NF-κB-induced NOX1 activation promotes gastric tumorigenesis through the expansion of SOX2-positive epithelial cells

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Supplementary Materials and Methods

Construction of *Noxo1* conditional knockout mice

BAC clones RPCI-23-124B17, RPCI-23-238L20 and RPCI-23-438P15 (Advanced GenoTechs Co, Tsukuba Japan) containing whole *Noxo1* gene of C57BL/6 mice were used as sources of genomic DNA. A 10 kb genomic DNA fragment including entire *Noxo1* exons and upstream sequences was cloned from BAC into PL253 vector. A PGK-Neo cassette was sandwiched between two FRT sequences and inserted into the intron 5 of *Noxo1* gene. Two loxP sites were inserted before the exon3 and after the exon 5. Thus, exons 3, 4 and 5 of *Noxo1* are deleted when Cre recombinase is expressed. The vector was electroporated to TT2 ES cells, and homologous recombinant clone was selected by G418 screening. Construction of the targeted allele was confirmed by genomic PCR and genomic Southern blotting. Germline transmitted *Noxo1*$^{flox\ now}$ heterozygous mice were crossed with CAG-FLPe transgenic mice (RBRC01834, RIKEN BRC, Japan) to obtain *Noxo1*^{flox} mice and subsequently crossed with CAG-Cre transgenic mice (Sakai K, Miyazaki J. *Biochem Biophys Res Commun* 1997; 237: 318-324) to generate conventional *Noxo1* knockout, *Noxo1*^{del}, mice. See also Fig. 7a for targeting strategy.

Organoid culture experiments

The primary organoid cultures of *K19-C2mE Noxo1*-/- and *K19-C2mE Noxo1*+/+ mouse gastric epithelial cells were prepared according to the protocol as described (Leushacke, et al. *Nat Cell Biol* 2017; **19**: 774-786.). Isolated gastric glands were seeded in Matrigel and cultured in medium supplemented with EGF, Gastrin, FGF10, Noggin, Wnt3 and R-spondin. Rock inhibitor (Y27632, Wako Chemical, Osaka, Japan) and GSK3 inhibitor (CHIR-00921, Tocris Bioscience, Bristol, UK) were added to the medium.

Primer sequences for RT-PCR (designed primers only)

	Gene name	Forward primer	Reverse priver
Human	*NOXO1*	AGATCAAGAGGCTCCAAACG	GGAAGGTCTCCTTGAGGGTCT
	GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
	NOTCH3	TGCAGCGTGACCGAGATA	CACCCATTATAAATAAAGGAAGACTGA
	KRT7	CAGGCTGAGATCGACAACATC	CTTGGCACGAGCATCCTT
	DUSP1	CGAGAGGGCTGGTCCTTAT	AAGTCATCACCATAACTGCTTAGAAA
	DUSP4	GGATGTCAAGGCGCTGTT	GGCTGTGGTTTCCACAAGA
	ALDH1A1	TTTGGTGGATTCAAGATGTCTG	CACTGTGACTGTTTTGACCTCTG
	SOX2	TTGCTGCCTCTTTAAGACTAGGA	TAAGCCTGGGGCTCAAACT

Mouse	*Noxo1*	CGGCTTCTTTGTACCCAAAC	GGTGTAGGCAGGATCACCAG
	Notch3	GACCGTGTGGCCTCTTTC	ATGACACAAGAGGCCTGTCTTC
	Krt7	GGCAGCAGCTCGAGACAC	GTCGGTTGATCTCCTCTTCATAC

Primer sequences for ChIP assay

NOXO1 promoter Fw: GCAAGAAAGCTGCAAAGGAC

NOXO1 promoter Rev: GAAAACCCCCTGGGATAGAA

shRNA sequence for lentivirus construction

The sequences for shRNA were designed by siDirect ver 2.0 (http://sidirect2.rnai.jp/)

Gene	shRNA sequence
luciferase	5'-GATTTCGAGTCGTCTTAATGT-3'
RELA #1	5'-GATGAGATCTTCCTACTGTGT-3'
RELA #2	5'-GGATTGAGGAGAAACGTAAAA-3'
RELA #3	5'-GACATTGAGGTGTATTTCACG-3'
NOXO1	5'-GAATTCAGGCAGCTCAAGACC-3'

Cell cycle analysis

MKN45 cells were plated in 6 well plates and cultured in the presence or absence of 100 μM apocynin (Sigma). After 3 days, apocynin was additionally added, and cells were trypsinized and fixed using cold ethanol at day 4. Cells were washed and treated with RNase A (0.25 mg/ml) at 37°C for 30min. DNA was stained with propidium iodide (PI) (Sigma) and analyzed by MACSQuant analyzer (Milteny BIotec). At least $5.0X10^3$ cells were analyzed.

