 mixture of IGF2BP1-3 antibodies and the corresponding IgG control antibody were conjugated to 1:1 mixed Dynabeads Protein A/G by incubation for 4 h at 4 °C, followed by 3× washing and incubation with pre-cleared cell lysate in RIPA buffer overnight at 4 °C. The beads conjugated with RNA-protein complex were washed and then subjected to digestion by DNase and Proteinase K. Input and co-immunoprecipitated RNAs were recovered by TRIzol (Invitrogen) extraction and used as RT-qPCR templates for qPCR analysis.

Immunoblotting. Protein extracts were obtained by washing the cells once with PBS and resuspending in RIPA buffer containing inhibitors for proteases and phosphatases, then sonicated. After quantifying protein concentrations through BCA assay, samples were heated for 10 min at 70 °C. Protein abundance was
Fig. 8 p62 is downregulated by arsenic through TNFR/NF-κB signaling and regulates FTO stability. a GO pathway analysis of downregulated genes shared in both As and As-T cells as compared with control cells. b GSEA of "TNF signaling pathway"-related genes in As-T cells versus control cells. ES: Enrichment score. NES: Normalized enrichment scores, p-value: FDR. False discovery rate. c qPCR analysis of NFκB, TNF, TNFRSF1A, TNFRSF13B, ATG5, ATG7, and p62 in control, As, and four As-T cells (n = 3). d Luciferase reporter analysis of NFκB activity in control, As, and four As-T cells (n = 3). e Luciferase reporter analysis of NFκB activity in As-T cells treated with or without TNFx for 10 h and 50 ng/ml for 20 h (n = 3). f Immunoblot analysis of NEDD4L, ATG5, ATG7, FTO, and p62 in As-T cells treated with or without TNFα for 20 h. g qPCR analysis of NFκB, p62, and NEDD4L mRNA levels in cells as if n (n = 3). h Immunoblot analysis of FTO, RELA, p62, and NEDD4L in As-T cells treated with or without TNF (50 ng/ml) following transfection with or without siRNA targeting p62 or RELA. qPCR analysis of p62 and NEDD4L mRNA levels in cells as if n (n = 3). j Proximity ligation assay (PLA) of the interaction between FTO and p62 in cells as if n. DAPI is used as a nuclear counterstain (blue). All samples are pretreated with BlnA1 for 6 h. Scale bar: 20 μm. k Quantification of the number of FLA red dots per cell in j (n = 30 cells from three biologically independent replicates). All data were performed on n ≥ 3 biologically independent samples. Error bars are shown as mean ± S.D. (c-e, g, i, k). p-values of all data by two-tailed unpaired t-test are indicated.

** Immunoblot analysis of FTO, RELA, p62, and NEDD4L in As-T cells treated with or without TNF (50 ng/ml) following transfection with or without siRNA targeting p62 or RELA. qPCR analysis of p62 and NEDD4L mRNA levels in cells as if n (n = 3). j Proximity ligation assay (PLA) of the interaction between FTO and p62 in cells as if n. DAPI is used as a nuclear counterstain (blue). All samples are pretreated with BlnA1 for 6 h. Scale bar: 20 μm. k Quantification of the number of FLA red dots per cell in j (n = 30 cells from three biologically independent replicates). All data were performed on n ≥ 3 biologically independent samples. Error bars are shown as mean ± S.D. (c-e, g, i, k). p-values of all data by two-tailed unpaired t-test are indicated.
w mice. When tissue size reached about 50 mm³, mice were treated with vehicle or 1 mg/ml Doxycycline (MP Biomedicals, Cat. ICN19895501) in acidified drinking water containing 5% sucrose (Sigma, S9324). Tumor volume was monitored and measured by a caliper, and tumor volume was calculated using the formula: tumor volume (mm³) = d² × 0.5/2, where d and D are the shortest and the longest diameters, respectively.

**Sphere formation assay.** Cells were first harvested in 10% FBS medium and passed through a 40 μm nylon mesh (Fisher Scientific, #19247). Then, cells were resuspended in a sphere medium (1–2 mL). Cells were next plated in 6-well ultra-low-attachment plates (Corning, 3471) at a density of 1,000 cells per well in culture medium DMEM/F12 (1:1) (In VitroGen) with 2% B27 serum-free supplement (17504-044, Invitrogen, Frederick, MD), 20 μg/ml EGF (E9644, Sigma, St. Louis), 0.4% bovine serum albumin (B4276, Sigma), 4 μg/ml insulin (Sigma, St. Louis, MO, #19278), and antibiotics, and cultured for 7–14 days at 37 °C with 5% CO₂. The number of tumorspheres per well was measured on day 7 [12,13].

