HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2016 March 10.

Published in final edited form as:

Oncogene. 2015 September 10; 34(37): 4879–4889. doi:10.1038/onc.2014.409.

NCOA3-mediated upregulation of mucin expression *via* transcriptional and post-translational changes during the development of pancreatic cancer

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Abstract

Pancreatic cancer (PC) is characterized by aberrant overexpression of mucins that contribute to its pathogenesis. Although the inflammatory cytokines contribute to mucin overexpression, the mucin profile of PC is markedly distinct from that of normal or inflamed pancreas. We postulated that *de novo* expression of various mucins in PC involves chromatin modifications. Analysis of chromatin modifying enzymes by PCR array identified differential expression of NCOA3 in MUC4-expressing PC cell lines. Immunohistochemistry analysis in tumor tissues from patients and spontaneous mouse models, and microarray analysis following the knockdown of NCOA3 were performed to elucidate its role in mucin regulation and overall impact on PC. Silencing of NCOA3 in PC cell lines resulted in significant downregulation of two most differentially expressed mucins in PC, MUC4 and MUC1 ($P<0.01$). Immunohistochemistry analysis in PC tissues and metastatic lesions established an association between NCOA3 and mucin (MUC1 and MUC4) expression. Spontaneous mouse model of PC (K-ras^{G12D}; Pdx-1cre) showed early expression of Ncoa3 during preneoplastic lesions. Mechanistically, NCOA3 knockdown abrogated retinoic acid-mediated MUC4 upregulation by restricting MUC4 promoter accessibility as demonstrated by micrococcus

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SK, SKB, SD designed the study. SK, SD, SR, S Kaur, SJ performed experiments, analyzed the data and wrote the manuscript. S Kaur performed MUC4 IHC and PMP performed NCOA3 chromatin immuno-precipitation. SR performed the animal experiments and SLJ analyzed the pathological samples. SK, MJ, SKB were involved in manuscript preparation and data analysis.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

nuclease digestion ($P<0.05$) and chromatin immuno-precipitation analysis. NCOA3 also created pro-inflammatory conditions by upregulating chemokines like CXCL1, 2, 5 and CCL20 ($P<0.001$). AKT, ubiquitin C, ERK1/2 and NF-κB occupied dominant nodes in the networks significantly modulated after NCOA3 silencing. In addition, NCOA3 stabilized mucins post translationally through fucosylation by FUT8, as the knockdown of FUT8 resulted in the downregulation of MUC4 and MUC1 at protein levels.

INTRODUCTION

Aberrant mucin expression is a hallmark of epithelial cancers.[1, 2] Pancreatic cancer (PC) is one of the most lethal malignancy that is compounded by late-stage diagnosis and has worst prognosis of all cancers.[3, 4] Aberrant expression of mucins, like MUC1, MUC4, MUC16 and MUC5AC occurs early in pancreatic intraepithelial neoplasia (PanIN) and increases with the progression of the disease. In addition to serving as potential diagnostic and prognostic biomarkers, mucins functionally contribute to the pathobiology of PC.[1, 2, 5]

Among the aberrantly expressed mucins, MUC4 a 930 kDa trans-membrane protein, is undetectable in the normal pancreas and pancreatitis,[6–8] but is one of the most differentially over-expressed mucin in PC appearing early in PanIN lesions and its expression increases progressively with disease advancement. MUC1 on the other hand, is expressed in normal pancreas but is overexpressed and aberrantly glycosylated in cancer.[9] The cytoplasmic tail of MUC1 activates multiple oncogenic signaling pathways (Wnt-β-catenin and NF-κB).[10] Similarly, we recently demonstrated that MUC16 is overexpressed and has a significant role in PC progression.[11] Under inflammatory and pre-neoplastic conditions mucin expression is influenced by inflammatory cytokines, especially Th2 type (interleukin (IL)-4, -9 or -13), tumour necrosis factor (TNF)-α, IL-1β, IL-5 and IL-10.[12–14] Studies from our laboratory demonstrated that interferon-γ (IFN-γ) and retinoic acid (RA) synergistically upregulate MUC4 in PC cells.[15] Similarly, MUC1 expression has been demonstrated to be regulated by IFN-γ, IL-1β, IL-6 and TNF-α.[14] Despite the similar profile of inflammatory cytokines during pancreatitis and PC, mucin expression patterns under the two conditions are very distinct, possibly because of the additional regulatory events like chromatin remodeling and altered promoter accessibility during PC development.

We hypothesized that *de novo* expression of MUC4 during the early stages of PC would require chromatin modifications to allow access of the transcriptional machinery to MUC4 promoters and identified nuclear receptor co-activator 3 (NCOA3 also known as AIB1, ACTR, RAC3, SRC3, TRAM-1) as one of the differentially upregulated chromatin remodeling enzymes in MUC4-expressing PC cell lines. NCOA3 belongs to the p160SRC family of proteins and interacts with nuclear receptors and transcriptional factors and possesses intrinsic histone-acetyltransferase activity to remodel chromatin for active transcription.[16–19] We observed that NCOA3 was undetectable in normal pancreas, but was expressed during early PanIN I lesions, coinciding with the appearance of MUC4. Furthermore, NCOA3 regulated MUC1 and MUC16 expression, both at transcriptional and post-translational levels. Our findings suggest that NCOA3 plays a vital role in mucin regulation, creates pro-inflammatory conditions and modulates tumor microenvironment to

promote growth and dissemination of pancreatic tumors. In this study, we focused primarily on NCOA3-mediated MUC4 regulation, and the clinical relevance of NCOA3 in PC.

RESULTS

NCOA3 is differentially upregulated in the MUC4 expressing cell lines and regulates mucin expression

In human PC, *de novo* expression of the MUC4 is an early event and is associated with the malignancy and poor prognosis.[7, 8] MUC4-expressing (Capan1) and non-expressing (Panc1) PC cells were profiled for the expression of 84 chromatin-modifying enzymes using a chromatin-modifying enzyme PCR array (PAHS-085; Figure 1a). Several genes were found to be differentially expressed in MUC4 expressing cells in comparison to non-expressing cells (Supplementary Table 1). The differentially upregulated (*PRMT6* (15.6-fold), *HDAC9* (11.4-fold), *HDAC* (8.8-fold), *KDM4C* (5.5-fold), *KDM6B* (4.9-fold) and *NCOA3* (4.3-fold)) and downregulated (*PRMT8* (0.02-fold), *AURKC* (0.10-fold), *KAT2B* (0.13-fold), *NCOA1* (0.17-fold), *MLL5* (0.20-fold), *UBE2A* (0.23-fold), *KDMA5B* (0.24-fold) and *ATF2* (0.30-fold)) genes were evaluated in a panel of MUC4 expressing (Capan1, CD18/HPAF, Panc10.05, QGP1 and T3M4) and non-expressing (ASPC1, Panc1, MIA PaCa-2) PC cell lines and immortalized normal pancreatic cell line (HPNE; Supplementary Figure 1A). Among various genes, *NCOA3* was found to be differentially upregulated in all MUC4-expressing cell lines compared with non-expressing (except ASPC1) cell lines both at transcript (two- to five-folds, $P<0.01$) and protein levels (Figures 1b and c).

To determine the role of NCOA3 in the aberrant overexpression of MUC4 and other mucins, NCOA3 was stably knocked down (KD) in CD18/HPAF and T3M4 PC cell lines. NCOA3 KD (80% in CD18/HPAF and 40% in T3M4) resulted in downregulation of MUC4 expression (60–70% in CD18/HPAF and 40–50% in T3M4) at the transcriptional level ($P<0.01$; Figure 1d and Supplementary Figure 1B, C). A recent study has demonstrated the regulation of MUC1 by NCOA3 in uterine epithelia[20] and we also observed 40–60% decrease in the MUC1 transcripts ($P<0.01$) after NCOA3 KD (Figure 1d). Consistent with the real-time PCR analysis, MUC4 and MUC1 protein levels were also downregulated (Figure 1e). Interestingly, NCOA3 KD resulted in decreased expression of MUC16 at the protein level but not at the transcriptional level (data not shown). These data suggest that NCOA3 is potentially a central regulator of mucin overexpression in PC. We have previously demonstrated[5] that MUC4 regulates EGFR family receptor stability and signaling. We observed decreased phosphorylation of EGFR and lower levels of HER2 in NCOA3 KD cells as compared with the control cells (Supplementary Figures 1C, D).

