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TNBC-Specific UGDH-AS1+ NK Cells Encode NSKM, Promoting Cancer Progression by Inhibiting NK Cells Activity

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TNBC-Specific UGDH-AS1+ NK Cells Encode NSKM, Promoting Cancer Progression by Inhibiting NK Cells Activity

Running title: NKSM Suppresses NK Cells to Promote TNBC

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Abstract

Deactivation of immune cells is a major characteristic in various malignant tumors, including breast cancer, a complex and malignant cancer. Here, using single-cell RNA sequencing, we identified UGDH-AS1^{+} NK cells specific to the triple-negative breast cancer(TNBC) subtype, which encode the micropeptide NKSM promoting cancer progression by inhibiting NK cell activity. NKSM was upregulated in UGDH-AS1^{+} NK cells and associated with TNBC-infiltrating (TINK) NK cells antitumor activity. Conditional NKSM knock-in into NK cells of mice resulted in NK cell deactivation and increased tumor growth. Targeted NKSM therapy effectively reduced tumor growth in TNBC mouse models. We found that UGDH-AS1^{+} NK cells are shaped by the tumor microenvironment (TME). Upregulated by the TGF-β signaling pathway, NKSM could bind to proto-oncogene c-Myc, inhibiting ERK1/2-mediated Ser62 phosphorylation and reducing its stability, thereby modulating the transcription of T-bet, a key protein involved in NK cell function, and leading to NK cell deactivation. TGF-β signaling pathway convert TINK cells into UGDH-AS1^{+} NK cells and targeting the expression of NKSM restrain cancer progression in TNBC.

Keywords: TNBC, UGDH-AS1^{+} NK cells , Micropeptide, Immunotherapy

Introduction

Breast cancer continues to be the most frequently diagnosed malignancy and the primary cause of cancer-related deaths among women worldwide[1]. Breast cancers are typically classified according to both molecular and histological subtypes[2]. Molecularly, tumors are divided into hormone receptor positive (HR^+), human epidermal growth factor receptor 2 positive ($HER2^+$), and triple negative breast cancers (TNBC)[3]. HR^+ breast cancers (luminal A and luminal B) are most common (60%-70%), characterized by estrogen receptor (ER) and progesterone receptor (PR) expression. Luminal A tumors have the best prognosis among all subtypes, while patients with luminal B tumors generally experience shorter overall and disease-free survival compared to Luminal A tumors[4, 5, 6]. HER2-enriched tumors are distinguished by their overexpression of the HER2 oncogene and typically show low-to-absent expression of ER/PR. $HER2^+$ disease is associated with a poor prognosis; however, advancements in anti-HER2 therapies have significantly enhanced outcomes for women diagnosed with $HER2^+$ breast cancer[7, 8]. Triple-negative breast cancer (TNBC) is a malignant subtype of breast cancer that accounts for approximately 10-24% of all breast cancer cases[9]. TNBC lacks expression of ER, PR, and HER2 and has low sensitivity to chemotherapy[10]; hence, traditional therapy and regular immunotherapy are ineffective in TNBC patients. Although several new antitumor immunotherapies have achieved great success, such as immune checkpoint inhibitors and chimeric antigen receptor (CAR)-T cells, TNBC patients still have a higher rate of recurrence

60 and a worse prognosis than patients with other forms of breast cancer[11]. Therefore,
61 TNBC remains a large challenge in clinical treatment, and novel therapeutic strategies
62 or improvements to existing therapies urgently need to be developed.
63 Natural killer (NK) cells are innate cytolytic and cytokine-producing lymphocytes that
64 play critical roles in antitumor and antiviral responses. Unlike T lymphocytes, NK
65 cells can efficiently kill MHC class I-deficient tumor cells, highlighting the possibility
66 of developing antitumor immunotherapy based on NK cells, such as CAR-NK cells
67 and immune checkpoint inhibitors (e.g., PD-1 inhibitors). However, due to the
68 complex tumor microenvironment, the state of tumor-infiltrating NK cells is
69 frequently dysfunctional, and these cells are deactivated in various solid tumors[12]. For
70 example, TGF-β can inhibit NK cell function by modulating the NK cell chemokine
71 receptor repertoire[13], tumor-associated fibroblasts in melanoma can modulate cell-to-
72 cell interactions and release PEG2 to inhibit NK cell function[14], and HIF1-α and c-
73 Myc control the activity of NK cells by regulating NK cell metabolism[15, 16]. Therefore,
74 restoring the function and increasing the activity of tumor-infiltrating NK cells will
75 provide a novel immunotherapeutic strategy for TNBC treatment.
76 In the past decade, long noncoding RNAs (lncRNAs) have been reported to be
77 involved in many biological processes, including cancer, the immune response, and
78 inflammation[17, 18]. Traditionally, lncRNAs are defined as RNA transcripts that are
79 longer than 200 nt and lack a protein-coding ability[19, 20], but recent studies have
80 reported that some RNAs previously defined as lncRNAs can encode functional
81 protein products[21, 22], such as *LINC00278*, which has been demonstrated to encode the

micropeptide YY1BM to promote apoptosis in esophageal squamous cell carcinoma (ESCC) cells[23]. In TNBC, the lncRNA-encoding polypeptide ASRPS can inhibit angiogenesis by blocking STAT3 phosphorylation, thereby suppressing the tumor growth of TNBC[24]. Although lncRNAs have been revealed to play critical roles in NK cells[25], no previous study has investigated the function of lncRNA-encoded peptides in NK cells.

In this study, we identified TNBC-specific UGDH-AS1^{+} NK cell clusters, which encode the micropeptide NKSM promoting cancer progression by inhibiting TNBC-infiltrating NK (TINK) cells activity. Compared to other breast cancer subtypes, we identified high expression of UGDH-AS1, an lncRNA, specifically in TNIK, which encodes the micropeptide NKSM. Based on transgenic mouse model in which NK cells conditionally expressed NKSM, we demonstrated that NKSM could suppress NK cell activity and promote TNBC tumor growth. We also showed that targeting NKSM is a potential antitumor immunotherapy in TNBC. Mechanically, in TINK cells, NKSM was shown to be upregulated by the TGF-β signaling pathway and to regulate the stability of the c-Myc protein by interfering with c-Myc phosphorylation. Our findings reveal the mechanism by which NKSM suppresses TINK cell activity and provide innovative clues for designing and improving NK cell-based immunotherapy for TNBC.

Results

ScRNA-seq analysis identifies a TNBC-specific UGDH-AS1^{+} NK cell subset in breast cancer

To construct a breast cacner single-cell transcriptome atlas for tumor-infiltrating NK cells, we analyzed single-cell RNA-sequencing (scRNA-seq) data derived from six TNBC patients, five HR^{+} patients, and three HER2^{+} patients. After quality control (QC) and data merging, a total of 1 209 single cells were used to construct a TNBC microenvironment atlas (Fig.S1A). These cells were classified into six main clusters, which contained epithelial cells, macrophages, fibroblasts, T cells, B cells, and NK cells (Fig.S1B). SingleR and known cell markers included in the CellMarker database were used for cell type identification (Fig.S1C). The top markers of the main clusters were visualized as a heatmap (Fig.S1D and Table S4). Interestingly, the lncRNA *UGDH-AS1* was highly upregulated in TINK cells (Fig.S1D). We next extracted NK cell data for further analysis. In addition, to compare TINK and non-TNBC infiltrating NK cells (HR^{+} infiltrating NK: HRINK, HER2^{+} infiltrating NK: HER2INK), we merged 213 TINK cells and 769 NK cells derived from HR^{+} and HER2^{+} scRNA-seq dataset (Fig.S2A-E, Fig.S3A, and Table S5). Through further analysis, we found the integrated NK cells can be further subdivided into 5 subsets: DNAJB1^{+} NK, GZMH^{+} NK, IL17R^{+} NK, ITGAE^{+} NK, UGDH-AS1^{+} NK (Fig.1A and B, Fig.S3A and B). Interestingly, compared to other breast cancer subtypes, UGDH-AS1^{+} NK cell clusters were predominantly found in TINK cells (Fig.1C). As shown in

122 Fig. 1D and Fig. S3C and D, UGDH-AS1$^+$ NK cells showed a deactivated status,
123 which were consistent with decreased expression of FCGR3A (CD16), KLRB1
124 (NK1.1), LAMP1 (CD107a) and CD244. Moreover, the expression of the
125 transcription factors TBX21 (T-bet) and EOMES (Eomesodermin), as well as that of
126 the genes encoding the cytolytic molecules granzyme B (GZMB), perforin (PRF1)
127 and IFN-γ (IFNG) were also significantly downregulated in UGDH-AS1$^+$ NK (Fig.
128 S3D). Subsequently, Kaplan-Meier survival curves show that patients with breast
129 cancer in the TCGA dataset with high UGDH-AS1$^+$ NK cells have shorter overall
130 survival (Fig.1E and Fig.3E). These data indicated that UGDH-AS1$^+$ NK cells are
131 closely associated with the occurrence, progression, and poor prognosis of TNBC.