Supplementary Table 1. Activation of NF-κB pathway in gastric lesions.

	human stomach cancer (TCGA)					
	intestinal			diffusive		
Upstream Regulator	Predicted Activation State	Activation z-score	p-value of overlap	Predicted Activation State	Activation z-score	p-value of overlap
NFkB (complex)	Activated[a]	4.841	3.02E-11	Activated	5.089	1.38E-13

	human gastritis (GSE60662)								
	intestinal metaplasia			mild gastritis			severe gastritis		
Upstream Regulator	Predicted Activation State	Activation z-score	p-value of overlap	Predicted Activation State	Activation z-score	p-value of overlap	Predicted Activation State	Activation z-score	p-value of overlap
NFkB (complex)	Activated	2.446	1.11E-04	Activated	5.518	9.32E-19	Activated	5.889	2.42E-13

[a], z-score > 2.0 is considered as significantly activated.

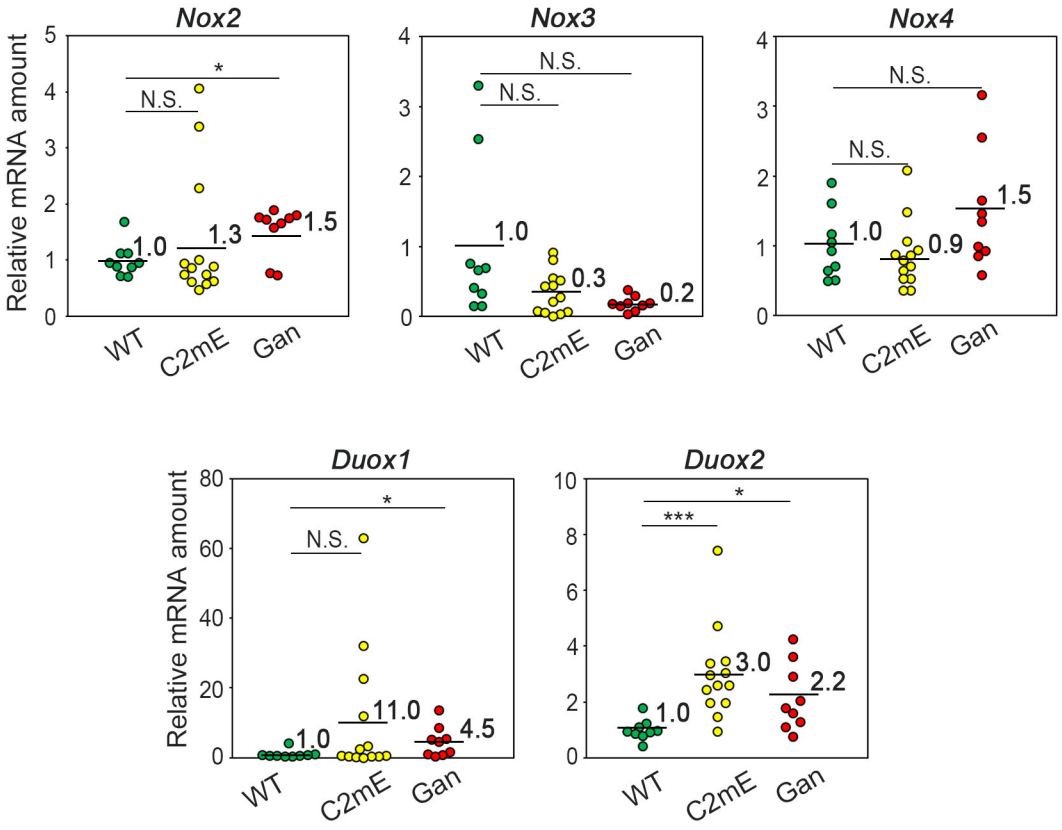
Supplementary Table 2. The results of upstream regulator analysis by IPA.