NCOA3 expression is associated with mucin expression in PC tissues

To explore the relationship between NCOA3 and mucin expression, tissue arrays containing primary pancreatic tumors and metastatic lesions were stained for NCOA3, MUC4 and MUC1. Expression of both NCOA3 and MUC4 in the normal pancreas was undetectable (Figure 2a). In PC tissue samples, there was strong ductal expression (with predominantly nuclear localization) of NCOA3 ($n = 34$, Mean composite score (MCS) 7.5, 94% positivity) whereas the expression of MUC4 ($n = 34$, MCS 3.4, 60% positivity) and MUC1 ($n = 34$,

MCS 5.6, 79.4% positivity) was both ductal and membranous, and was rarely observed in the cytoplasm (Figure 2b). A positive association was observed between the expression of the mucins and NCOA3 (Table 1). In the primary tumors 53% of the MUC4-positive samples were also positive for the NCOA3 nuclear expression, and similar association was observed (27.2–64.7%) in the metastatic lesions of PC. MUC1 expression also showed strong correlation with NCOA3 in the primary tumors (79.4%) and metastatic lesions (71.4–76.4%). Further, majority of the metastatic lesions showed NCOA3 expression; liver (n = 22, MCS 4.9, 77.2% positivity), lung (n = 14, MCS 6.78, 85.6% positivity), lymph node (n = 17, MCS 5.66, 94% positivity) and omentum (n = 12, MCS 6.5, 88.3% positivity; Figure 2c).

Ncoa3 is upregulated in the spontaneous PC mouse model The well-defined mouse model of PC (K-ras^{G12D}; Pdx-1cre, KC) recapitulates histopathology and mucin expression of human PC. The KC mouse model[21] was used to analyze Ncoa3 and mucin expression from 10 weeks of age (earliest precancerous lesions) to 40 weeks of age (when majority of animals develop high grade PanIN). Immunohistochemistry analysis of normal pancreatic ducts from 30-week-old control mice (LSL-K-ras^{G12D}) were negative for Ncoa3 and Muc4 expression, but showed weak Muc1 expression (Figure 3a). However, pancreas of 30-week-old KC mice showed strong nuclear localization of Ncoa3 in the ducts which exhibited higher Muc4 and Muc1 expression (Figure 3b). Real-time RT-PCR analysis from pancreatic RNA of KC mice also showed Ncoa3 upregulation (~1.8-fold) at 10 weeks of age (Figure 3c). Concurrently, we have recently reported 2.9- and 2.5-fold upregulation in Muc1 and Muc4 expression, respectively, in pancreas of 10-week-old KC mice compared with control LSL-Kras^{G12D} mice.[22] No further increase in Ncoa3 transcripts was observed at 30 or 40 weeks and it is conceivable that Ncoa3 possibly triggers chromatin modification to open up the mucin gene locus and subsequent upregulation is driven by inflammatory cytokines and other modulators that are upregulated during disease progression. Given the differential profile of mucin expression in pancreatitis and PC, we studied the expression of Ncoa3 in cerulein based pancreatitis model in the wild type and mutant K-ras background to discriminate between its expression during sterile inflammation and oncogene-induced/associated inflammation (Supplementary Figure 2). Ncoa3 expression was only observed in the presence of K-ras^{G12D} mutation (KC animals) compared with wild type animals suggesting a link between Ncoa3, inflammation and PC through constitutively active K-ras mutation.

NCOA3 KD abrogates RA-mediated upregulation of MUC4 and modulates MUC4 promoter accessibility

NCOA3 interacts with RA,[23] estrogen, progesterone[24] and vitamin-D receptors.[25] In the presence of RA, retinoic acid receptors, translocates to the nucleus and binds to the RA response elements in gene promoters to modulate gene expression. MUC4 promoter contains multiple potential RA response elements and we have previously demonstrated that RA upregulates MUC4 expression.[15] To determine if NCOA3 regulated MUC4 in RA dependent manner, the impact of RA on MUC4 expression was studied in control and NCOA3 KD cells. RA treatment upregulated MUC4 both at the RNA (~3-fold, P<0.01, CD18/HPAF) and protein levels in CD18/HPAF, Capan1 and QGP1 PC cell lines (Supplementary Figures 3A, B, C). Further, RA treatment increased NCOA3 expression in

both nuclear and cytoplasmic fractions of MUC4-expressing (CD18/HPAF and T3M4) cell lines compared with MUC4 non-expressing (Panc1) cell line (Supplementary Figure 3D). Silencing of NCOA3 in CD18/HPAF cells resulted in significant decrease in the MUC4 transcripts and protein and abrogated RA-dependent upregulation of MUC4 (Figures 4a and b).

The role of NCOA3 in modulating MUC4 promoter accessibility was examined using chromatin immuno-precipitation (ChIP) assay with NCOA3 and methylated histone H3 (me3H3K4) antibodies, and micrococcal nuclease protection assay. ChIP using NCOA3 antibody demonstrated that NCOA3 binds in the region between − 3.46 kb to − 3.27 kb from the translational start site at the distal region of MUC4 promoter (Figure 4c). Further, there was a decrease in active transcriptional mark at the MUC4 locus following the NCOA3 KD as compared with the control cells as indicated by poor enrichment of MUC4 promoter region, which could not be rescued by RA treatment (Figure 4d). Micrococcal nuclease protection assay was performed to ascertain the impact of NCOA3 on accessibility at the MUC4 promoter. The nuclei from the CD18/HPAF scrambled and NCOA3 KD cells were treated with micrococcal nuclease (Supplementary Figure 4A) and the primers spanning MUC4 transcriptional start site showed significantly higher amplification (Figures 4e and f; $P<0.05$) in NCOA3 KD cells (CD18/HPAF) compared with the scrambled control. These results indicate that the absence of NCOA3 caused greater protection, hence, poor accessibility of the MUC4 promoter region.

NCOA3 modulates pathways regulating inflammation and tumor microenvironment

In addition to mucins, NCOA3 can also modulate the expression of other genes which can directly or indirectly impact the mucin expression. To identify the networks and canonical pathways (CPs) modulated by NCOA3 and to determine its potential impact on PC biology, we studied the changes in global gene expression profiles following NCOA3 KD. The top differentially up and downregulated genes (Supplementary Table 2) were validated by real-time RT-PCR analysis (Supplementary Figure 4B). NCOA3 KD caused significant downregulation of chemokines, such as CXCL2, CXCL1, CXCL5, CCL20 and IL8, which recruit neutrophils, macrophages and lymphocytes (Supplementary Table 2). Pathway analysis following NCOA3 KD, indicated that AKT (score 32), ubiquitin C (score 28), ERK1/2 (score 25) and NF-κB (score 20) (Figure 5 and Supplementary Figures 5A and B) occupied dominant nodes in the predominantly modulated networks. Further, significantly altered CPs like integrin signaling, IGF-1 signaling, role of IL-17A and stellate cell activation have also been shown to have critical roles in PC progression and upregulation of mucin expression (Figure 5 and Supplementary Figure 6A).[26–29] Together, in precancerous lesions, these significantly altered immune effectors, networks and CPs create inflammatory microenvironment, provide proliferative advantage and inhibit apoptosis. In addition, NCOA3 may also participate in the building up of the extracellular matrix by upregulating genes like lysyl oxidase-like-2 (LOXL2) which is involved in the crosslinking of collagen type I and hardening of the desmoplasia.