132 ***UGDH-AS1* suppressed NK cell activity by blocking IFN-γ expression**

133 Considering that *UGDH-AS1* is one of the top markers of deactivated UGDH-AS1$^+$
134 NK cells, we hypothesized that *UGDH-AS1* may suppress the activity of UGDH-
135 AS1$^+$ NK cells. Hence, we isolated NK cells from peripheral blood mononuclear
136 cells (PBMCs; PBNK cells) or breast cancer tissues (TINK cells，HRINK cells, and
137 HER2INK cells) (Fig.S4A and B). QPCR data showed that *UGDH-AS1* expression
138 was significantly upregulated in TINK cells compared with PBNK or non-TINK cells
139 (Fig.S4C and D).

140 The protein levels of GZMB, PRF1, and IFN-γ were decreased in *UGDH-AS1*
141 overexpressed NK-92MI cells (Fig.2A). We also showed that *UGDH-AS1* could
142 downregulate the expression of GZMB, PRF1, and IFN-γ in NK-92MI cells without

affect the proliferation or survival of NK-92MI cells (Fig.S5A). In addition, we showed that the cytotoxicity of *UGDH-AS1*-overexpressing NK-92MI cells to TNBC cells was less than that of control NK-92MI cells (Fig.2B and Fig.S5B). These data indicated that *UGDH-AS1* expression is associated with NK cell activity.

***UGDH-AS1*$^{+}$ NK cells encoded a micropeptide**

We next biologically characterized *UGDH-AS1*. The *UGDH-AS1* locus is located on chromosome 4 and spans from 39,528,019 to 39,594,707. A nuclear/cytoplasmic fractionation experiment indicated that *UGDH-AS1* was mainly located in the cytoplasm (Fig.S5C). Interestingly, using ribosome profiling (Ribo-seq) data obtained from the GWIPS-viz database, we found that *UGDH-AS1* might contain two small opening reading frames (sORFs) interacting with ribosomes (Fig.2C). Polysome profiling assays also suggested that *UGDH-AS1* could bind to ribosomes (Fig.S5D-F). These data indicated that *UGDH-AS1* is a lncRNA with coding potential.

To validate the coding ability of *UGDH-AS1*, we subsequently cloned the above two sORFs with an in-frame FLAG epitope tag at the C terminus to construct expression vectors. After transfection into 293T cells for 48 h, we determined sORF expression using western blotting with an anti-FLAG antibody. As shown in Fig.2D, western blotting confirmed that sORF1 of *UGDH-AS1*, which encodes a micropeptide of 76 amino acids, has coding ability. Furthermore, to further determine the coding ability of sORF1, we mutated the initiation codon (ATG to ATT) of sORF1 of *UGDH-AS1* and the GFP-ORF to construct FLAG-tagged and GFP-fusion expression vectors

164 (Fig.2E-J). Then, we transfected wild-type (GFPwt and sORF1-FLAG) or mutant
165 (GFPmut, sORF1-GFPmut, sORFmut-GFPmut and sORFmut-FLAG) expression
166 vectors into 293T cells for 48h. Using western blotting and immunofluorescence, we
167 showed that the sORF1-FLAG expression vector, but not the sORF1mut-FLAG
168 expression vector, could express the FLAG-tagged micropeptide (Fig.2F and G). As
169 shown in Fig.2I and J, expression of the GFP fusion protein was observed in the cells
170 transfected with the GFPwt and sORF1-GFPmut expression vectors but not in the
171 cells transfected with GFPmut and sORFmut-GFPmut.

172 To determine whether the micropeptide encoded by *UGDH-AS1* is endogenously
173 expressed in human cells, we generated a rabbit polyclonal antibody against the
174 micropeptide. Using western blotting and immunofluorescence with the anti-
175 micropeptide antibody, we showed that the micropeptide level detected by the anti-
176 micropeptide antibody was significantly increased when UGDH-AS1 was introduced
177 into 293T cells or NK cells (Fig.2K and L, and Fig.S5G and H). We also detected the
178 micropeptide in 293T cells transfected with GFPwt, sORF1-FLAG, GFPmut, sORF1-
179 GFPmut, sORFmut-GFPmut, and sORFmut-FLAG expression vectors by western
180 blotting with the anti-micropeptide antibody (Fig.2F and I). Moreover, we detected
181 the micropeptide in TINK, HRINK, HER2INK, and PBNK cells, and the data showed
182 that the expression of the micropeptide was much higher in the TINK cells (Fig.2M
183 and N and Fig.S5I). These data validated the specificity of the antibody and the
184 endogenous expression of the *UGDH-AS1*-encoded micropeptide.

185 ***UGDH-AS1*-encoded micropeptide inhibited the antitumor activity of NK cells**
186 **and promoted TNBC tumor growth**

187 Because *UGDH-AS1* could suppress NK cell activity and encode a micropeptide, it
188 was necessary to elucidate how *UGDH-AS1* suppresses NK cell activity, either
189 through its RNA transcript or through its encoded micropeptide. Hence, we next
190 sought to determine how *UGDH-AS1* functions as an NK cell suppressor. We
191 overexpressed full-length *UGDH-AS1* (*UGDH-AS1*-OE) and sORF1 of *UGDH-AS1*
192 (sORF1-OE) in NK-92MI cells and injected these cells into TNBC xenograft mice. As
193 shown in Fig.3A, compared with mice injected with control NK-92MI cells, the mice
194 injected with *UGDH-AS1*-OE or sORF1-OE NK-92MI cells had significantly
195 increased tumor growth, and the tumor growth inhibition effects of *UGDH-AS1*-OE
196 and sORF-OE NK-92MI cells were not significantly different. We further knocked
197 down *UGDH-AS1* in *UGDH-AS1*-OE (*UGDH-AS1*-OE-KD) and sORF-OE (sORF-
198 OE-KD) NK-92MI cells using *UGDH-AS1*-specific shRNAs and injected the cells
199 into TNBC xenograft mice. Compared with the *UGDH-AS1*-OE NK-92MI cell-
200 injected mice, the *UGDH-AS1*-OE-KD NK-92MI cell-injected mice showed
201 decreased tumor growth (Fig.3B). However, the sORF1-OE-KD and sORF1-OE NK-
202 92MI cell-injected mice did not show a significant difference in tumor growth
203 (Fig.3C). These data suggested that *UGDH-AS1* suppresses NK cell activity through
204 its encoded micropeptide but not its RNA transcript. Therefore, we named the *UGDH-*
205 *AS1*-encoded micropeptide NK cell suppressor micropeptide (NKSM).