Upstream regulator	K19-C2mE/wild-type[a]		Apo-treated/control[b]	
	z-score	*p*-value	z-score	*p*-value
MYC	4.89	1.53E-19	0.941	3.78E-06
PDGF BB	4.485	1.36E-09	2.658	5.01E-10
TNF	4.462	1.41E-39	0.628	3.17E-05
SOX2	3.615	2.07E-08	-0.505	2.20E-05
PRKCD	1.786	1.45E-02	0.05	4.15E-05
TREM1	1.688	1.24E-10	2.714	7.70E-05
CCND1	1.463	1.26E-02	1.982	2.43E-05
TP63	1.313	4.99E-22	0.714	4.49E-10
CREBBP	1.309	7.17E-09	N.D.	2.45E-05
MGEA5	1.192	3.25E-16	-0.775	9.23E-05
TGFB1	1.176	7.28E-28	2.294	3.78E-05

[a], Upregulated pathways in K19-C2mE mouse gastritis tissues compared with wild-type normal stomach are listed (z-score > 1).

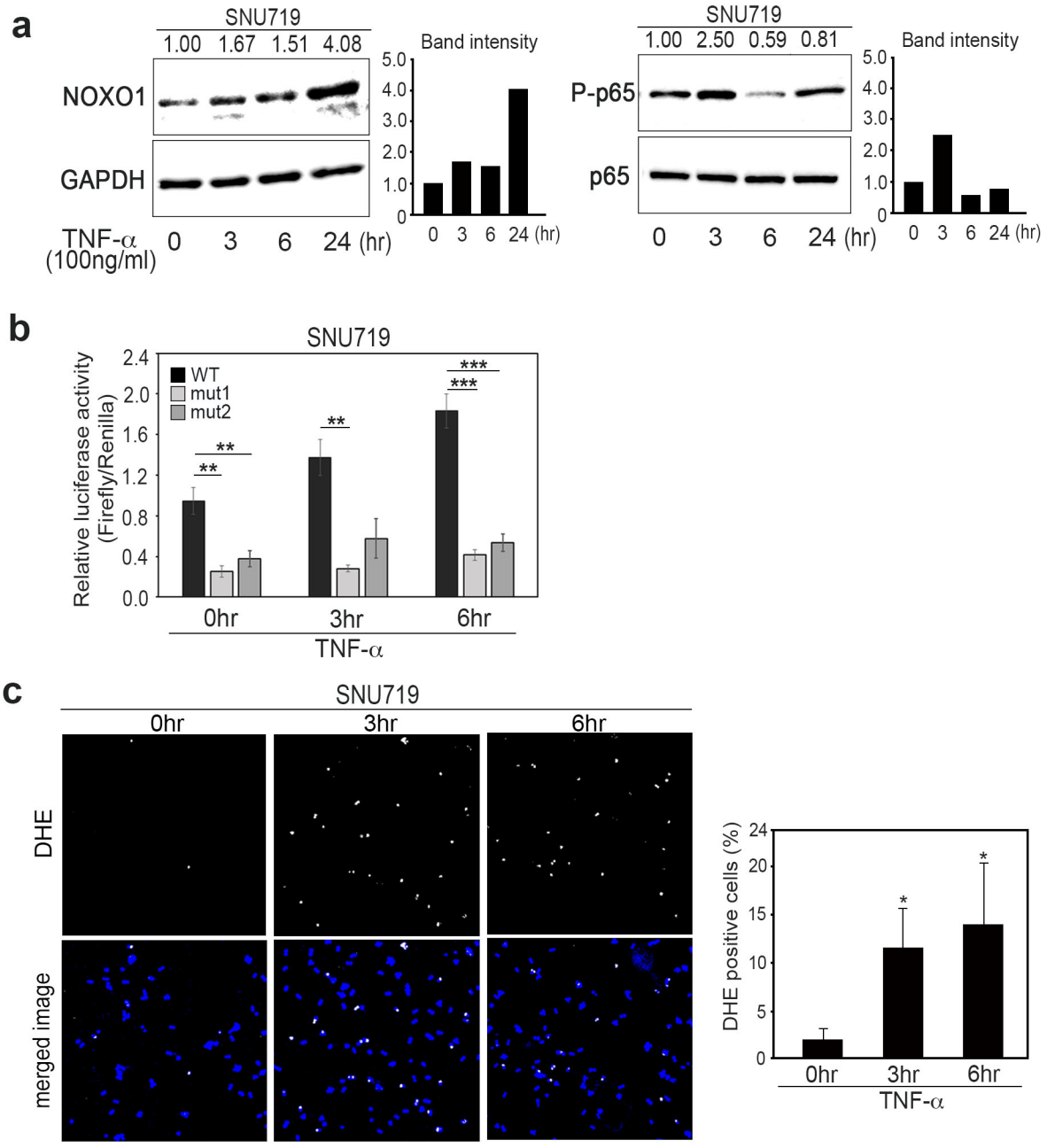
[b], These listed pathways were further examined using differentially expressed genes in apocynin-treated MKN45 cells compared with untreated control cells.