NCOA3 regulates post-translational stability of MUC4 through glycosylation

Microarray analysis showed significant downregulation of fucosyl-transferase 8 (FUT 8) following NCOA3 KD (Supplementary Table 2). Glycosylation can affect the stability of MUCs and other glycoproteins. Downregulation of FUT8 after NCOA3 KD suggests that it may have a possible involvement in post-translational stability of the mucins. Stable knockdown of FUT8 in CD18/HPAF PC cell line (Supplementary Figure 6B) resulted in downregulation of MUC4 and MUC1 proteins (Figure 6a); however, their transcript levels remained unchanged (Figure 6b). These results indicate the additional indirect role of NCOA3 in post-translational stability of MUCs and suggest that NCOA3 can modulate mucin expression at multiple levels (Figure 6c).

DISCUSSION

Aberrant and *de novo* mucin overexpression is a hallmark of PC. While the inflammatory cytokines and RA have been demonstrated to upregulate mucin expression (particularly MUC4) in cancer cells,[13–15] mucin overexpression is not observed under non-malignant inflammatory pathologies of the pancreas despite the presence of inflammatory cytokines and activated stellate cells.[6–8] The *de novo* expression and/or silencing of tumor-associated genes require chromatin modifications that alter transcription factor accessibility to their promoter-binding sites. In our search for chromatin-modifying factors that contribute to mucin overexpression, we found that NCOA3 was differentially upregulated in MUC4-expressing PC cell lines. NCOA3 is a p160SRC family homolog that functions as a nuclear receptor co-activator by interacting with steroid receptors and possesses an intrinsic histone acetyltransferase activity.[16, 17] Studies have demonstrated several roles for NCOA3, it is involved in the maintenance of pluripotency,[30] angiogenesis, wound healing,[31] metabolic defects[32] and immunomodulation.[33] NCOA3 locus is amplified in breast (9.5%)[34] and colorectal cancers (27.5% gain in copy number),[35] that highlights its oncogenic potential. A previous study reported NCOA3 amplification in PC cells and suggested that NCOA3 may interact with CBP/p300 to modulate signaling pathways that give proliferative advantage to hormone-insensitive tumors, like PC.[36] The current study substantiates and extends the observations made by Henke *et al.*[37] that NCOA3 is undetectable or rarely expressed in normal pancreatic ducts, but its expression progressively increases in pancreatitis (>14%) and in low grade PanINs (>25%) to PDAC (>65%). We observed even higher incidence of NCOA3 overexpression in primary pancreatic tumors and metastatic lesions, suggesting its critical role in PC progression. In breast cancer, high level of NCOA3 is associated with the high-grade tumor, shorter disease-free interval, worse disease outcome and high lymph node positivity.[38, 39] Further, Ncoa3 ablation decreased the incidence, growth and metastasis of v-Ha-Ras-induced mammary tumors and complete disappearance of tumor in ovariectomized mice.[40]

To understand the regulation of mucin genes by NCOA3, we explored MUC4 because unlike MUC1 its expression is strictly associated with malignant transformation.[6, 7] The role of NCOA3 in modulating MUC4 promoter accessibility was clearly evident from the fact that NCOA3 knockdown led to a greater protection at MUC4 promoter from micrococcal nuclease digestion and there was a significant decrease in active transcriptional mark

(me3H3K4) even in the presence of RA. Binding of NCOA3 close to the transcriptional start site further highlights its importance in regulation of MUC4 transcription. We also performed microarray expression studies to understand how NCOA3 might function in the larger context of PC and mucin regulation. Critical signaling molecules like AKT, ERK1/2, ubiquitin C and NF-kB, which are involved in proliferation, pro-inflammatory microenvironment and PC progression,[41] occupied the central position in the most significantly altered networks identified following NCOA3 silencing. Furthermore, LOXL2, the most significant gene down-regulated by NCOA3 KD, has been shown to participate in fibroblast activation and hardening of desmoplasia,[42] a characteristic feature of PC.

The intrinsic inflammatory cascade initiated by oncogenes like K-ras creates an extracellular milieu of pro-inflammatory cytokines like TNF-α, IFN-γ, IL-6, IL-1β and IL-10, previously shown to upregulate MUC2, MUC1, MUC5AC and MUC4 expression.[12–14, 43] In addition, this pro-inflammatory microenvironment contributes significantly to the activation of quiescent pancreatic stellate cells. Under quiescent conditions pancreatic stellate cells store vitamin A in lipid droplets and upon activation by a cytokine (TNF-α, IL-6, CXCL8, IL-1) release the vitamin-A store as *all-trans* RA, which has been shown previously by us to activate and synergize with IFN-γ to upregulate the MUC4 expression in PC cell lines.[15] While there is a commonality in stellate-cell activation and elevated cytokine profile in PC and pancreatitis, MUC4 expression however, is only observed in PC. Our studies provide evidence that NCOA3 mediates this differential response of MUC4 promoter to inflammatory cytokines and RA during oncogene-associated inflammation. In the cerulein-induced inflammation model, Ncoa3 expression was only observed in the presence of constitutively active K-ras. Presumably, Ncoa3 participates in perpetuating these inflammatory conditions during PC progression through cytokine upregulation. However, a study has also demonstrated that ablation of NCOA3 in macrophages enhances the synthesis of pro-inflammatory cytokines,[44] suggesting the context-dependent relationship between inflammation and NCOA3. In addition to transcriptional regulation, NCOA3 appears to modulate the post translational modifications of mucins and enhance their stability. FUT8 KD downregulated both MUC4 and MUC1, and may influence their interactions with stroma through differential fucosylation. FUT8-mediated fucosylation of haptoglobin and high levels of fucosylated haptoglobin in serum samples have already been identified as a novel diagnostic marker for PC.[45]

EGFR in PC has been shown to be essentially required to maintain K-ras initiated transformation events.[46] NCOA3 has been demonstrated to be an important regulator of the activity of EGFR and HER2 signaling.[47, 48] We and others have previously demonstrated that mucins, particularly MUC4, regulate HER2 and other EGFR family receptor stability and this modulates their signaling.[5] Reduced phosphorylation status of EGFR[48] and stability of HER2 following NCOA3 knock down is in line with these observations. These effects could be attributed to the direct action of NCOA3 or may be because of indirect effects. The reduced EGFR phosphorylation can also indirectly be attributed to reduced FUT8 levels in the NCOA3 knock down cells. Deletion of FUT8 in alveolar-carcinoma cell lines (A549) resulted in significant reduction in TGF-β1, EGF and VEGFR-2 receptor expression[49] and EGFR dimerization in lung cancer cells.[50]

In conclusion, we have demonstrated that NCOA3 regulates critical mucins (MUC4 and MUC1) in PC play have a central role in the progression and metastasis of PC. Owing to the additional involvement of NCOA3 in promoting oncogene-associated inflammation and possibly desmoplasia, targeting NCOA3 expression by small molecular inhibitors like goosypol[51] and bufalin[52] can provide novel avenues to target PC. Understanding the regulation and/or amplification of NCOA3 locus in various cancers including PC and the role of tumor-initiating mutations like K-ras in regulating NCOA3 expression will be of interest to elucidate its role in oncogenesis.

MATERIALS AND METHODS

Cell culture and tissue specimen

PC cell lines were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G and streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. RNA and protein samples were prepared at 70–80% confluence of the cells. Formalin-fixed and paraffin-embedded PC tissue (8) and Rapid Autopsy tissue array (consisting of 7 normal pancreas, 29 primary PC and matching metastasis to liver (22), lungs (14), lymph nodes (17) with colon and kidney as controls) were obtained from University of Nebraska Medical center Rapid Autopsy Program (IRB-091-01) for immunohistochemistry analysis (IHC). Tissues harvested from the mice were embedded in paraffin wax after incubation in 10% formalin for 72 h. Five micrometer sections were sliced and prepared for IHC analysis.

Chromatin-modifying enzyme PCR array

RNA isolated from MUC4-expressing (Capan 1) and non-expressing PC cell lines (Panc1) were reverse transcribed using RT2 first strand kit (SA Bioscience, Valencia, CA, USA). cDNA was mixed with RT2 SYBR qPCR master mix and 25 μl aliquot of mix was added in 96-well PCR array kit containing lyophilized gene-specific primer set (PAHS-0085F, SA Bioscience). Threshold values (Ct) were used to calculate fold change using web server RT2 Profiler PCR Array Data Analysis (Figure 1a).