206 **Decreasing NK cell activity resulted in faster tumor growth in *NKSM*$^{+/+}$ mice**

207 To investigate NKSM function in NK cells and TNBC tumors, we generated *NKSM*$^{+/+}$
208 mice, which harbor a conditional knock-in of NKSM specifically in NK cells
209 (Fig.S6A and B). *NKSM*$^{+/+}$ mice did not exhibit changes in the numbers of NK cells in
210 different organs (Fig.S6C and D) or the proliferation and survival of NK cells
211 (Fig.S6E). Our data showed that the production of IFN-γ, GZMB, and PRF1 in
212 *NKSM*$^{+/+}$ mouse NK cells isolated from the spleen was significantly reduced (Fig.3D).
213 Then, we examined the antitumor activity of *NKSM*$^{+/+}$ NK cells in vivo. We
214 subcutaneously transplanted 1 x 10^6 E0771 cells into wild-type and *NKSM*$^{+/+}$ mice
215 and found that the tumor growth in *NKSM*$^{+/+}$ mice was significantly increased
216 (Fig.3E). We also established lung metastasis mouse models by tail vein injection of 5
217 x 10^5 E0771 cells and found that tumor lung metastasis was increased in *NKSM*$^{+/+}$
218 mice (Fig.3F). We further conditionally knocked in NKSM in *MMTV-PyMT* mice
219 (*MMTV-PyMT*;*NKSM*$^{+/+}$), a spontaneous breast cancer mouse model. Consistent with
220 the results for *NKSM*$^{+/+}$ mice, we observed similar tumor growth in *MMTV-*
221 *PyMT*;*NKSM*$^{+/+}$ mice (Fig.3G), and tumor lung metastasis was also increased in
222 *MMTV-PyMT*;*NKSM*$^{+/+}$ mice (Fig.3H). These data demonstrated that NKSM inhibits
223 the antitumor activity of NK cells and thereby promotes TNBC progression.

224 **TGF-β signaling pathway promoted the formation of UGDH-AS1^{+} NK cells and**
225 **the expression of NKSM**

226 To explore the mechanism of NKSM upregulation in UGDH-AS1^{+} NK cells, we

227 predicted the transcription factor binding sites in the *UGDH-AS1* promoter.
228 Surprisingly, we found that *UGDH-AS1* has a potential TGF-β signaling pathway key
229 protein (SMAD2/3/4) binding site in the -184 to -172 region of its promoter (Fig.S7A
230 and B). Because the TGF-β signaling pathway has been reported to play a critical role
231 in NK cells[26], we hypothesized that the TGF-β signaling pathway may upregulate the
232 expression of NKSM in a manner involved in NK cell biological processes. Therefore,
233 we analyzed SMAD2/3/4 chromatin immunoprecipitation (ChIP)-sequencing (ChIP-
234 seq) data derived from different human cells. We showed that these transcription
235 factors could bind to the *UGDH-AS1* promoter (Fig.4A). These data were consistent
236 with our prediction, suggesting that the TGF-β signaling pathway regulates the
237 transcription of *UGDH-AS1*. Indeed, the expression of NKSM was significantly
238 increased after stimulation with TGF-β (Fig.4B).
239 We further performed ChIP experiments using anti-SMAD2/3/4 and confirmed that
240 SMAD2/3/4 could precipitate the *UGDH-AS1* promoter -200 to +300 region (Fig.4C).
241 Moreover, we constructed *UGDH-AS1* promoter luciferase reporters with or without
242 the SMAD2/3/4 binding site (Fig.4D). Luciferase assays showed that *UGDH-AS1*
243 transcription was upregulated by SMAD4 overexpression and downregulated by
244 SMAD4 knockdown in TGF-β-stimulated cells, while the absence of the SMAD2/3/4
245 binding site abolished the above changes (Fig.4E). When we inhibited SMAD3/4
246 using the SMAD3 phosphorylation inhibitor SIS3 or SMAD4-specific small
247 interfering RNAs (siRNAs), respectively, TGF-β-stimulated NKSM upregulation was
248 significantly abolished (Fig.4F and G). Taken together, our results demonstrated that

249 the TGF-β signaling pathway was responsible for the upregulation of NKSM
250 expression by promoting *UGDH-AS1* transcription.

251 **NKSM interacted with c-Myc**

252 To explore how NKSM is involved in NK cell biological processes, we identified
253 NKSM-interacting proteins using coimmunoprecipitation (co-IP) with an anti-FLAG
254 antibody coupled with mass spectrometry (MS) analysis of NK-92MI and 293T cells
255 transfected with an NKSM-FLAG expression vector (Fig.5A and B). The subsequent
256 co-IP and western blot results indicated that NKSM could interact with the proto-
257 oncogene protein c-Myc (Fig.5C). To further confirm this interaction, we co-
258 transfected NKSM-FLAG and c-Myc-HA expression vectors into 293T and NK-92MI
259 cells and performed co-IP assays using anti-FLAG and anti-HA antibodies, and the
260 corresponding western blot results confirmed the interaction between NKSM and c-
261 Myc (Fig.5D). Moreover, we showed that an anti-c-Myc antibody could precipitate
262 endogenous c-Myc and NKSM in NK-92MI cells (Fig.5E).

263 **NKSM reduced the stability of the c-Myc protein by blocking Ser62**
264 **phosphorylation**

265 We next sought to map the NKSM binding domain in the c-Myc protein. We
266 constructed vectors expressing HA-tagged c-Myc truncation variants and
267 cotransfected them with the NKSM-FLAG expression vector into 293T cells (Fig.5F).
268 Co-IP with an anti-FLAG antibody and western blotting with anti-FLAG and anti-HA
269 antibodies indicated that NKSM was bound to the 1-144 region of c-Myc (Fig.5G).

270 Because the 1-144 region of c-Myc contains the conserved phosphorylation site
271 Ser62[27], which increases the stability of the c-Myc protein[28], and c-Myc is crucial for
272 NK cell function[16], we hypothesized that NKSM could negatively regulate c-Myc
273 Ser62 phosphorylation. Interestingly, we found that both the protein level and Ser62
274 phosphorylation of c-Myc were decreased in NKSM-overexpressing cells (Fig.5H).
275 To exclude another conversed phosphorylation site, Thr58, of c-Myc, we also co-
276 transfected NKSM-FLAG with c-Myc-WT or the c-Myc mutants S62A, T58A or
277 S62A/T58A into 293T cells. The results showed that NKSM decreased only c-Myc
278 Ser62 phosphorylation (Fig.5I). Furthermore, to exclude the effect of protein stability
279 on c-Myc Ser62 phosphorylation, we treated NKSM-transfected cells with the
280 proteasome inhibitor MG132 to inhibit protein degradation. As shown in Fig.5J,
281 NKSM inhibited c-Myc Ser62 phosphorylation in both the absence and presence of
282 MG132 but decreased the c-Myc protein level only in the absence of MG132,
283 suggesting that NKSM decreased the c-Myc protein level by reducing its stability. We
284 also performed cyclohexamide (CHX) assays and measured the c-Myc protein level.
285 Our data indicated that NKSM could significantly reduce the half-life of the c-Myc
286 protein (Fig. 5K and L).
287 Furthermore, because c-Myc Ser62 phosphorylation is mediated by ERK1/2[28], we
288 next used co-IP to determine whether the interaction between ERK1/2 and c-Myc
289 could be altered by NKSM. As shown in Fig.5M, the interaction of c-Myc and
290 ERK1/2 was blocked in NKSM-overexpressing cells. We also showed that NKSM
291 could not regulate phosphorylation and protein level of c-Myc when cells were treated

292 with SCH772984, a selective ERK1/2 inhibitor (Fig.5N). These data suggested that
293 NKSM regulates the c-Myc protein level in NK cells by blocking ERK1/2-mediated
294 c-Myc Ser62 phosphorylation and reducing c-Myc protein stability.

295 **NKSM regulated NK cell activity by downregulating c-Myc-mediated T-bet**
296 **expression**

297 Given that the transcription factor T-bet controls the expression of the key cytokines
298 for NK cell function[29], we next examined T-bet expression and IFN-γ, GZMB and,
299 PRF1 production in NKSM-overexpressing and control NK-92MI cells. As shown in
300 Fig.6A and B, the expression of T-bet and the production of GZMB, PRF1, and IFNG
301 were significantly reduced by NKSM overexpression. To investigate whether NKSM-
302 regulated c-Myc controls the transcription of T-bet, we predicted a potential c-Myc
303 binding site in the promoter of T-bet (Fig.S7C and D). Then, we analyzed public c-
304 Myc ChIP-seq datasets and visualized c-Myc occupancy on the T-bet promoter. The
305 data suggested that c-Myc potentially bound to -149 to -160 regions of the T-bet
306 promoter (Fig.6C), which was subsequently confirmed by ChIP assays with an anti-c-
307 Myc antibody (Fig.6D and Fig.S7E). Moreover, we constructed T-bet promoter-
308 containing luciferase reporters with or without c-Myc binding sites and showed that T-
309 bet transcription was inhibited in both NKSM-overexpressing cells and c-Myc-
310 knockdown cells using luciferase assays (Fig. 6E and Fig.S7F). Such changes were
311 abolished by c-Myc binding site deletion (Fig.6E). Furthermore, when c-Myc and T-
312 bet were knocked down, the expression of IFN-γ, GZMB and PRF1 in NKSM-
313 overexpressing and control NK-92MI cells was not significantly different (Fig.S7G).