Supplementary Figure 1



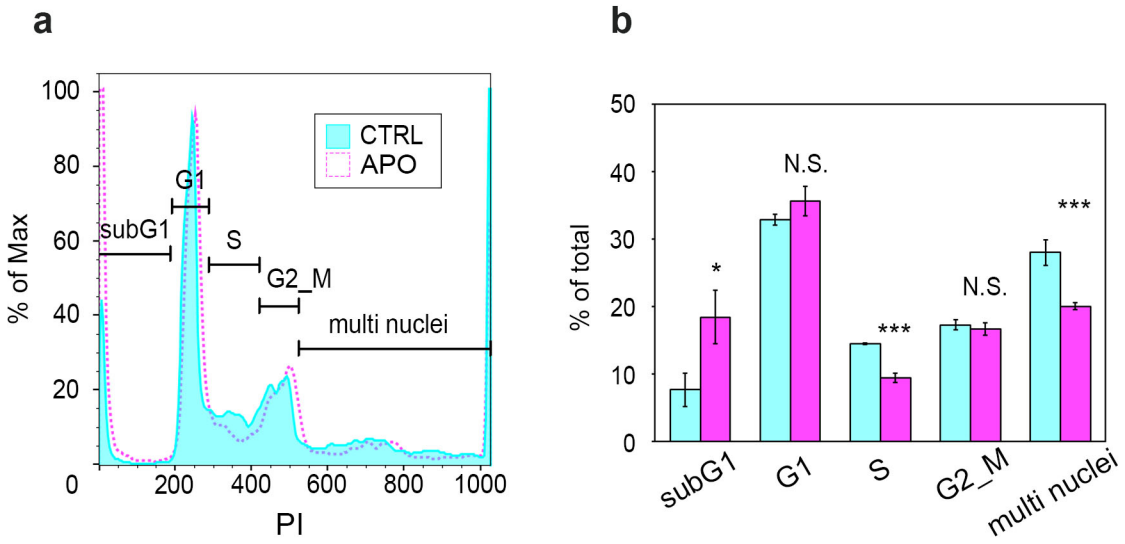
Supplementary Figure 1. The expression of NOX family member genes in gastritis and gastric tumors. The relative mRNA levels of *Nox2*, *Nox3*, *Nox4*, *Duox1* and *Duox2* in wild-type mouse stomach (WT) (n = 9), *K19-C2mE* mouse gastritis-associated hyperplasia (C2mE) (n = 13) and *Gan* mouse gastric tumors (Gan) (n = 9) are shown with mean values. *, $p < 0.05$; ***, $p < 0.001$; N.S., not significant.