Stable knock down of NCOA3 and FUT8

The pSUPER.retro.puro vector was digested with *Bgl*II and *Hin*dIII restriction enzymes and dephosphorylated with calf intestinal alkaline phosphatase. Two complementary oligonucleotides specific for NCOA3 (5′-GACAGGCACTTGAATTGAA-3′) and FUT8 (5′-GGCTTCAAACATCCAGTTA-3′) were synthesized (IDT, San Jose, CA, USA), annealed and cloned using *Bgl*II and *Hin*dIII restriction sites. The presence of the insert was confirmed by sequencing and digestion with EcoRI and *Hin*dIII restriction enzymes. The vectors were transfected in Phoenix cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The supernatant was collected after 48 and 72 h and was used to transfect CD18/HPAF and T3M4 cell lines. After 72 h of incubation, cells were selected with 5 μg/ml of puromycin; clones were pooled and maintained at the concentration of 2 μg/ml of puromycin.

RNA isolation and real-time analysis

Total RNA from the cells and tissue was isolated using Qiagen RNeasy and *mir*Vana miRNA kit (Ambion, Austin, TX, USA), respectively. RNA was reverse transcribed using 1 μg of total RNA with random hexamer oligos (500 μg/ml), 1 μl of 10m$_M$ dNTPs, 5x first-strand reverse transcriptase buffer buffer, 1 μl of 0.1M dithiothreitol and 1 μl of (50 unit) SuperScript as described previously.[53] The reaction mixture was diluted to final concentration of 10 ng/μl. The expressions of various genes were profiled using gene-specific primers (Supplementary Tables 3 and 4) and the relative expression was calculated using $2^{-\Delta\Delta CT}$ method.[54] The data was expressed as mean ± s.e.m. and statistical significance was set at $P \leq 0.05$. Statistical comparisons of two groups were made using a Student's *t*-test.

Western blot analysis

Western blot analysis was performed as previously described.[55] Briefly, whole-cell lysate was prepared in the lysis buffer (RIPA), protein was quantified and equal amount (20–60 ug) of total protein was resolved by 10% SDS-polyacrylamide gel electrophoresis and/or 2% horizontal SDS agarose (for mucin) electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane and incubated overnight with the primary antibody after blocking with 5% skim milk. Next day, blot was washed three times with PBST (0.1% Tween 20; (phosphate-buffered saline (PBS) with Tween 20)) and incubated with horseraddish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were visualized by enhanced chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA). The primary antibodies used were NCOA3 (sc25742, Santa Cruz Biotechnologies, Dallas, TX, USA) PGK (sc-17943, Santa Cruz Biotechnologies), PARP (sc8007, Santa Cruz Biotechnologies), MUC4 (8G7, developed in our laboratory previously), EGFR (sc-03, Santa Cruz Biotechnologies) p-EGFR (S1046, sc-101665, Santa Cruz Biotechnologies), p-EGFR (Y1068, 3777, Cell Signaling Technologies, Danvers, MA, USA), Her-2 (2165, Cell Signaling Technology) and MUC1 (HMFG2, kindly gifted by Dr Hollingsworth).

Immunohistochemistry (IHC) analysis

IHC analysis was performed as described previously.[53, 56] Briefly, slides were baked overnight at 56 °C, deparaffinized with xylene and tissues were rehydrated. Endogenous peroxidase activity was blocked by incubating for 30 min (1 h for mouse sections) with 3% H_2O_2: methanol solution. Antigens were retrieved by boiling sections in 0.5% citrate buffer for 15 min. Tissue sections were blocked with 2.5% horse serum for 1 h and incubated with NCOA3, MUC1, MUC4, Muc1 (ab15481, Abcam, Cambridge, MA, USA) and Muc4 (4A, Rabbit polyclonal developed by our laboratory and described previously[22]) primary antibody over night at 4 °C. Slides were washed with PBS and incubated with horseraddish peroxidase-conjugated secondary antibody for 1 h, washed and developed for colorimetric detection by 3.3' diaminobenzidine kit (Vector laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. Tissues were dehydrated, dried and mounted with Permount and evaluated by pathologist. MCS of the molecule was calculated by multiplying the intensity and percentage positivity.

ChIP

ChIP analysis was carried out as described previously with modifications.[20] Briefly, PC cells (CD18/HPAF) were serum starved for 24 h and treated with RA (all-*trans* RA) for 1 h, fixed with 1% formaldehyde for 10 min at room temperature followed by addition of glycine to a final concentration of 125mM. Cells were harvested and washed twice with PBS and lysed in lysis buffer (5mM PIPES, 85mM KCl, 0.5% IGEPAL-CA630, pH8.0). Nuclei were pelleted down and re-suspended in SDS buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.0) followed by sonication (1 min, at high amplitude, Bioruptor UCD-200, Diagenode, New York, NY, USA). Lysate was pre-clarified and incubated with anti-H3 (tri-methly) K4 (ab 8580, Abcam) and anti-NCOA3 antibodies overnight at 4 °C. Immune complex was precipitated with protein A/G agarose beads and washed twice, first with low-salt (20mM Tris-HCl, 2mM EDTA, 1% SDS, 1% TritonX-100, 150mM NaCl, pH8.0) followed by high-salt buffer (20mM Tris-HCl, 2mM EDTA, 1% SDS, 1% TritonX-100, 500mM NaCl, pH8.0). Beads were washed twice with TE buffer and the complex was eluted in elution buffer (100mM $NaHCO_3$, 1% SDS); crosslinking was reversed by heating at 65 °C overnight followed by treatment with RNase A (Invitrogen, Grand Island, NY, USA) and proteinase K (Qiagen, Valencia, CA, USA). Fragmented DNA was isolated using ChIP DNA clean and concentrator Kit (ZYMO Research, Irvine, CA, USA). MUC4 promoter targeted primers (Supplementary Table 5) were used to amplify and study the enrichment of the fragmented DNA.

Micrococcal nuclease assay

Micrococcal nuclease assay was performed as described previously.[57] Briefly, nuclei from PC cells (CD18/HPAF) were isolated and treated with 100 gel units of micrococcal nuclease enzyme at 37 °C for 5 min followed with overnight proteinase-K treatment. DNA was isolated with phenol: chloroform extraction method and re-suspended in nuclease-free water. 100 ng of genomic DNA was used for the real-time analysis using MUC4 promoter-targeted primers (Supplementary Table 5) and the data was expressed as mean ± s.e.m. and statistical comparisons of two groups were made using a Student's *t*-test. The $P \leq 0.05$ was considered statistically significant.

Microarray and pathway analysis

RNA was isolated from the scrambled and NCOA3 KD CD18/HPAF cell lines. The purity and quality of the RNA from three biological replicates was analyzed by bioanalyzer before submitting for the microarray analysis. RNA was converted to cDNA, tagged to Cy3 and Cy5 and subjected to microarray using Phalanx Biotech Spotted Microarray (HOA_005.1). The fluorescence intensities after background correction were analyzed using LOWESS Normalization protocol using BRB Array Tools version 3.1 (National Cancer Institute, Bethesda, MD, USA). The microarray data was independently validated through qRT-PCR (primer sequence in Supplementary Table 3) and the probe sets with two-fold change and greater were included in the Ingenuity Pathway Analysis (Ingenuity System, Redwood City, CA, USA). The CPs and networks were generated from the Ingenuity Pathway Knowledge database (GEO submission: GSE51492). The genes were represented as nodes and their

relationship with other genes was depicted by lines (direct relation by solid lines and indirect relation by dotted lines).