314 Our data suggested that T-bet was transcriptionally downregulated by NKSM-
315 regulated c-Myc in NK cells, leading to reduced production of GZMB, PRF1, and
316 IFN-γ, which in turn inhibited NK cell activity.

317 **NKSM was a potential target in TNBC immunotherapy**

318 Based on our findings, we hypothesized that blocking NKSM expression to prevent
319 NK cells from being deactivated by the TGF-β signaling pathway in the TNBC tumor
320 microenvironment may provide a novel strategy for TNBC NK cell-based antitumor
321 immunotherapy. Therefore, we generated an NKSM knockout (NKSM-KO) NK-
322 92MI cell line (Fig.S8A-C). Our data indicated that NKSM-KO NK-92MI cells could
323 produce more IFN-γ, GZMB, and PRF1 and had a higher cytotoxicity to K562 cells in
324 culture medium from TNBC cells with high TGFB1 expression (MDA-MB-231 and
325 Hs578T cells) (Fig.6F and Fig.S8D). We next injected WT or NKSM-KO NK-92MI
326 cells into TNBC xenograft mice to determine their antitumor effect in vivo. We found
327 that the injection of NKSM-KO NK-92MI cells significantly reduced tumor growth
328 and improved mouse survival (Fig.6G and Fig.S8E, and Fig.6H). Therefore, pre-
329 blocking NKSM expression has the potential to improve the efficacy of NK-cell based
330 antitumor immunotherapy.

331 **Discussion**

332 NK cells are innate lymphocytes which play a crucial role in antitumor immune
333 responses. However, the deactivation of NK cells is a common phenomenon in the

334 tumor microenvironment[12]; hence, enhancing the activity of NK cells is a major NK-
335 cell-based immunotherapeutic strategy for multiple solid and hematologic cancers. In
336 the present study, compared to other breast cancer subtypes, we identified UGDH-
337 AS1$^+$ NK cells specific to TNBC, UGDH-AS1$^+$ NK cell clusters were predominantly
338 found in TINK cells. UGDH-AS1$^+$ NK cells encoded the micropeptide NKSM, which
339 promoting cancer progression by inhibiting NK cell activity. NKSM was
340 transcriptionally regulated by the TGF-β signaling pathway and could block the Ser62
341 phosphorylation of the proto-oncogene protein c-Myc to reduce the stability of c-Myc,
342 which in turn inhibited the expression of T-bet, a key transcription factor for NK cell
343 function, and ultimately led to decreased GZMB, PRF1, and IFN-γ production in
344 UGDH-AS1$^+$ cells. Inspired by the function of NKSM, we also propose silencing
345 NKSM expression as a potential NK-cell-based immunotherapy for TNBC.
346 In recent decades, due to the development of clinical diagnosis and antitumor therapy
347 approaches, the survival of breast cancer patients has significantly improved. Luminal
348 A tumors have the best prognosis among all subtypes, while patients with luminal B
349 tumors generally experience shorter overall and disease-free survival compared to
350 Luminal A tumors[4, 5, 6]. HER2$^+$ breast cancer is associated with a poor prognosis;
351 however, advancements in anti-HER2 therapies have significantly enhanced outcomes
352 for women diagnosed with HER2$^+$ breast cancer[7, 8]. Nevertheless, TNBC, the
353 aggressive subtype of breast cancer that accounts for approximately 10-24% of all
354 breast cancer cases[9], still lacks efficient therapy, resulting in a poor prognosis for
355 TNBC patients. To date, the common approaches for TNBC treatment are

356 chemotherapy and radiotherapy, and a novel targeted agent for TNBC treatment,
357 sacituzumab govitecan, which targets the Trop-2 protein, was recently approved by
358 the FDA; however, the development of efficient TNBC therapies is still an urgent
359 need. Moreover, immunotherapy for TNBC is drawing attention because TNBC
360 presents more neoantigens for recognition by the immune response than other breast
361 cancers, but TNBC frequently evades the immune system[30]. Therefore, exploring the
362 characteristics of TNBC-infiltrating immune cells holds promise for improving
363 therapeutic strategies for TNBC patients. Inspired by immunotherapy based on T cells,
364 such as CAR-T cells, which have been widely studied and registered in clinical trials
365 for several solid and hematologic cancers, including TNBC[31, 32], NK-cell-based
366 immunotherapy is recognized as having the potential to improve the outcomes of
367 TNBC patients. Here, we identified a deactivated cluster of UGDH-AS1^{+} NK cells in
368 TNBC tumor tissues by analyzing integrated scRNA-seq data. The lncRNA UGDH-
369 AS1, a coding lncRNA from which the NK cell suppressor NKSM is expressed, acts
370 as a marker gene for deactivated TINK cells. We showed that NKSM but not the
371 lncRNA UGDH-AS1 could inhibit the activity of NK cells and promote TNBC tumor
372 growth.

373 To elucidate the mechanism by which NKSM is upregulated in deactivated UUGDH-
374 AS1^{+} NK cells, we analyzed the promoter of UGDH-AS1 by bioinformatic prediction
375 and ChIP-seq data integration. Our data strongly suggested that SMAD2/3/4, the key
376 transcription factors of the TGF-β signaling pathway, interacted with the UGDH-AS1
377 promoter. Subsequent ChIP assays and luciferase reporter assays validated that

378 SMAD2/3/4 were responsible for UGDH-AS1 upregulation and confirmed the
379 SMAD2/3/4 binding region of the UGDH-AS1 promoter. TGF-β is a pleiotropic
380 cytokine that widely regulates biological processes in cancers, such as migration,
381 invasion, apoptosis, and angiogenesis[33]. In TNBC patients, a high level of TGF-β has
382 been reported to be associated with a worse prognosis[34]; this phenomenon may be due
383 to the negative regulatory effect of TGF-β on NK cell functions mediated by
384 influencing several signaling pathways[35, 36, 37] or changing the metabolic pattern of
385 NK cells[26]. Hence, improving the ability of NK cells to protect against TGF-β-
386 induced deactivation would benefit TNBC NK cell-based immunotherapies. Our
387 study identified a novel mechanism by which TGF-β regulates NK cell functions,
388 which improved the current knowledge of the mechanism that results in deactivated
389 tumor-infiltrating immune cells, particularly TINK cells.

390 Furthermore, through co-IP assays and mass spectrometry, we demonstrated that
391 NKSM could endogenously interact with the proto-oncogene protein c-Myc and block
392 c-Myc Ser62 phosphorylation by reducing the interaction of c-Myc and ERK1/2,
393 thereby reducing the protein stability of c-Myc and decreasing c-Myc protein level in
394 NK cells. Interestingly, it is known that TGF-β inhibits c-Myc expression in many cell
395 types[38, 39], which is consistent with our findings, supporting the conclusion that
396 NKSM connects the TGF-β and c-Myc signaling pathways. To illuminate the
397 mechanism by which TGF-β inhibits c-Myc expression and suppresses NK cell
398 functions, we performed transcription factor binding prediction and ChIP-seq data
399 analyses, as well as ChIP and luciferase reporter assays, on the promoter of T-bet, the

400 critical transcription factor involved in NK cell functions and regulation of the
401 production of the cytokine IFN-γ, GZMB and PRF1[29]. Our data indicated that TGF-β-
402 NKSM-mediated c-Myc could promote T-bet transcription, in turn regulating the
403 production of GZMB, PRF1, and IFN-γ and the activity of NK cells.