Supplementary Figure 2



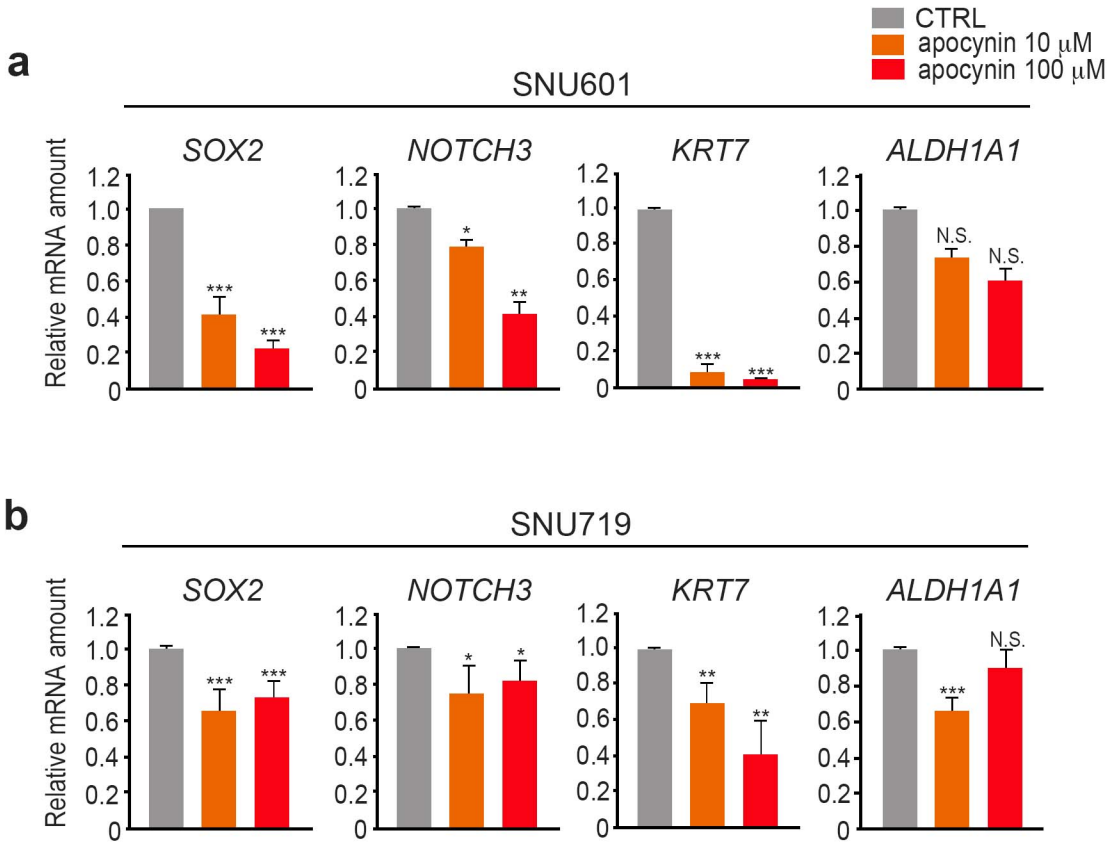
Supplementary Figure 2. The induction of the NOXO1 expression and ROS production by the TNF-α/NF-κB pathway. **a** Immunoblotting for NOXO1 (left) and phosphorylated p65 (right) in the SNU719 cells after TNF-α stimulation. The relative band intensities are shown as bar graphs (right). **b** The relative luciferase activities in SNU719 cells after TNF-α stimulation (mean ± s.d.). **, $p < 0.01$; ***, $p < 0.001$. **c** Representative photographs of DHE staining (top) and merged images with DAPI (bottom) of SNU719 cells at the indicated time after TNF-α stimulation. The mean ratio of the DHE-positive cells (mean ± s.d.). *, $p < 0.05$.