Preparation of cytoplasmic and nuclear extract

The cytoplasmic and nuclear extracts were prepared by methods described previously.[57] Briefly, cells were rinsed with ice-cold PBS and incubated on ice for 1h in cytoplasmic-extraction buffer (10mM Hepes, pH 7.4, 10mM KCl, 0.2% NP-40, 0.1mM EDTA, 10% glycerol, 1.5mM MgCl2, supplemented with protease inhibitor, 1mM DTT, 1mM PMSF, 5mM Na_3VO_4, 5mM NaF). Cells were centrifuged at 16 000 × g and the supernatant was used as cytoplasmic extract. The remaining pellet was washed with PBS, incubated with the nuclear extraction buffer (20mM Hepes, (pH 7.6), 420mM NaCl, 1mM EDTA, 20% glycerol, 1.5mM MgCl2, 1mM DTT, 1mM PMSF, 5mM Na3VO4, 5mM NaF) for 1 h, sonicated at 60% amplitude for 10s, subjected to centrifugation and supernatant was collected as a nuclear extract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Lynette Smith, Dr Joshua Souchek, Ms Kavita Mallya, Dr Neeru Sharma and Dr Parama Dey for help in statistical analysis and technical support. Microarray analysis was performed using BRB-Array Tools developed by Dr. Richard Simon and BRB-Array Development Team. The work was supported, in parts, by grants from the NIH (TMEN U54 CA163120, RO1 CA183459, EDRN UO1, CA111294, SPORE P50 CA127297 and RO3 CA167342).

ABBREVIATIONS

CP	canonical pathways
EGFR	epidermal growth factor receptor
ERK1/2	extracellular-signal-regulated kinases
FUT8	fucosyltransferase 8
IFN	interferon
KD	knock down
LOXL2	lysyl oxidaselike-2
NCOA3	nuclear receptor co-activator-3
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate-buffered saline
PC	pancreatic cancer
RA	retinoic acid

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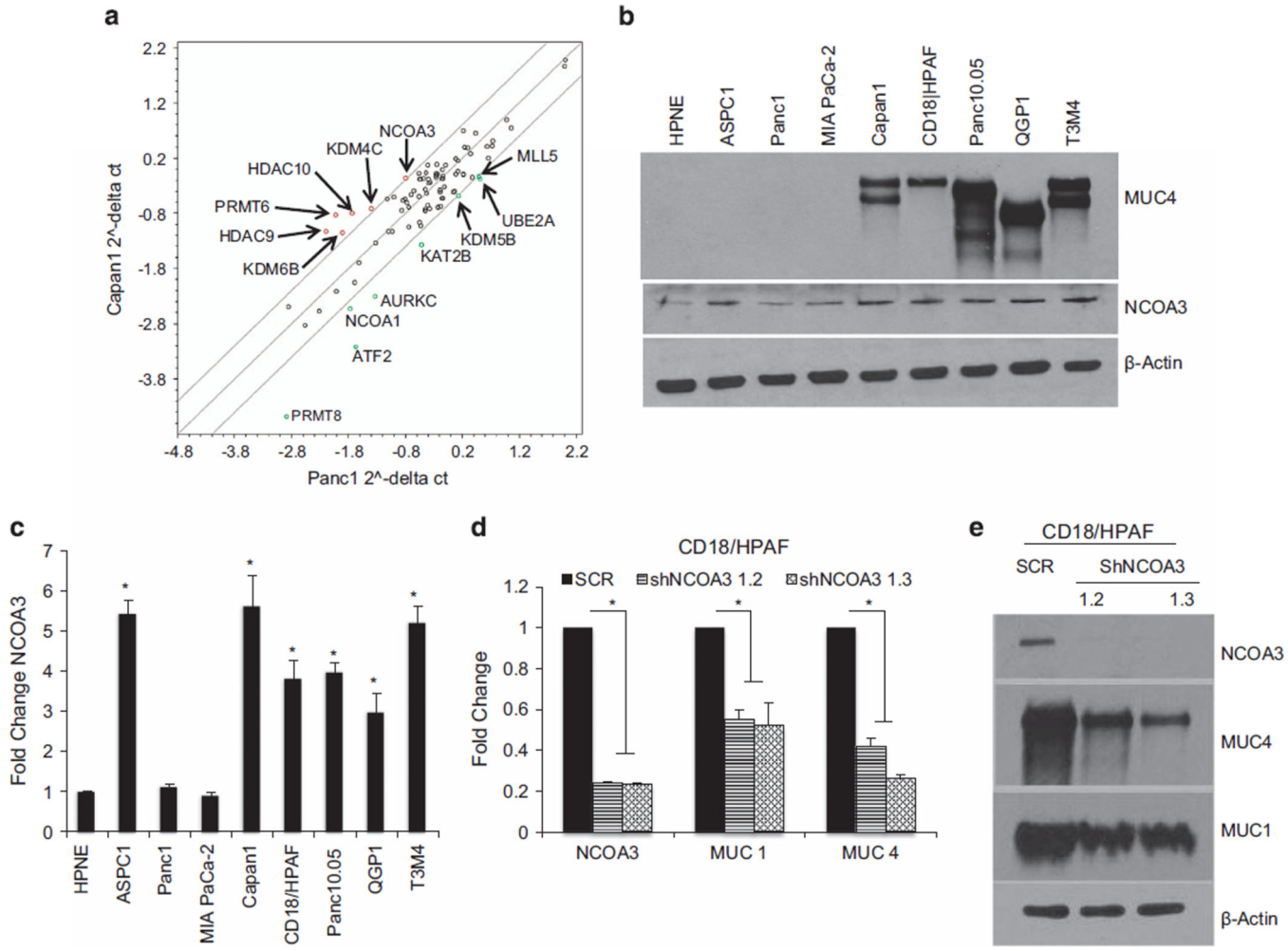

Figure 1.

NCOA3 is differentially unregulated in MUC4-expressing cell lines and regulates mucin expression. (**a**) Scatter plot of PCR array analysis of the MUC4 expressing (CAPAN1) and non-expressing (Panc1) cell lines (red circles indicate the upregulated and green circles indicate the downregulated genes on the scatter plot). (**b**) Expression profile of MUC4 and NCOA3 in MUC4 expressing and non-expressing cell lines. (**c**) NCOA3 was differentially upregulated in MUC4 expressing cell lines both at protein and RNA (**P*<0.01) levels. (**d**) Stable knock down (KD) of NCOA3 in CD18/HPAF (ShNCOA31.2 and ShNCOA31.3 are two pooled populations) and T3M4 (Supplementary Figure 1B) cell lines significantly downregulates MUC4 and MUC1 expression at the transcriptional level (**P*< 0.01). (**e**) Western blot analysis confirming downregulation of the levels of MUC4 and MUC1 proteins after NCOA3 KD.