404 In recent years, due to the limitations of T cell-based immunotherapies, NK cell-based
405 immunotherapies have been considered a new-generation immunotherapeutic[40].
406 Adoptive transfer of autologous NK cells activated in vitro is one of the main
407 strategies for NK cell-based immunotherapies[41]. However, the tumor
408 microenvironment may induce the deactivation of preactivated NK cells through
409 multiple immunosuppressive pathways, including the TGF-β signaling pathway[42],
410 thereby impairing the efficiency of adoptively transferred NK cells.

411 In summary, we identified TNBC-specific UGDH-AS1^{+} NK cells encode
412 micropeptide NSKM, which promoting TNBC progression by inhibiting NK cell
413 activity. We found the lncRNA-encoded micropeptide NKSM, which was upregulated
414 in TINK cells by the TGF-β signaling pathway and could reduce the c-Myc protein
415 level by blocking ERK1/2-mediated c-Myc Ser62 phosphorylation; these events
416 negatively regulated T-bet transcription and ultimately reduced the production of
417 GZMB, PRF1, and IFN-γ in NK cells (Fig.7). Our study reveals a novel mechanism
418 by which the micropeptide NKSM connects TGF-β and c-Myc in the biological
419 processes of NK functions and highlights that deletion of NKSM could serve as a
420 novel strategy for TNBC immunotherapy.

421 **Materials and Methods**

422 **Human study subjects**

423 Breast cancer tissues (TNBC, n=57; HR^+, n=29; $HER2^+$, n=33) were obtained from
424 patients who underwent tylectomies at the Affiliate Hospitals of Soochow University.
425 None of these patients received anticancer treatment, including chemotherapy or
426 radiotherapy, before surgery. The Ethics Committees of Soochow University approved
427 this study.

428 **Animals and cell cultures**

429 NOD-$Prkdc^{em26}Il2rg^{em26}$/Nju (NCG) female mice (4 - 8 weeks of age) were purchased
430 from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing,
431 China). *MMTV-PyMT* mice were obtained from Zhongjun Dong's laboratory at the
432 Institute for Immunology and School of Medicine, Tsinghua University. All mouse
433 experiments were carried out in accordance with guidelines approved by the
434 Laboratory Animal Center of Soochow University. For xenograft experiments, a total
435 of 0.1 ml of cell suspension (1×10^6/ml) was injected subcutaneously into the hind
436 flank of mice. For NK cell cytotoxicity experiments, different NK-92MI cell lines
437 were injected via the mouse tail vein (twice a week for 4 weeks) when the xenograft
438 tumor volume reached ~100 mm^3.

439 All cell lines were purchased from Procell Life Science & Technology Co., Ltd
440 (Wuhan). These cell lines were all characterized by DNA fingerprint analysis and

441 passaged less than 6 months in this study. DMEM and fetal bovine serum (FBS) were
442 purchased from Invitrogen. MDA-MB-231 and 293T cells were grown in DMEM
443 supplemented with 10% FBS; Hs578T cells were grown in DMEM supplemented
444 with 10% FBS, 0.01 mg/mL bovine insulin; NK-92MI cells were grown in MEM-
445 alpha supplemented with 12.5% FBS, 12.5% Horse serum, 0.2 mM inositol, 0.02 mM
446 folic acid, 0.1mM beta-mercaptoethanol. All cell lines were grown in
447 penicillin/streptomycin-containing medium at 37°C in a humidified atmosphere with
448 5% CO_2.

449 **NK cell isolation**

450 For human NK cell isolation, PBMCs were isolated by centrifugation over a Ficoll400
451 cell separation solution (density 1.077 g/mL; Biocoll, VWR). NK cells were isolated
452 by magnetic cell sorting with the NK Cell Isolation Kit (Miltenyi, 130-092-657) from
453 PBMCs. Fresh tumor tissues were harvested, cut into small pieces, and digested into
454 single-cell suspensions with the Tumor Dissociation Kit (Miltenyi, 130-095-929)
455 using gentleMACS (Miltenyi). NK cells ($CD3^-$ $CD56^+$) were sorted using a BD
456 FACSAria III. For mouse NK cell isolation, NK cells were isolated from mouse
457 spleens by negative selection with the NK Cell Isolation Kit (Miltenyi,130-115-818).

458 **Data acquisition**

459 The scRNA-seq count matrix of TNBC tissues was published in Karaayvaz et al.[43].
460 The scRNA-seq count matrix of HR^+ and $HER2^+$ tissues were published in Liu T et
461 al[44].ChIP-seq data were obtained from the Gene Expression Omnibus (GEO) database

462 (GSE104352, GSE41580, GSE51510, GSE29422, GSE61475, GSE42958, GSE51011,
463 GSE36354). The database of IP contaminants was obtained from the Contaminant
464 Repository for Affinity Purification (CRAPome)[45].

465 **RNA extraction and qRT-PCR**

466 Total RNA was isolated from cells and tissues using TRIzol Reagent (Invitrogen).
467 First-strand cDNA was synthesized with the Superscript II-reverse transcriptase kit
468 (Invitrogen, Carlsbad). All qRT-PCR primers are listed in Table S1.

469 **Cell transfection**

470 Small interfering RNA (siRNA) and non-targeting siRNA control (Table S2) were
471 purchased from GenePharma. Transfections with siRNA were performed with
472 Lipofectamine 3000 (Life Technologies). For overexpression or RNA interference of
473 target genes in NK cells, 10 micrograms of plasmids were mixed with NK-92MI cells
474 and incubated on ice for 20 minutes in sterile electroporation cuvettes (0.2 cm). After
475 electroporation, the NK-92MI cells were immediately cultured in complete culture
476 medium.

477 **ScRNA-seq data analysis**

478 The scRNA-seq data of six TNBC samples were obtained from GEO database
479 (GSE118389). The quality control process was performed by Seurat. Briefly, single
480 cells that expressed less than 200 genes were considered low-quality cells and
481 discarded. Then, the filtered data were merged using the SCTransform and

482 IntegrateData functions in Seurat. The main cell clusters were identified using the
483 FindClusters function (resolution = 0.2) of Seurat and visualized using t-distributed
484 stochastic neighbor embedding (t-SNE), and the results were plotted by the DimPlot
485 function of Seurat. Subsequently, the markers of each main cell cluster were identified
486 by the FindAllMarkers function. The top 10 cluster markers are listed in Table S3.
487 Cell type was recognized by SingleR and combined with markers obtained from the
488 CellMarker database. NK cells were extracted from TNBC cells using the subset
489 function of Seurat for further analysis.
490 The scRNA-seq data of non-TNBC breast cancer tissues (HR^+ and $HER2^+$) were
491 obtained from GEO database (GSE167036). Single cells with less than 100 unique
492 feature counts (nFeature_RNA), with more than 20% mitochondrial UMl or with
493 more than 50% ribosomal UMl counts were considered low-quality cells and
494 discarded. Delete the double cells annotated by the DoubletFinder package (version:
495 2.0.3). We exclude genes detected in fewer than three cells, as well as mitochondrial
496 and ribosomal genes. Seurat package (version: 4.3.0) was used for cell normalization
497 and regression to obtain the scaled data. We selected the top 3000 largest variable
498 genes as highly variable genes (HVGs). We then used the "RunPCA" function to
499 perform the principal component analysis (PCA). The number of significant principal
500 components is determined by the "Elbow" function, and PC is selected as 25. The
501 main cell clusters were identified using the FindClusters function (resolution = 1.0)
502 and visualized using t-SNE, with the results plotted by the DimPlot function.
503 Subsequently, the markers of each main cell cluster were identified by the

504 FindAllMarkers function. The top 10 cluster markers are listed in Table S4. Based on
505 these marker genes, we manually annotated cell clusters, identifying 10 cell
506 populations including T cells, Plasma B cells, Naive B cells, NK/T cells, Myeloid
507 cells,Epithelial cells, Endothelial cells, Fibroblasts, myoepithelial cells, and cycling
508 cells. For NK/T cells, we used a similar dimensionality reduction clustering method.
509 Unlike before, Harmony package is used to remove batch effects on patient_id. The
510 selected PC dimension is 30 and the resolution is 1.5. Based on cell cluster specific
511 marker genes, 976 NK cells were obtained by manual annotation. NK cells were
512 extracted using the subset function of Seurat and merged with NK cells from TNBC
513 by the SCTransform and IntegrateData functions in Seurat. The merged NK cells were
514 clustered by the FindClusters function (resolution = 0.2) and visualized using t-SNE.
515 A hexbin plot of gene expression in single cells was generated with the schex package
516 (version: 1.14.0).