**Immunofluorescence.** Cells were first fixed with 4% paraformaldehyde/PBS for 25 min and permeabilized in 0.5% (v/v) Triton X-100 in PBS for 20 min at 4 °C. Then, cells were washed with PBS. PBS supplemented with 5% normal goat serum (Invitrogen, Carlsbad, California) was used as a blocking solution for 60 min at room temperature. After removal of the blocking solution, cells were incubated at 4 °C overnight with the following primary antibodies prepared in 1× PBS/1%BSA: anti-LC3B (Abcam, ab192890, 1:200), and anti-p62 (Sigma-Aldrich, P0067, 1:400). Next, cells were washed with PBS, incubated at 37 °C for 1 h in fluorescence-activated secondary antibody (Jackson ImmunoResearch Lab, 715-545-150; Jackson ImmunoResearch Lab, 711-585-152; 1:200) prepared in 1× PBS/1%BSA, followed by washing with PBS three times in the dark. Then, cells were fixed in Prolong Gold Antifade with DAPI (Invitrogen) to visualize the cell nuclei and observed under a fluorescence microscope (Olympus IX71) [14].

For tissue IF staining, formalin-fixed, paraffin-embedded tissue sections were pre-treated by antigen retrieval and incubated with blocking solution with 3% albumin from chicken egg white (Sigma-Aldrich, A9503) in PBS after antigen retrieval. For RNA-seq mRNA staining, DNA on the tissue slide was removed by DNase treatment. Tissue sections were incubated at 4 °C with primary anti-FTO (Abcam, ab92821, 1:200), and anti-p62 (Sigma-Aldrich, P0067, 1:400), anti-NEDD4L (Cell signaling, #4013, 1:100), anti-p62 (Sigma, P0067, 1:100), anti-m6A (Synaptic Systems 1: 200), and anti-cytokeratin (ORIGENE, BP5069, 1:200) antibodies. After removing the primary antibodies, slides were washed with PBS solution with 0.1% Triton X-100 (Sigma-Aldrich, T8778). Slides were then incubated at room temperature with Alexa Fluor 594-conjugated secondary antibody rabbit IgG (Jackson ImmunoResearch, 715–585–152, 1:100), Alexa Fluor 488-conjugated secondary mouse IgG (Jackson ImmunoResearch, 715–545–150, 1:100), or DyLight 405 AffiniPure secondary Guinea Pig IgG (Jackson ImmunoResearch, 706–475–148, 1:200) for 1 h, then washed 3 times with PBS solution with 0.1% Triton-X100. Slides were mounted with Fluoromount (Jackson ImmunoResearch, F4680). Stained slide samples were analyzed using a fluorescence microscope (Olympus IX71, Olympus Life Science, Japan). For statistical analysis by ImageJ, five areas of the same size were randomly selected from each sample.

**In situ proximity ligation (PLA) assay.** PLA assay was performed according to the manufacturer’s instructions (Sigma). Briefly, cells were first fixed with 4% paraformaldehyde/PBS and then permeabilized in 0.5%(v/v) Triton X-100. After blocking with 5% normal goat serum (Invitrogen, Carlsbad, California), cells were incubated overnight with the following primary antibodies at 4 °C in 1×PBS/1%BSA: anti-p62 (Rabbit) (Sigma-Aldrich, P0067, 1:400), anti-LC3B (rabbit) (Abcam, ab92890, 1:200), and anti-FTO (mouse) (Abcam, ab92821, 1:200). In situ PLA detection was performed using a Duolink Detection Kit (Sigma-Aldrich) with a pair of nucleotide-labeled secondary antibodies. After ligation and amplification of PLA probes, signals were examined using a fluorescence microscope (Olympus IX71).

**m6A IP seq.** 100–150 μg total RNA was extracted from cells using TRIzol following the manufacturer’s protocol. mRNA was purified using a Dynabeads mRNA DIRECT Kit (Thermo Fisher, # 61012). 1 μg mRNA was fragmented to ~200 nt using a Biornyper Pico Sonication System, and 5% of the fragmented mRNA was saved as input. m6A mRNA fragments containing m6A were enriched with an EpiMark M6A Methyladenosine Enrichment Kit (NEB, E1610S) and then extracted using RNA Clean and Concentrate (Zymo Research). RNA libraries were prepared for both input and IP samples using TruSeq® Stranded mRNA Library Prep (Illumina, 20020594) following the manufacturer’s protocol. Sequencing was performed at the University of Chicago Genomics Facility on an Illumina NextSeq 4000 machine in single-read mode with 50 bp per reading at around 25 M to 30 M sequencing depth [15].