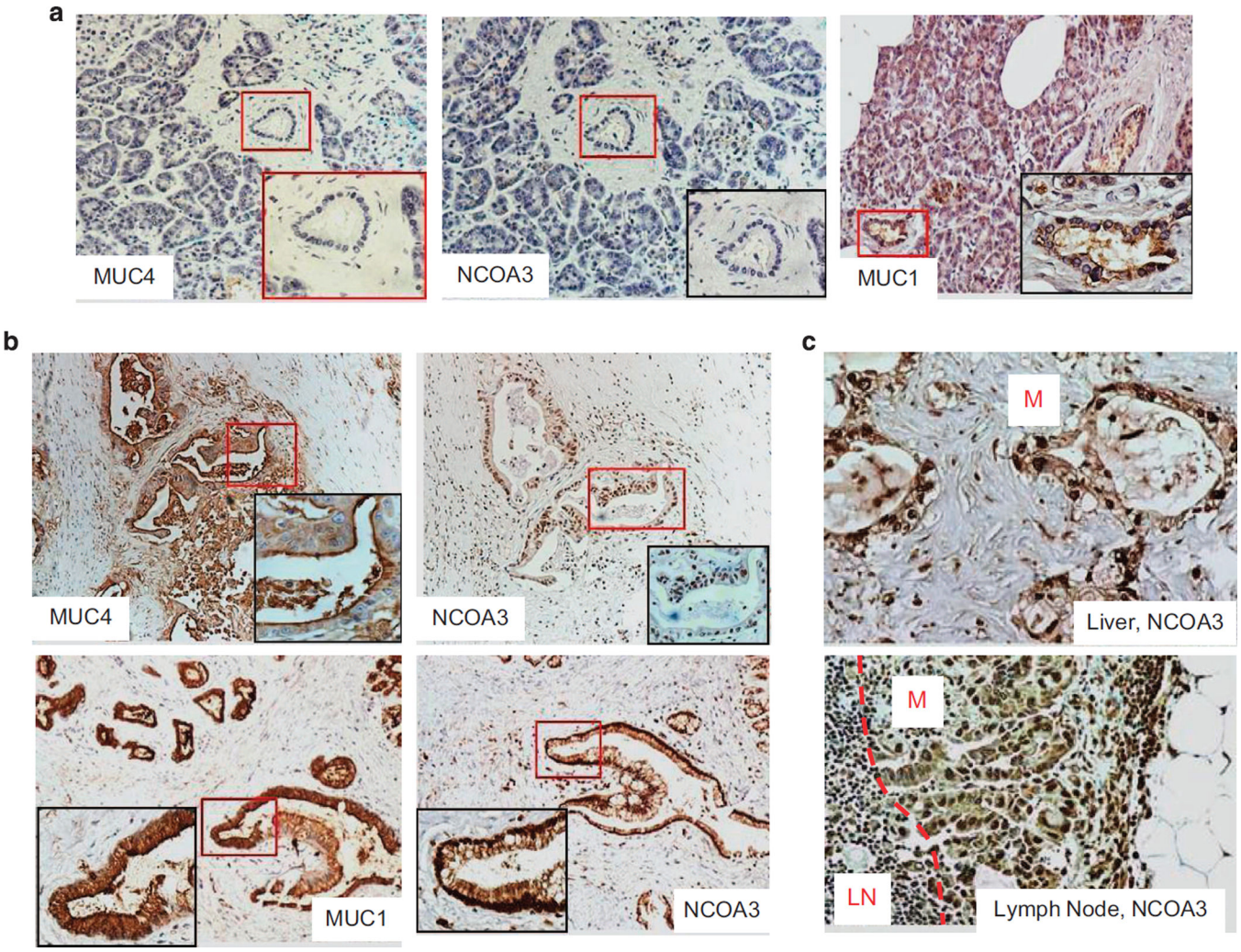

Figure 2.

Association between NCOA3 and mucin expression in PC tissue samples. (**a**) Immunohistochemistry analysis of NCOA3, MUC4 and MUC1 in normal pancreas after staining with respective antibodies. Normal pancreatic ducts were negative for NCOA3 and MUC4 expression and there was weak MUC1 expression. (**b**) IHC of the pancreatic cancer tissue samples acquired from the pancreatic cancer patients and Rapid Autopsy Program. Pancreatic tumor ducts positive for MUC4 and MUC1 expression showed strong nuclear staining for NCOA3 (magnification x100 and inset x400). (**c**) NCOA3 expression in the primary cancer matched metastatic lesion (liver and lymph nodes) from rapid autopsy tissue arrays. There was strong NCOA3 nuclear staining in the metastatic lesions, M (metastatic lesion), LN (lymph node). Red line indicates the boundaries of metastatic lesion and normal tissue (magnification x100).

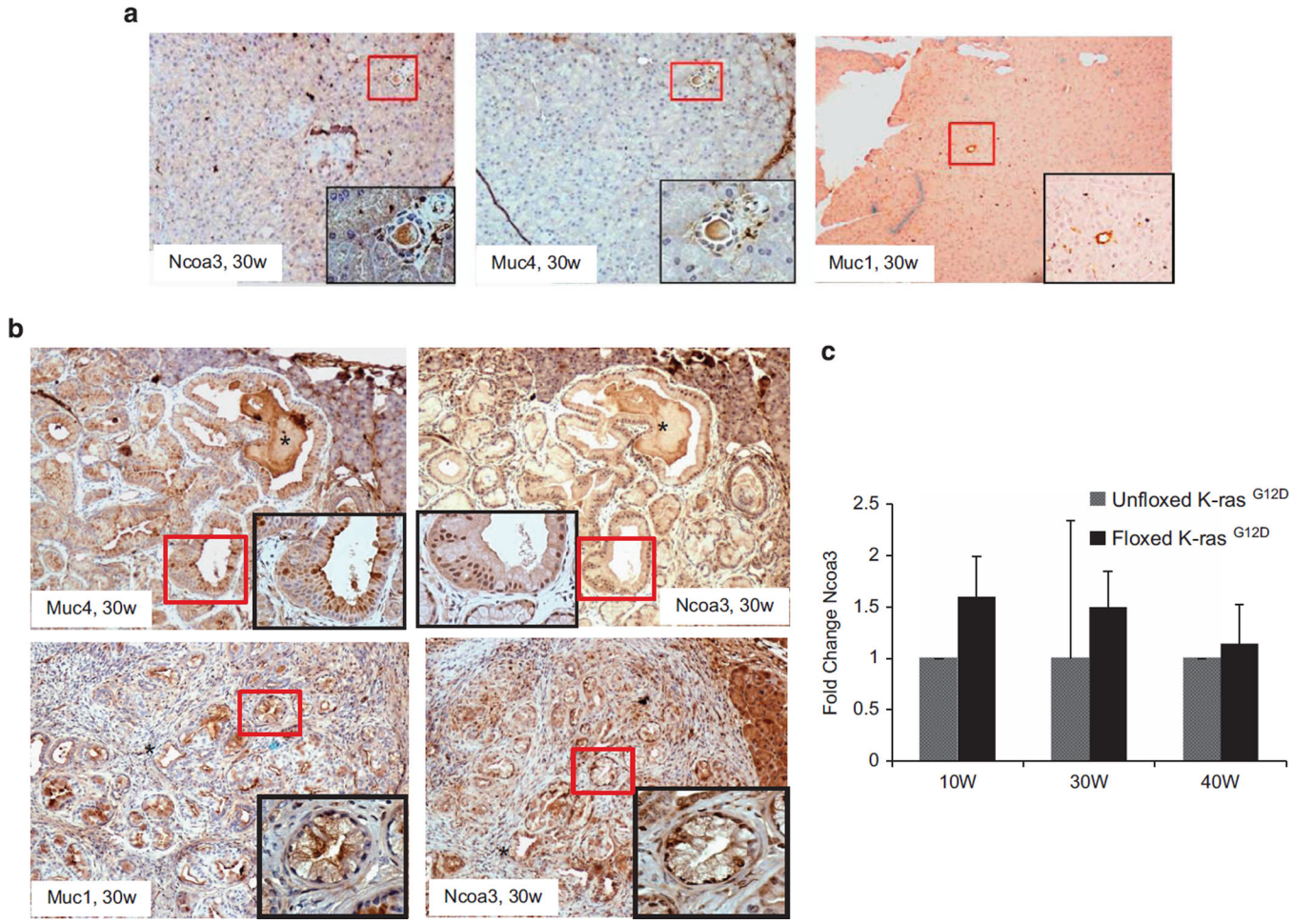

Figure 3.

Expression of Ncoa3 and mucins in precancerous lesions in spontaneous PC mouse model (K-ras^{G12D}; Pdx-1cre). (**a**) Expression of Ncoa3, Muc4 and Muc1 in normal pancreatic ducts from LSL-Kras^{G12D} mouse. Normal pancreatic ducts were negative for Ncoa3 and Muc4 expression. (**b**) IHC analysis of Ncoa3, Muc4 and Muc1 expression in precancerous lesions (PanIN II, PanIN III) in 30-week-old K-ras^{G12D}; Pdx-1cre mice. Pancreatic ducts positive for Muc4 and Muc1 expression showed strong nuclear staining for Ncoa3 ((black asterisk denotes the reference point in the serial sections) magnification x100 and inset x400 (focused to duct)). (**c**) Real-time RT-PCR analysis form the RNA isolated from the pancreas of 10, 30 and 40 weeks old (n=2–4) LSL-Kras^{G12D} and K-ras^{G12D}; Pdx-1cre mice (primer sequence in Supplementary Table 4). There was upregulation of Ncoa3, expression very early (10 week, PanIN I stage) in the PC progression.