517 **ChIP-seq data analysis**

518 ChIP-seq reads were preprocessed by Fastp software for filtering low-quality reads
519 and aligned to GRCh38 by Bowtie2 with default parameters; the mapped reads of
520 ChIP-seq were preprocessed by SAMtools and then submitted to MACS2 for peak
521 calling with default parameters. The peaks were visualized by Integrative Genomics
522 Viewer (IGV) software.

523 **Transcription factor binding site prediction**

524 For transcription factor binding site prediction for the promoters of *UGDH-AS1* and

525 TBX21, promoter sequences from -2000 to +300 were obtained from Ensembl. Then,
526 the SMAD2/3/4 or MYC binding sites were identified by the R package TFBSTools [46].
527 SMAD (MA0513.1) and MYC (MA0147.3) motifs obtained from JASPAR were used
528 for the analyses.

529 **Cell cytotoxicity assay**

530 TNBC cells were seeded at a density of 1×10^4 cells per well in a 96-well plate. After
531 48 h of culture, the supernatants were collected and centrifuged for further use. Then,
532 1×10^4 K562 cells were cultured in collected TNBC cell medium, and different NK-
533 92MI cell lines were added at various ratios (1:1, 1:5, and 1:15; target cells: effector
534 cells). After 4 h of coculture, the 96-well plate was centrifuged at 250 g for 3 min, and
535 the supernatants were removed to a new flat-bottom 96-well plate for detection using
536 the CyQUANT™ LDH Cytotoxicity Assay (Thermo Fisher Scientific, C20301)
537 according to the manufacturer's instructions. NK cell cytotoxicity was defined as:

538 $$\%\text{Cytotoxicity}=\frac{\text{Experimental value - Effector Cells Spontaneous Control - Target Cells Spontaneous Control}}{\text{Target Cell Maximum Control - Target Cells Spontaneous Control}}*100$$

539 **Immunofluorescence staining**

540 Cells were plated on glass coverslips, fixed with 4% paraformaldehyde (PFA) at room
541 temperature (RT) for 15 min, rinsed three times with PBST (0.3% Triton), incubated
542 with primary antibodies overnight at 4°C, washed four times with TBSTx and
543 incubated with FITC-conjugated secondary IgG antibodies (Beyotime) at RT for 1 h
544 in the dark. Cell nuclei were stained with DAPI.

Western blot

Cells were collected and lysed in RIPA buffer. Proteins were separated on a SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting of membranes was performed using the following primary antibodies: anti-FLAG (sc-807, Santa Cruz), anti-HA (ab9110, Abcam), anti-GFP (2956, CST), anti-phospho-SMAD2 (3108, CST), anti-SMAD2 (5339, CST), anti-phospho-SMAD3 (9520, CST), anti-SMAD3 (9523, CST), anti-SMAD4 (38454, CST), anti-HSPA1A (4873, CST), anti-GCN1 (ab86139, Abcam), anti-c-Myc (ab32072, Abcam), anti-phospho-c-Myc (ab185656, Abcam), anti-DIS3 (ab68570, Abcam), anti-RACK1 (ab129084, Abcam), anti-p-ERK1/2 (ab76299, Abcam), anti-T-bet (5214, CST), anti-IFN-γ (sc-8423, Santa Cruz), anti-GZMB (4275, CST), anti-PRF1 (sc-374346, Santa Cruz) and anti-β-actin (A5441, Sigma-Aldrich). Signals were revealed after incubation with the recommended secondary antibody coupled to peroxidase using enhanced chemiluminescence.

Anti-NKSM antibody preparation

Peptide synthesis and anti-NKSM antibody generation were performed as previously described with some modifications[47]. Briefly, BSA and the OVA-coupled peptide WHEHVSLQPQPPRLK were synthesized, and a polyclonal antibody against NKSM was obtained from inoculated rabbits. The antibody was purified using affinity chromatography on columns containing the corresponding peptide.

565 **Polysome profiling**

566 Polysome profiling was performed to measure the translation of UGDH-AS1, with
567 monitoring by qRT-PCR. We performed polysome profiling following a procedure
568 described in[48]. The primers for qRT-PCR are listed in Table S1.

569 **Cell fractionation of RNA and proteins**

570 To determine the cellular localization of RNAs and proteins, cytosolic and nuclear
571 fractions were collected from cells according to the manufacturer's instructions for the
572 Nuclear/Cytoplasmic Isolation Kit (BioVision).

573 **Co-IP assay**

574 Co-IP assays were performed using the Pierce™ Co-Immunoprecipitation Kit
575 (Thermo Scientific) according to the manufacturer's instructions. Briefly, lysates were
576 applied to columns containing 10 μg of immobilized antibodies covalently linked to
577 an amine-active resin and incubated overnight at 4°C. Then, the coimmunoprecipitate
578 and controls were eluted and analyzed by SDS-PAGE or mass spectrometry. Co-IP
579 assays were performed using the following antibodies: anti-FLAG (sc-807, Santa
580 Cruz), anti-HA (ab9110, Abcam), and anti-c-Myc (ab32072, Abcam).

581 **Mass spectrometry analysis**

582 Samples were analyzed on a Thermo Fisher LTQ Obitrap ETD mass spectrometer.
583 Briefly, samples were loaded onto an HPLC chromatography system (Thermo Fisher
584 Easy-nLC 1000) equipped with a C18 column (1.8 mm, 0.15×1.00 mm). Solvent A

585 contained 0.1% formic acid, and solvent B contained 100% acetonitrile. The elution
586 gradient was from 4% to 18% in solvent A for 182 min and 18% to 90% in solvent B
587 for 13 min at a flow rate of 300 nL/min. Mass spectrometry analysis was carried out
588 at AIMS Scientific Co., Ltd. (Shanghai, China) in the positive-ion mode with
589 automated data-dependent MS/MS analysis with full scans (350-1600 m/z) acquired
590 using Fourier transform mass spectrometry at a mass resolution of 30,000, and the ten
591 most intense precursor ions were selected for MS/MS. MS/MS results were acquired
592 using higher-energy collision dissociation at 35% collision energy and a mass
593 resolution of 15,000.

594 **ChIP**

595 ChIP assays were performed with an EZ-ChIP kit (Millipore, Bedford, MA) according
596 to the manufacturer's instructions. Chromatin from cells was immunoprecipitated
597 with antibodies against SMAD4 (38454, CST) and c-Myc (ab32072, Abcam).

598 **Dual-luciferase reporter assay**

599 Cells were seeded in 24-well plates (1×10^5 cells per well) and cultured to 60–70%
600 confluence before transfection. Then, the cells were transfected with 800 ng of the
601 reporter plasmids described above using Lipofectamine 3000 (Invitrogen). After
602 transfection for 24 h, the cells were collected using 100 µl of passive buffer, and
603 Renilla luciferase activity was detected using the Dual-Luciferase Reporter Assay
604 System (Promega) and a TD-20/20 illuminometer (Turner Biosystems).

605 **Production of *UGDH-AS1*-overexpressing and NKSM-overexpressing cells**

606 To produce cells with overexpression, full-length human UGDH-AS1 or NKSM
607 cDNA was synthesized by GeneWiz (Beijing, China) and cloned into the lentiviral
608 expression vector pLVX-IRES-neo (Clontech Laboratories Inc.). To produce
609 lentiviruses containing a full-length UGDH-AS1 or NKSM targeting sequence, 293T
610 cells were cotransfected with the vector described above and a lentiviral vector
611 packaging system using Lipofectamine 3000. Infectious lentiviruses were collected at
612 48 h and 72 h after transfection and filtered through 0.45-μm filters. These
613 lentiviruses were designated UGDH-AS1-overexpressing and NKSM-overexpressing
614 recombinant lentiviruses, respectively. We used the empty plenty-pLVX-IRES-neo
615 vector to generate negative control lentiviruses. Recombinant lentiviruses were
616 concentrated by centrifugation. The virus-containing pellet was resuspended in
617 DMEM, and aliquots were stored at -80°C until use. Cells were infected with
618 concentrated virus in the presence of polybrene (Sigma-Aldrich). The supernatant was
619 replaced with complete culture medium after 24 h, followed by selection with 800
620 μg/ml G418, and the expression of UGDH-AS1 and NKSM in infected cells was
621 verified by qRT-PCR.

