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# Impact of gamma-aminobutyric acid receptor-associated protein (GABARAP) on carcinogen-induced tumorigenesis

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**31-12-2018**

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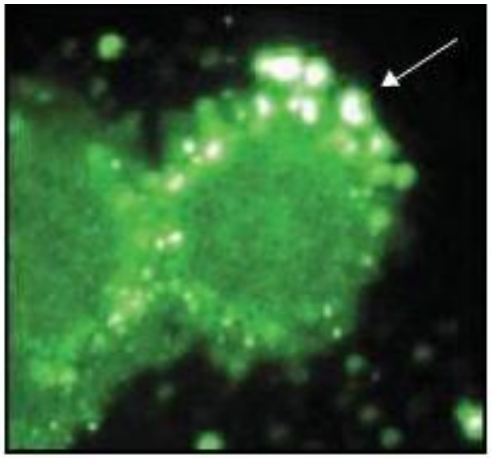
Gamma (γ)-aminobutyric acid type A ($GABA_A$) receptor-associated protein (**GABARAP**) is an evolutionary highly conserved gene family from yeast to mammals.

➢100% identity at amino acid level for mammalian forms.

➢ Previous study indicated a tumor suppressive effect of GABARAP in a breast cancer cell line (***in vitro***).



**However, the precise role and mechanism that GABARAP played to inhibit cell growth was not elucidated.**

***Klebig et al., Cancer Res. 2005; 65: 394-400.***

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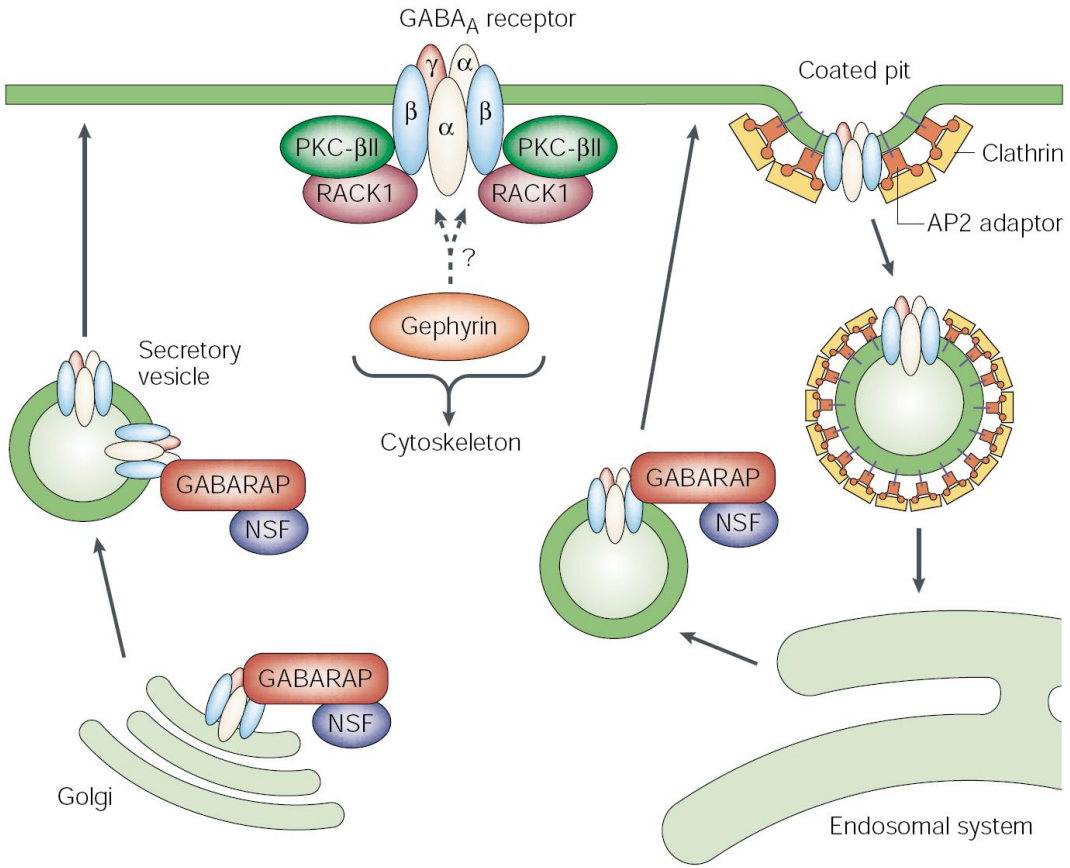
GABARAP regulates the intracellular trafficking of $GABA_A$ receptor, a major inhibitory neurotransmitter in cortical neurons.