**Sequencing data analysis.** The adapters were removed by using cutadapt for m6A-seq, and reads were aligned to the reference genome (hg38) in TopHat v2.0.14 using the parameter -g 1–library-type = fl-firststrand. RSeqF Gene structure annotations were obtained from the UCSC Table Browser. If a gene had multiple isoforms, the longest isoform was used. In order to eliminate the interference caused by introns in peak calling, aligned reads were extended to 150 bp (average fragment size) from genome-based coordinates to isoform-based coordinates. The method used for peak calling was adapted from published work with modifications [16]. For calling m6A peaks, a gene’s longest isoform was scanned using a sliding window (100 bp) with a step of 10 bp. To minimize bias from potential inaccuracy in gene structure annotation and/or the longest isoform, windows with read counts of less than 1/20 of the top window in both m6A IP and the input were excluded. The read counts in each window for each gene were normalized by the median count of all windows for the gene. The differential windows between IP and input samples were identified using a Fisher exact test. If the FDR < 0.01 and log2(Enrichment Score) ≥ 1, the window was considered positive. Overlapping positive windows were merged. To obtain the enrichment score of each peak (or window), the following four numbers were calculated: (1) read counts of the IP samples in the current peak/window, (2) median read counts of the IP sample in all 100 bp windows on the current mRNA, (3) read counts of the input sample in the current peak/window, and (4) median read counts of the input sample in all 100 bp windows on the current mRNA. For each window, the enrichment score was calculated as (a × d)/(b × c) [12].

**Human skin and tumor samples.** All human specimens were studied after approval by the University of Chicago Institutional Review Board. Normal human skin samples were obtained from the archives in the tissue bank of the Section of Dermatology (Department of Medicine, University of Chicago). The arsenical keratoses were obtained from the clinical follow-up for arsenic-exposed cohorts that evaluate the health effects of exposure, including skin cancer, in Bangladesh.

**GO datasets and analysis of differentially expressed genes (DEG).** Two RNA-seq gene expression profiles (GSE84292, GSE84293) [13] were acquired from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) [104], a public functional genomics data repository of high-throughput gene expression data. GE84292 consisted of 6 paired mouse chronically irradiated skin (CHR) and cutaneous squamous cell carcinoma (cuSCC) and GE84283 consisted of 7 cases of human normal skin (NS) and 9 cases of human cuSCC. The DEG was performed by NetworkAnalyst (http://www.networkanalyst.ca), and R programming language-based tool for comprehensive gene expression profiling [105]. The edgeR method was selected with adjusted P < 0.05 to identify the DEGs between mouse and human chronically irradiated skin (CHR) and cuSCC.

**GO, KEGG, GSEA, Venn diagram, and heatmap analysis.** To understand the function of the differentially expressed genes, gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using Metascape and Networkanalyst. P < 0.05 was considered to indicate a significantly enriched DEG. Metascape (http://metascape.org) is a free gene annotation and analysis resource that helps biologists make sense of one or multiple gene lists [106]. Gene Set Enrichment Analysis (GSEA) and the heatmap of relative significant genes were performed using Networkanalyst and Webgestalt (http://www.webgestalt.org) [102]. Venn diagrams were generated using the following web tool: https://bioinfogp.cnb.csic.es/tools/venny/index.html [107]. The following statistical cutoff: p-value <0.05 in combination with log2 (fold change) >0.5 or <−0.5 for human cuSCC/CHR and mouse cuSCC/NS (GSE84292, GSE84293), or log2 (TPM) >0.5 or <−0.5 for As-T cells/Control cells.

**Statistical analyses.** Statistical analyses were carried out using Prism 7 and 9 (GraphPad). Data were expressed as the mean of at least three independent experiments. Error bars indicate the SDs or SEs of the means. P < 0.05 was considered statistically significant.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** m6A IP sequencing and RNA sequencing data are accessible at the GEO repository, under accession number GSE145923. Other data from this study are available from the corresponding author upon request. Source data are provided with the paper. Source data are provided with this paper.

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Author contributions
Y.Y.H. conceived the project. Y.C., S.Y., J.W., C.H., and Y.Y.H. designed the research. Y.C., S.Y., and J.W. carried out most of the experiments and/or performed data analyses with input from Y.Y.H. and C.H. C.R.S. performed the histological analysis of the human samples. W.Z., F.W., and P.S. helped with some of the experiments. M.G.K. and H.A. provided the human samples. X.C. contributed m(6)A seq and RNA seq data analyses. Y.C. and Y.Y.H. wrote the manuscript with input from S.Y., J.W., C.R.S., M.G.K., H.A., and C.H. All authors reviewed the results and the manuscript.

Competing interests
C.H. is a scientific founder and a member of the scientific advisory board of Accent Therapeutics, Inc. The remaining authors declare no competing interests.

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