622 **CRISPR/Cas9 mediated gene knockout**

623 NK92MI cells were infected with lentivirus to stably knock out NKSM (hU6-sgRNA-
624 EF1a-SpCas9-NLS-FLAG-P2A-Puro). The gRNA sequence designed specifically for
625 the NKSM initiation codon was 5’-CACTCACTGGTCCAGTCATGAGG-3’. Cells

626 seeded in 96-well plates were spin transduced at 1000 x g for 120min at 37℃ with 5
627 ug/mL polybrene supplemented. The transfected cells were continuously screened
628 with interval puromycin (2.5 ug/mL) screening, taking 7 days as a screening cycle.
629 The expanded cells that survived from puromycin screening were used for western
630 blot analysis, sequencing, and further experiments.

631 **Generation of *NKSM*$^{+/+}$ and *MMTV-PyMT;NKSM*$^{+/+}$ mice**

632 We generated an NKSM-overexpressing mouse model using a gene-targeting
633 construct. A single copy of NKSM cDNA was inserted downstream of the STOP
634 cassette in the ROSA26 (R26) locus through homologous recombination, so
635 exogenous NKSM cDNA was transcribed under the control of the R26 promoter,
636 allowing reproducible and stable overexpression of NKSM. This gene-targeting
637 construct was electroporated into embryonic stem (ES) cells, and microinjection of
638 the recombinant ES clones led to the generation of chimeric animals. To analyze the
639 in vivo consequences of NKSM overexpression, conditional knock-in animals were
640 crossed with the Ncr1-Cre strain to obtain *NKSM*$^{+/-}$ heterozygous mice. The Ncr1-Cre
641 mice were purchased from Biocytogen (China). *NKSM*$^{+/+}$ homozygous animals were
642 generated by intercrossing heterozygous mice. Finally, *NKSM*$^{+/+}$ mice were crossed
643 with C57BL/6 *MMTV-PyMT* mice to generate *MMTV-PyMT;NKSM*$^{+/+}$ mice on the
644 C57BL/6 background.

645 **Mouse xenografts**

646 To generate xenograft models, cells were diluted to a concentration of 1×10^6/mL in

647 PBS, and mice were injected subcutaneously with 0.1 mL of the suspension in the
648 back flank. Tumor volume was calculated according to the following formula: volume
649 = length×width^2×0.5.

650 **Flow cytometry**

651 Non-specific binding of mouse and human cells were blocked by FcR blocking
652 reagent for mice (Cat# 130-092-575, Miltenyi) and human (Cat# 130-059-901,
653 Miltenyi) respectively. Dead cells were excluded using the Zombie Aqua Fixable
654 Viability Kit (BioLegend), and intracellular cytokine staining was performed with the
655 FIX&PERM Kit (FcMACS). Cells were stained for 20 min at 4°C with conjugated
656 antibodies. Flow cytometric analyses were performed with a CytoFLEX
657 (BECKMAN), and data were analyzed using FlowJo software (Treestar).

658 **Statistical analysis**

659 All experiments were repeated at least 3 times unless stated in the figure legend. Data
660 analysis was performed using SPSS 19.0 software for Windows. The significance of
661 differences between datasets is expressed as p values, and $p<0.05$ was considered
662 statistically significant. Survival curves were obtained using the Kaplan-Meier
663 method and compared using the log-rank test. Paired or unpaired Student's t-tests
664 were used for comparisons of various types of data.

665 **Ethics Declarations**

666 Ethical consent was given by Soochow University Committee for Ethical Review of

667 Research Involving Human Subjects. The use of human breast cancer tissue
668 specimens was evaluated and approved by the Ethical Committee of the Affiliate
669 Hospitals of Soochow University, and written informed consents were obtained from
670 all participants or their appropriate surrogates. All animal studies were conducted with
671 the approval of Soochow University Institutional Animal Care and Use Committee
672 and were performed in accordance with established guidelines.

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678 **Declaration of interests**

679 The authors declare no competing interests.

680 **Reference**

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840 **Figure Legends**

841 **Fig. 1 Overview of single cells and NK cells derived from breast cancer tissues**
842 **(TNBC, HR$^+$, HER2$^+$).**
843 (A) T-SNE plot of the integrated NK cells derived from TNBC, Luminal and HER2$^+$
844 breast cancer tissues. (B) Heatmap plot of the top 10 marker genes among major cell
845 types. The colors from gray to red indicate the gene expression levels from low to
846 high. (C) Bar plot depicting the distribution of the NK cells assigned to specific cell
847 clusters, by sample origin. (D) T-SNE plot of the integrated NK cells derived from
848 TNBC, HR$^+$ and HER2$^+$ breast cancer tissues. (E) Kaplan–Meier curves of overall
849 survival by stratifying the patients by high and low proportion of the UGDH-AS1$^+$
850 NK cell type. High fractions of UGDH-AS1 are associated with poor prognosis in the
851 TCGA-BRCA cohort.

852 **Fig. 2 *UGDH-AS1*$^+$ NK cells show immune exhaustion and encode a micropeptide.**
853 (A) Flow cytometry (FACS) analysis of IFN-γ, GZMB, and PRF1 in *UGDH-AS1*
854 overexpressed (OE) and control (empty vector) NK92-MI cells after 16 hours of
855 stimulation with IL-12 and IL-15 (n = 3). (B) Cytotoxic activity of *UGDH-AS1*
856 overexpressed and control NK-92MI cells against MDA-MB-231 cells (n = 3). (C) An
857 overview of predicted sORFs and Ribo-seq data of GWIPS-viz database at *UGDH-*
858 *AS1* genomic region. (D) The sORFs were cloned into the pcDNA3.1 vector and
859 transfected into 293T cells. The sORFs-FLAG fusion proteins were detected by
860 western blotting using an anti-FLAG antibody in the cell lysate. (E) The schematic
861 diagram of the FLAG-tag fusion protein expression plasmids. The initiation codon
862 ATG of the *UGDH-AS1* sORF1 was mutated to ATT. (F and G) ORF-FLAG fusion
863 protein was detected by western blotting (F) with anti-FLAG or anti-micropeptide and
864 immunofluorescence (G) with anti-FLAG. (H) The schematic diagram of the GFP-
865 fusion protein expression plasmids. The initiation codon ATG of the GFP (GFPwt)
866 gene was mutated to ATT (GFPmut). The initiation codon ATG of the *UGDH-AS1*
867 sORF1 was mutated to ATT. (I and J) The specified plasmids were transfected into
868 293T cells. The proteins translated by these plasmids were identified by western
869 blotting (I) using anti-GFP or anti-micropeptide antibodies and the GFP fluorescence
870 (J) was detected using fluorescence microscope. (K) The micropeptide expression
871 level in control and *UGDH-AS1* overexpressed 293T cells was determined by western
872 blotting with anti-micropeptide antibody. (L) Immunofluorescence with anti-
873 micropeptide in control and *UGDH-AS1* overexpressed 293T cells. (M) The
874 micropeptide expression level in infiltrating NK cells from TNBC, Luminal, and
875 HER2 cancer tissues was determined by western blotting using an anti-micropeptide

876 antibody. (N) The micropeptide expression level in TINK and PBNK was determined
877 by western blotting using an anti-micropeptide antibody. (mean ± SD, *p<0.05;
878 **p<0.01 and ***p<0.001).