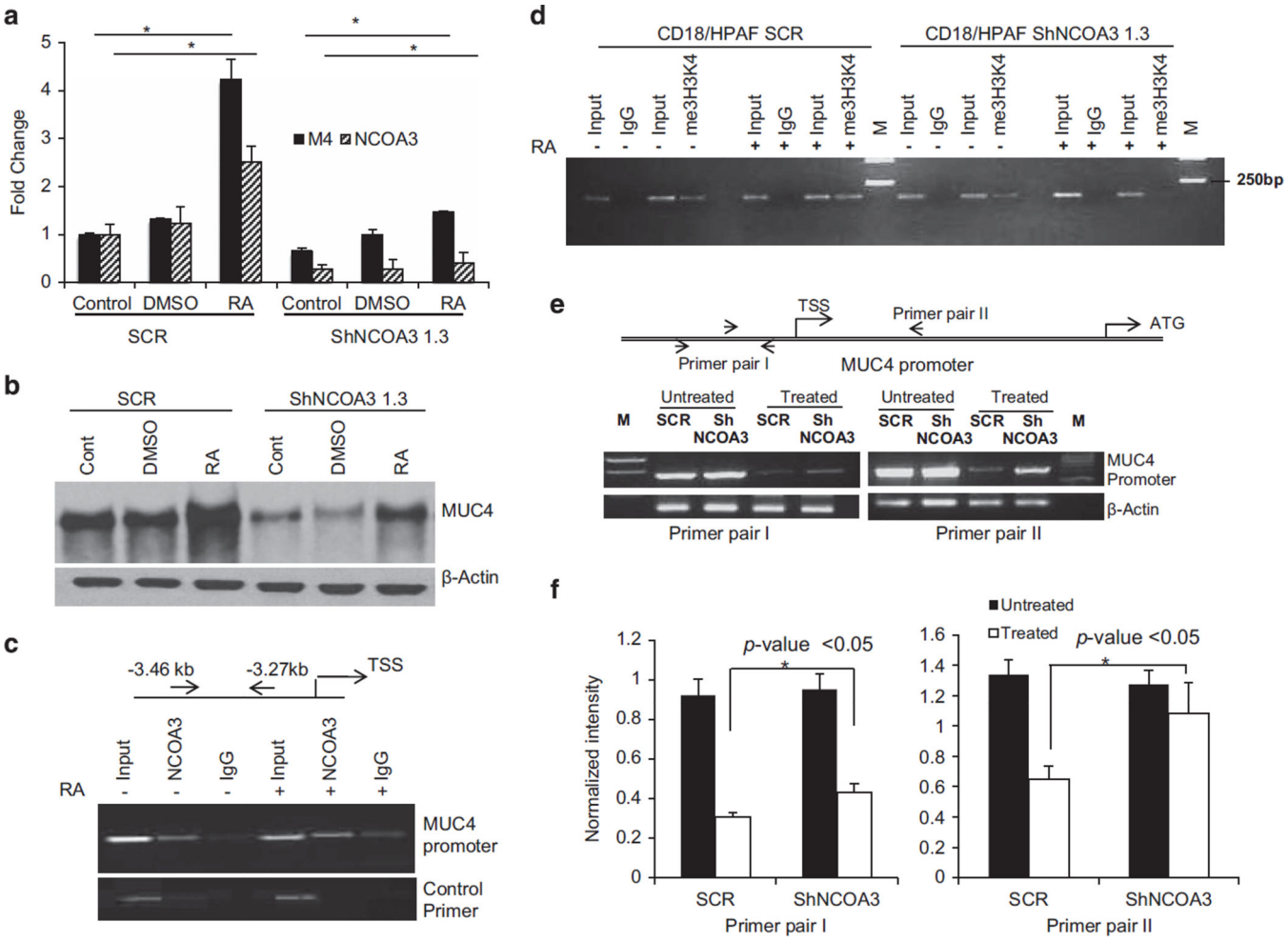

Figure 4.

NCOA3 abrogates RA-mediated MUC4 upregulation. (**a**) RA mediated upregulation of MUC4 was diminished after NCOA3 KD compared with the scramble control both at RNA (**P*<0.01) and (**b**) protein levels. (**c**) Chromatin immunoprecipitation (ChIP) analysis was performed on CD18/HPAF cells after 24 h serum starvation, followed by 1 h of RA treatment using NCOA3 antibody. There was enrichment of the fragment in the MUC4 distal promoter region close to the transcriptional start site (TSS). (**d**) ChIP analysis was performed with me3H3K4 antibody in NCOA3 KD CD18/HPAF cells after 24 h of serum starvation followed by treatment with RA for 1 h. The primers positioned close to TSS at MUC4 locus were used to analyze the enriched fractions. There was reduction in the active transcriptional mark in NCOA3 KD cells even after RA treatment. (**e**) PCR amplification by two sets (I, II) of primers targeting MUC4 promoter region from the DNA isolated from scrambled and NCOA3 KD clones after micrococcus nuclease digestion. (**f**) Quantification of PCR products showed significantly (**P*<0.05) higher amplification in ShNCOA3 clone reflecting the poor accessibility of MUC4 promoter after NCOA3 KD.