Supplementary Figure 3



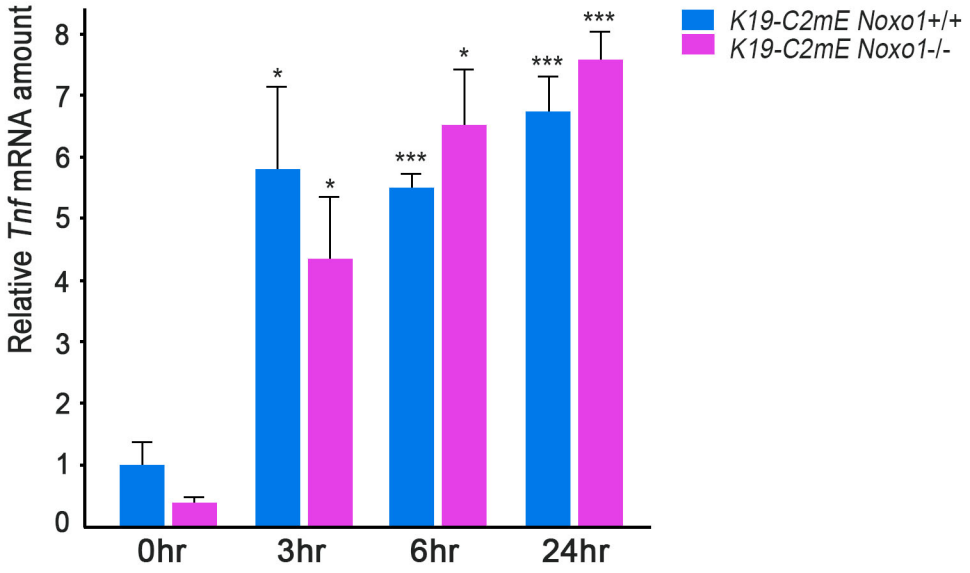
Supplementary Figure 3. A cell cycle analysis of apocynin-treated MKN45 cells. **a** Representative histograms of the flow cytometry analysis for PI staining of control (CTRL, *blue*) and apocynin-treated MKN45 cells (APO, *red dashed line*) are shown. **b** Cells were gated and counted. The results are shown as the average of three wells for control cells (blue) and apocynin-treated MKN45 cells (red) (mean ± s.d.). *, $p < 0.05$; ***, $p < 0.001$; N.S., not significant.

Supplementary Figure 4



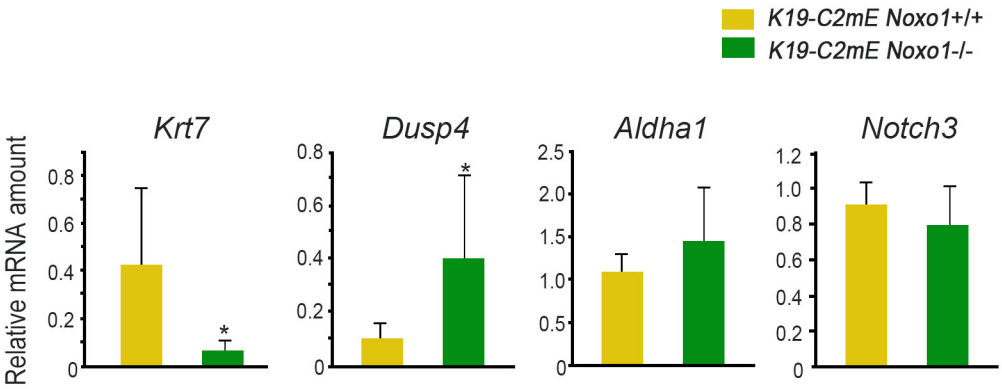
Supplementary Figure 4. The suppression of the SOX2 pathway by NOX inhibition in SNU601 cells (**a**) and SNU719 cells (**b**). The relative mRNA levels of SOX2 and SOX2-target genes (indicated by asterisks in Figure 5C) in the apocynin-treated (10 and 100 μM) and control (CTRL) cells are shown (mean ± s.d.). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N.S., not significant.