879 **Fig. 3 The UGDH-AS1-encoding micropeptide NKSM promotes TNBC tumor**
880 **growth and inactivates the NK cells**

881 (A-C) The UGDH-AS1-encoding micropeptide inhibits the NK cells induced TNBC
882 tumor regression in the xenografts NCG mice. Six-week-old female NCG mice were
883 injected subcutaneously in the left side with TNBC cells until the tumors volume
884 reached ~100mm^3. The mice were then randomly grouped and intravenously injected
885 with indicated cells (1×10^6 in 100 μL PBS) twice a week for 4 weeks (n = 5).
886 (D) FACS analysis of IFN-γ, GZMB, and PRF1 in NK cells isolated from the spleen
887 of WT or *NKSM* $^{+/+}$ mice. Before detection, splenic NK cells were stimulated with
888 IL-2, IL-12, and IL-18 for 18 hours (n = 5). (E) Growth of E0771 tumors in WT or
889 *NKSM* $^{+/+}$ mice (n = 5). (F) Representative images of mice lungs in WT and *NKSM*$^{+/+}$
890 mouse lung metastasis model (left) and the quantification of lung metastatic
891 colonization (right) (n = 5). (G) The tumor growth in *MMTV-PyMT* and *MMTV-*
892 *PyMT;NKSM* $^{+/+}$ mice (n = 5). (H) Representative images of mice lungs in *MMTV-*
893 *PyMT* and *MMTV-PyMT;NKSM* $^{+/+}$ mice (left) and the quantification of lung
894 metastatic colonization (right) (n = 5). (mean ± SD, *p<0.05; **p<0.01 and
895 ***p<0.001).

896 **Fig. 4 TGF-β signaling pathway promotes the formation of UGDH-AS1$^+$ NK cells,**
897 **which express NKSM.**

898 (A) Overview of CHIP-seq data of SMAD2, SMAD3 and SMAD4 occupancy at
899 UGDH-AS1 promoter region. (B) Immunoblotting analysis of expression of the
900 indicated proteins in NK-92MI and PBNK cells cultured with or without TGF-β. (C)
901 Chromatin immunoprecipitation showing SMAD4 occupancy at the *UGDH-AS1* locus
902 in NK-92MI and 293T cells. Co-precipitated DNA was analyzed for amplicons A–E
903 showed in the upper schematic diagram through qPCR. Values represent the
904 enrichment of bound protein fractions relative to input. (D) Schematic diagrams of
905 luciferase reporter constructs with wild-type and mutant *UGDH-AS1* promoter. (E)
906 Luciferase reporter assays were performed in 293T cells following co-transfection
907 with wild-type or mutant UGDH-AS1 promoter fragment and SMAD4 overexpressed,
908 or knocked down and respective controls for 36h and treatment with TGF-β for 24h.
909 The reporter constructs were expressing the luciferase gene under UGDH-AS1
910 promoter segment or UGDH-AS1 promoter deleted -200 to +300 region. (F) NK-
911 92MI and PBNK cells were pretreated with specific antagonist against SMAD3 (SIS3,
912 3μM) for 1h and then cultured with or without TGF-β. The indicated proteins were

913 determined by western blotting. (G) SMAD4 siRNA and corresponding control
914 transfected NK-92MI cells were cultured with or without TGF-β. The indicated
915 proteins were determined by western blotting. (mean ± SD, *p<0.05; **p<0.01 and
916 ***p<0.001).

917 **Fig. 5 NKSM interferes c-Myc phosphorylation**

918 (A) UpSet plot of mass spectrometry results for Co-IP assays in NK-92MI and 293T
919 cells that transfected NKSM-FLAG expression plasmid. The highlighting block with
920 yellow indicate indicates proteins precipitated by anti-FLAG but not IgG, and do not
921 be included in the contaminant database (CRAPome). (B) Top 5 proteins of the
922 proteins precipitated by anti-FLAG but not IgG, and do not be included in the
923 contaminant database. (C) Co-IP assay was performed in the cell lysis of NK-92MI
924 cells that transfected NKSM-FLAG expression plasmid. The indicated proteins were
925 detected by western blot in NKSM-FLAG precipitated complex that precipitated by
926 anti-FLAG. (D) C-Myc-HA and NKSM-FLAG plasmids were co-transfected into
927 NK-92MI and 293T cells. The c-Myc-HA and NKSM-FLAG precipitated complex
928 was analyzed by western blotting. (E) NK-92MI cells were pre-treated with TGF-β,
929 then NKSM and c-Myc were detected by western blotting in c-Myc precipitated
930 complex. (F) Schematic diagram for c-Myc truncated constructs. (G) Co-IP and
931 western blot indicating NKSM interacted with c-Myc at 1-144 of the N-terminal. (H)
932 The expression of NKSM, c-Myc and phosphorylated c-Myc were determined in
933 NKSM overexpressed or control NK-92MI and 293T cells by western blotting. (I)
934 NKSM overexpression promoted protein degradation of wild-type c-Myc as well as c-
935 Myc mutants T58A, but not c-Myc mutants S62A and T58A/S62A. (J) The
936 proteasome inhibitor MG132 blocks NKSM-induced c-Myc degradation. The NKSM
937 overexpressed or control NK-92MI and 293T cells were transfected with HA-c-Myc
938 and treated with 20μM MG132 for 6h before protein harvest. (K and L) NKSM
939 reduces c-Myc protein stability. 293T cells were transfected with NKSM-FLAG or
940 empty vector. The half-life of endogenous c-Myc was detected using a CHX chase
941 assay and were analyzed by western blotting (K) and quantification (L). (M) Co-IP
942 assay was performed in the cell lysis of NKSM overexpressing and control NK-92MI
943 or 293T cells using anti-c-Myc and anti-ERK1/2 antibodies. The ERK1/2 proteins
944 were detected by western blot in c-Myc precipitated complex that precipitated by anti-
945 c-Myc, and the c-myc protein was detected by western blot in ERK1/2 precipitated
946 complex that precipitated by anti-ERK1/2. (N) The levels of c-Myc protein and
947 phosphorylation were determined by western blot. Cells were culture with TGF-β and
948 IL-12/IL-15 for 1 day.

949 **Fig. 6 NKSM deregulates T-bet expression via c-Myc to inactivate UGDH-AS1^{+}**

950 **NK cells.**
951 (A) FACS analysis of IFN-γ, GZMB, and PRF1 in NKSM overexpressed (OE) and
952 control (empty vector) NK92-MI cells (*n* = 3). (B) The expression of T-bet in NKSM
953 overexpressed and control NK-92MI cells. (C) Overview of ChIP-seq data of c-Myc
954 occupancy at *TBX21* promoter region. (D) Chromatin immunoprecipitation showing
955 c-Myc occupancy at the *TBX21* locus in NK-92MI and 293T cells. Co-precipitated
956 DNA was analyzed for amplicons A–E through qPCR. Values represent the
957 enrichment of bound protein fractions relative to input. (E) Luciferase reporter assays
958 were performed in 293T cells following co-transfected reporter constructs with wild-
959 type or mutant *TBX21* promoter fragment and constructs of NKSM overexpression, c-
960 Myc knockdown, or respective controls. The reporter constructs were expressing the
961 luciferase gene under the *TBX21* promoter segment or *TBX21* promoter deleted -200
962 to +300 region (*n* = 3). (F) Cytotoxic activity of NKSM knockout and wild-type NK-
963 92MI cells towards K562 cells in the culture medium of MDA-MB-231 cells (*n* = 3).
964 (G) NKSM knockout enhanced the NK cells induced TNBC tumor regression in the
965 xenografts NCG mice. Six-week-old female NCG mice were injected subcutaneously
966 in the left side with MDA-MB-231 cells until the tumors volume reached ~100mm^3.
967 The mice were then randomly grouped and intravenously injected with indicated cells
968 twice a week for 4 weeks (*n* = 5). (H) The survival data of mice injected
969 subcutaneously in the left side with MDA-MB-231 cells and injected with indicated
970 cells twice a week (*n* =10). (mean ± SD, *p<0.05; **p<0.01 and ***p<0.001).

971 **Fig. 7 TNBC-specific UGDH-AS1$^+$ NK cells encoded micropeptide, NSKM,**
972 **which functions as a NK cells suppressor in TNBC.**

Supplementary Files

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