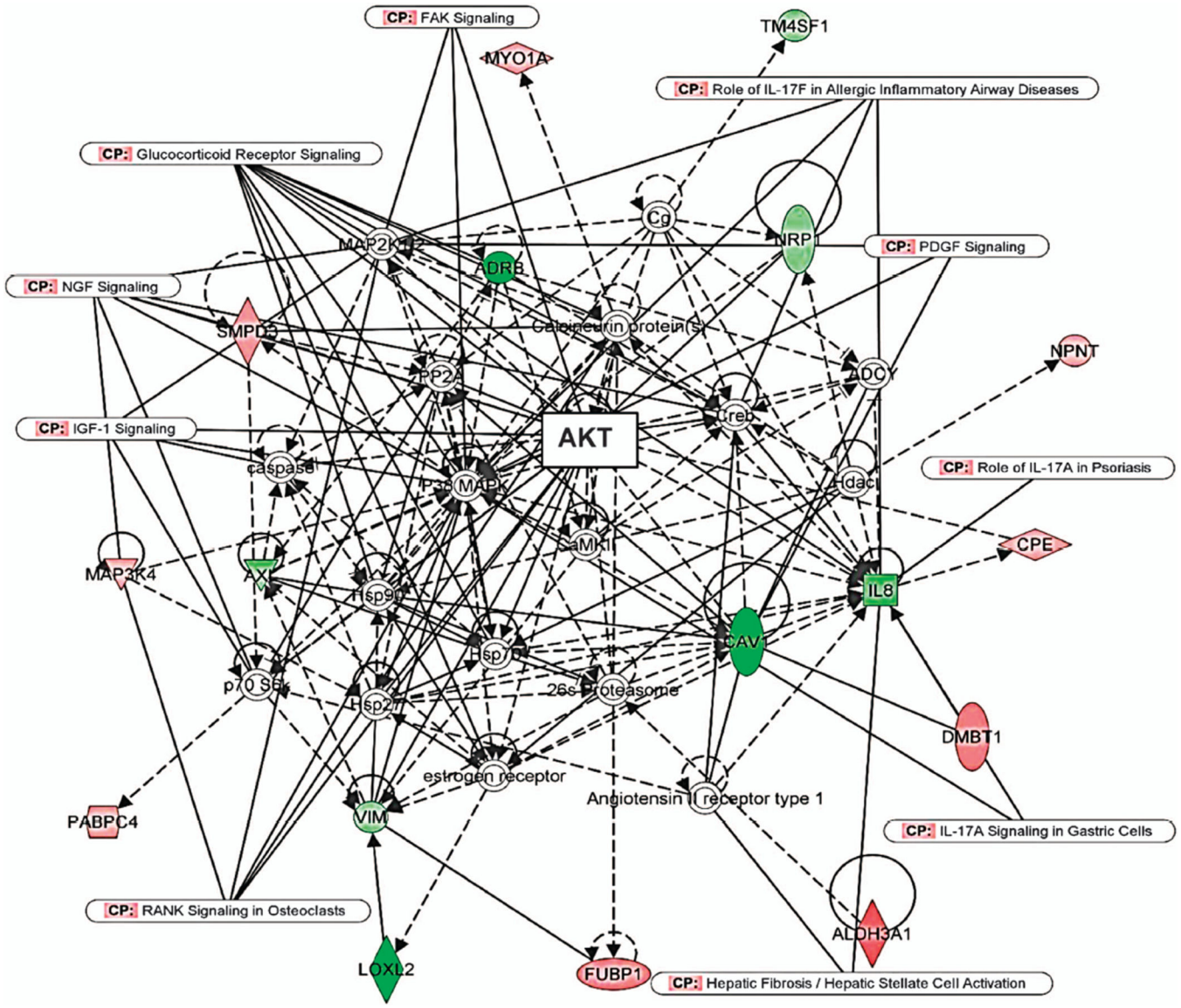

Figure 5.

NCOA3 upregulation initiates a self-perpetuating mechanism of PC progression. Microarray data was analyzed by IPA software (Ingenuity System, Redwood City, CA, USA) to reveal the top differentially regulated genes, CP and networks modulated by NCOA3 KD. The most significant network revealed after NCOA3 KD includes AKT and P38 MAPK as the dominant nodes and involves disease and functions like cellular movement, cancer and tumor morphology. Other networks including ERK1/2, ubiquitin C and NF-kB were also influenced by NCOA3 expression (Supplementary Figure 5). The genes upregulated and downregulated in the network are shown in red and green colors, respectively. The network is overlaid by CPs differentially regulated after NCOA3 KD.

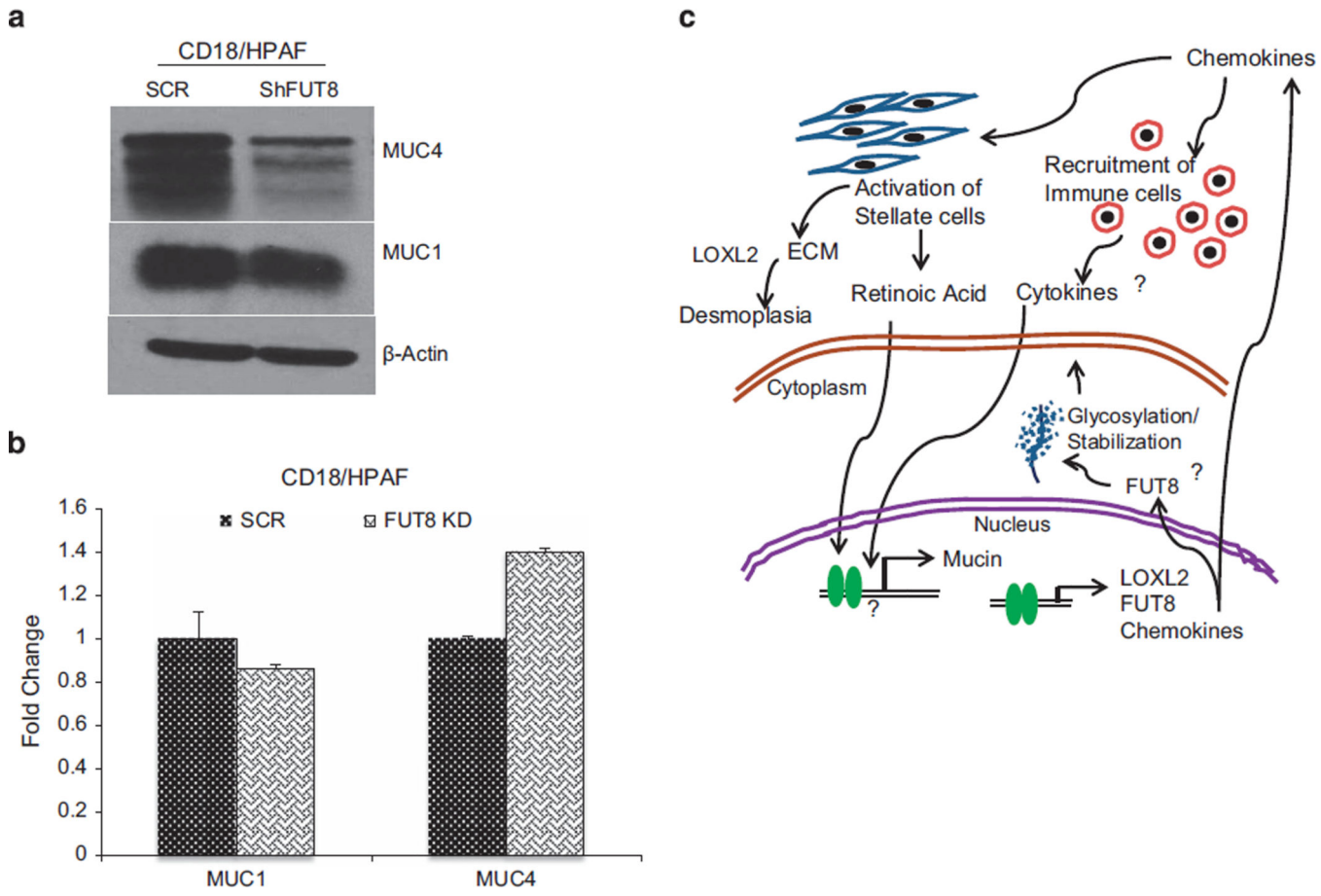

Figure 6.

NCOA3 regulates mucin stability through glycosylation. (**a**) Stable KD of FUT8 in the CD18/HPAF PC cell lines (Supplementary Figure 6B) resulted in the downregulation of MUC4 and MUC1 levels, however, real-time RT-PCR analysis showed no significant difference in the expression of MUC4 and MUC1 at transcriptional levels (**b**), reflecting the indirect regulation of mucins by NCOA3 through protein stability. (**c**) Schematic presentation of the overall regulation of mucins by NCOA3. NCOA3 upregulated the expression of chemokines, LOXL2 and FUT8. Chemokines participate in the recruitment of immune cells, perpetuation of pro-inflammatory conditions and activation of pancreatic stellate cells; LOXL2 crosslinks collagen to harden desmoplasia and FUT8 is responsible for the post-translational stability of mucins through glycosylation/fucosylation. Taken together, NCOA3 regulates mucin expression at transcriptional and post-translational levels during the progression of PC.

Table 1

Incidence of NCOA3 expression in the context of MUC1 and MUC4 positivity in primary tumors and metastatic lesions of pancreatic cancer

Type of tissue	MUC4 positive	MUC4 negative	MUC1 positive	MUC1 negative
Pancreatic tumor, n=34				
NCOA3 positive	18 (53.0%)	14 (41.1%)	27 (79.4%)	5 (14.7%)
NCOA3 negative	1 (2.9%)	1 (2.9%)	0	2 (5.8%)
Liver metastasis, n=22				
NCOA3 positive	6 (27.2%)	11 (50%)	16 (72.7%)	1 (4.5%)
NCOA3 negative	1 (4.5%)	4 (18.1%)	3 (13.6%)	2 (9.0%)
Lung metastasis, n=14				
NCOA3 positive	6 (50%)[a]	3 (25%)	10 (71.4%)	2 (14.2%)
NCOA3 negative	1 (8.3%)	2 (16.6%)	2 (14.2%)	0
LN metastasis, n=17				
NCOA3 positive	11 (64.7%)	5 (29.4%)	13 (76.4%)	3 (17.6%)
NCOA3 negative	1 (5.88%)	0	0	1 (5.8%)

Abbreviations: LN, Lymph Node; NCOA3, nuclear receptor co-activator 3.

[a]Only 12 spots for the MUC4 staining were evaluable.