Supplementary Figure 5



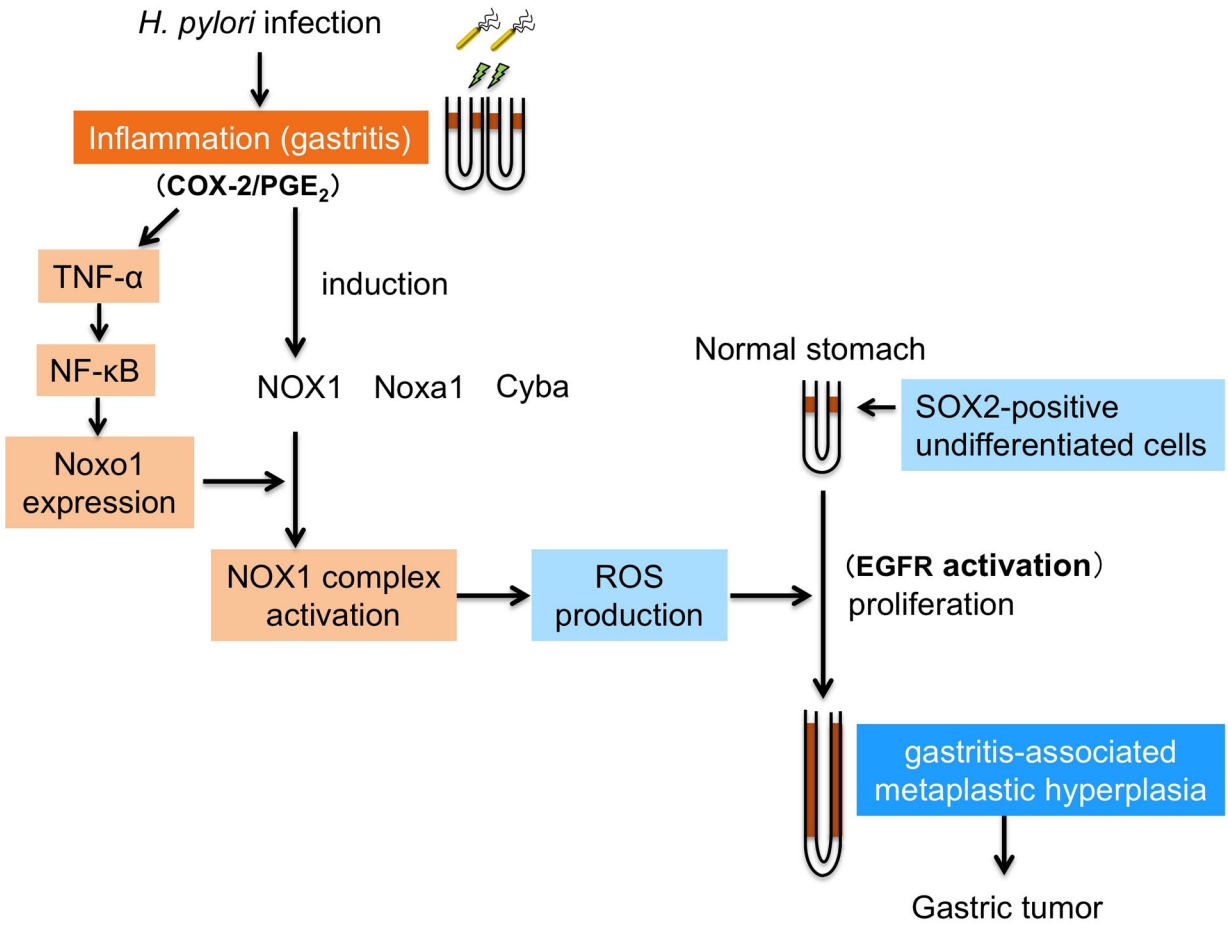
Supplementary Figure 5. The induction of TNF-α expression in the TNF-α-stimulated gastric epithelial organoids. The relative TNF-α (*Tnf*) mRNA levels in *K19-C2mE Noxo1*+/+ and *K19-C2mE Noxo1*-/- mouse-derived organoid cells at the indicated time point after TNF-α stimulation are shown (mean ± s.d.). *, $p < 0.05$; *** $p < 0.001$.

Supplementary Figure 6



Supplementary Figure 6. The expression of the SOX2-target genes in *K19-C2mE Noxo1+/+* and *K19-C2mE Noxo1-/-* mouse gastric epithelial cells. Gastric epithelial cells were isolated by LMD from frozen sections and examined expression by RT-PCR. Relative mRNA levels of SOX2-target genes are shown (mean ± s.d.). *, $p < 0.05$.

Supplementary Figure 7



Supplementary Figure 7. A schematic illustration of the roles of Noxo1 and NOX1 in inflammation-associated tumorigenesis gastric tumorigenesis. The TNF-α/NF-κB pathway induces *Noxo1* expression in the inflamed gastric mucosa, resulting in ROS production, which causes the further increase in SOX2-expressing undifferentiated epithelial cells and the development of gastric hyperplasia, a potent precursor of gastric cancer.