***Moss SJ and Smart TG, Nat Rev Neurosci. 2001; 2(4): 240-50.***

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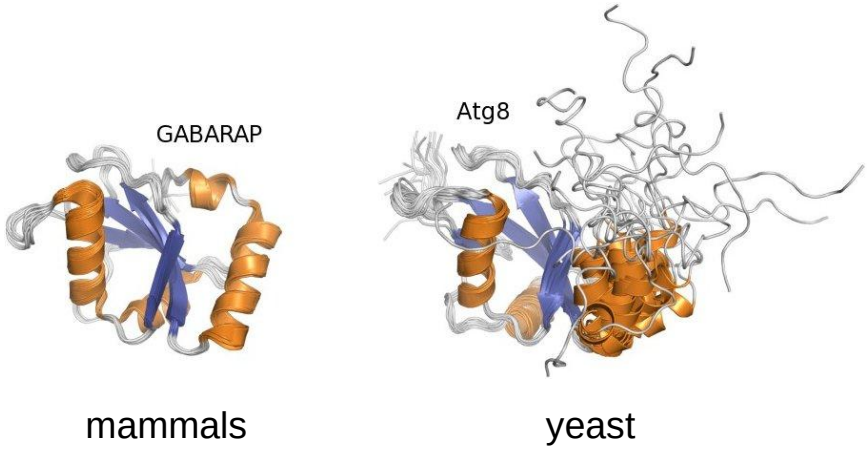
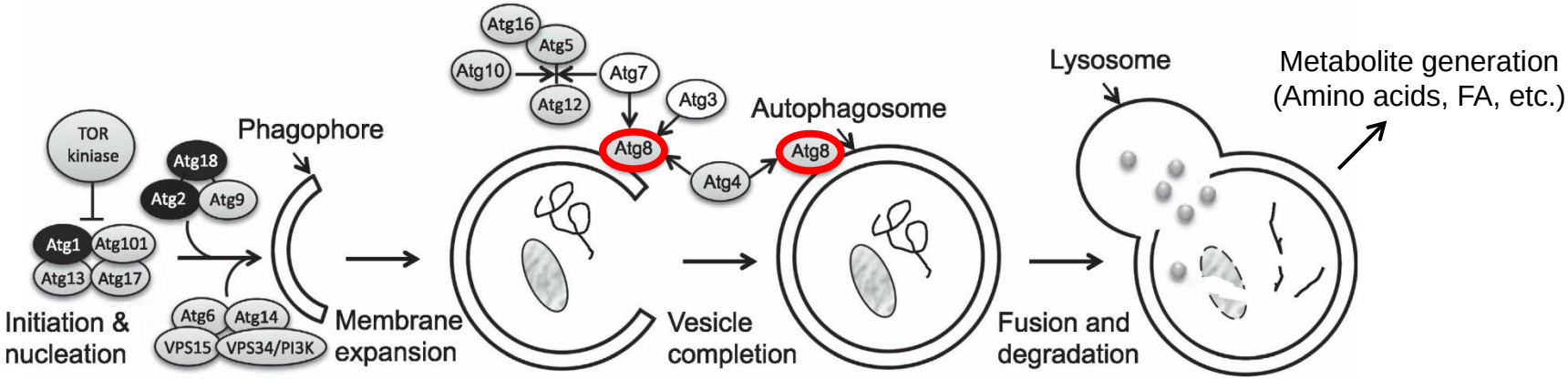
In mammals, there are several Atg8 homologues; grouped into two subfamilies:

- LC3 (microtubule-associated protein-1 light chain 3)
- GABARAP

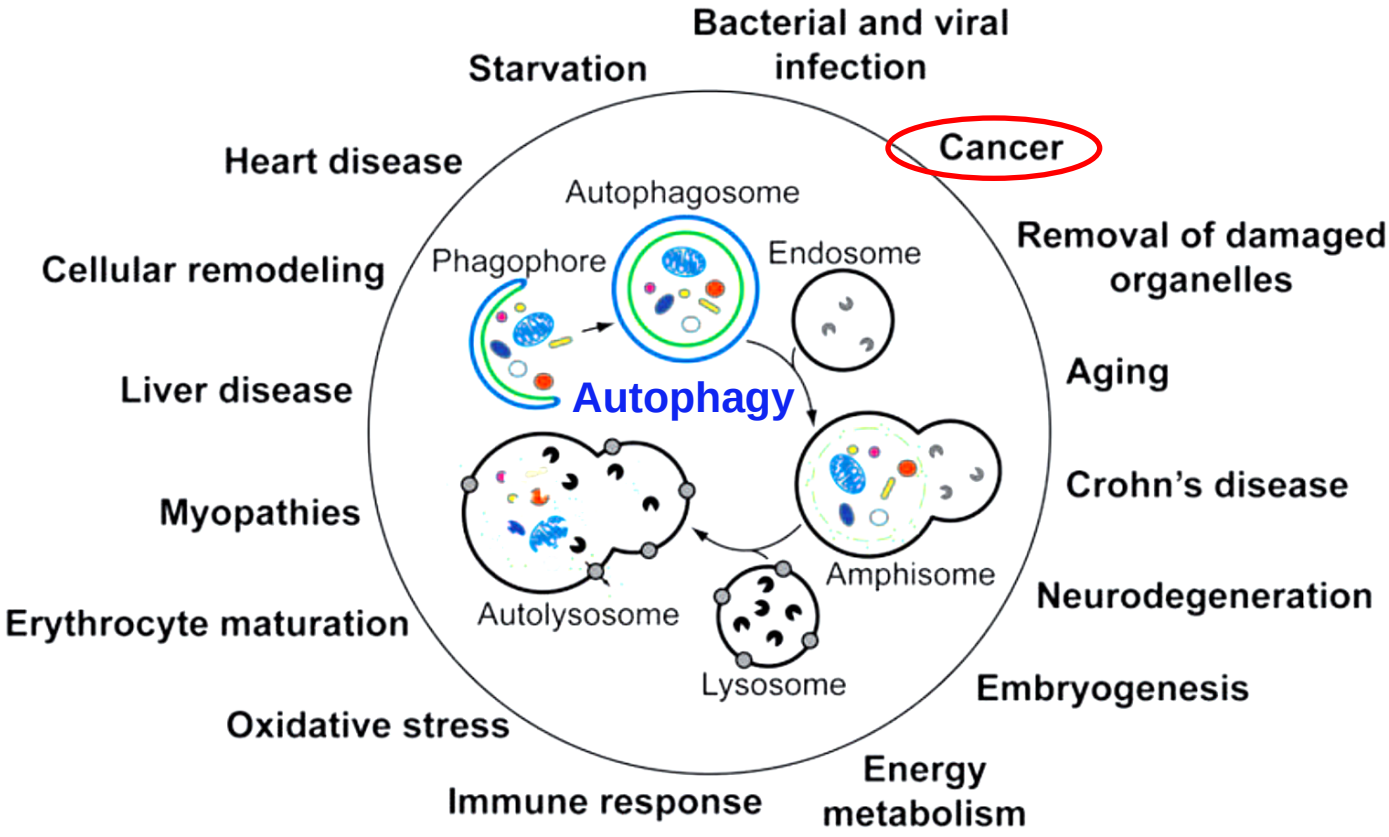
- Atg8 has a crucial role in the autophagic process.
- GABARAP has essential role for a later stage of autophagosome maturation.




mammals yeast




*Denton et al., Immunol Cell Biol. 2015; 93(1): 35-42.*




*Klionsky DJ, Dev Cell. 2010;19(1):11-12.*

The role of autophagy in cancer is complex and likely tissue and genetic context-dependent.

- ***Mouse models for autophagy-deficient gene**:*
  - Beclin1: tumor suppression function
  - ATG5, ATG7, and FIP200: No malignant tumor development *in vivo*.

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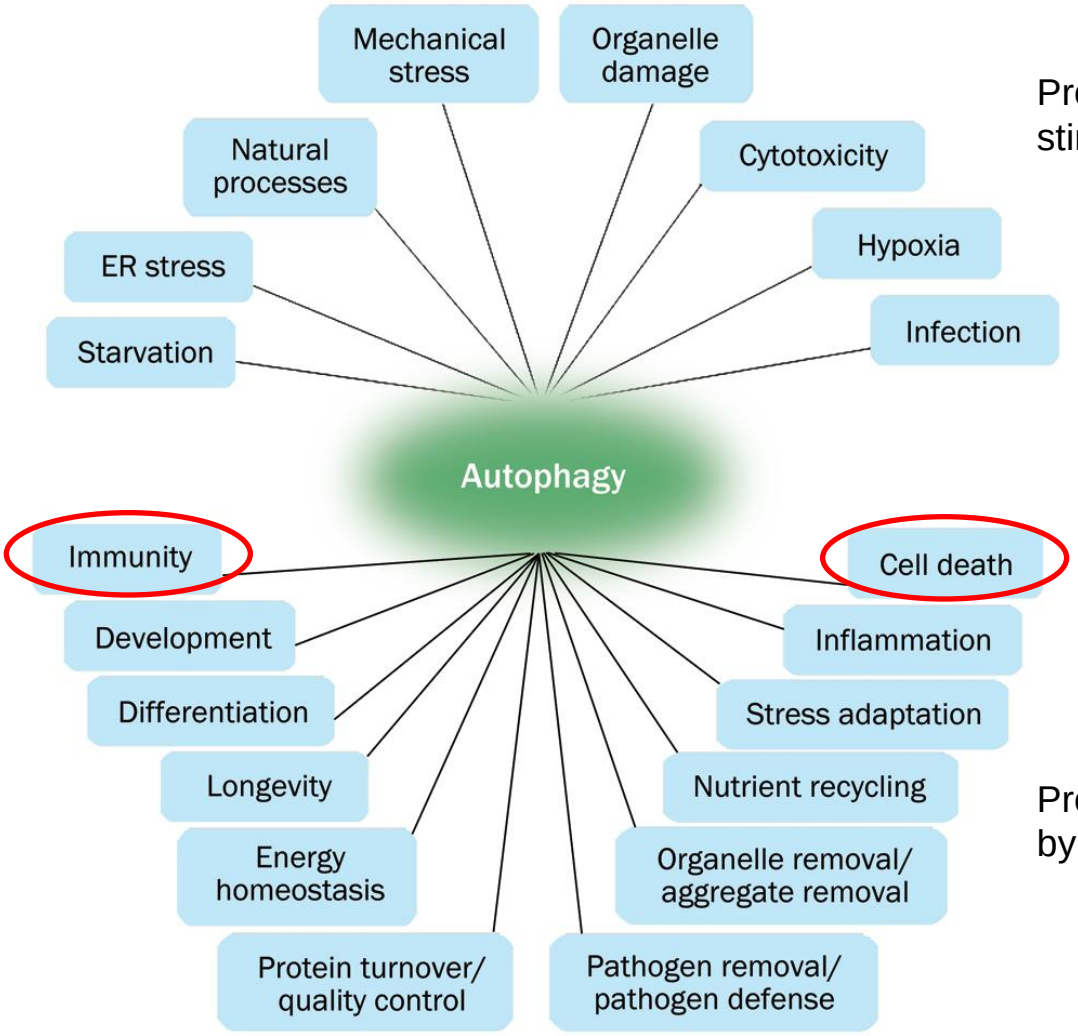
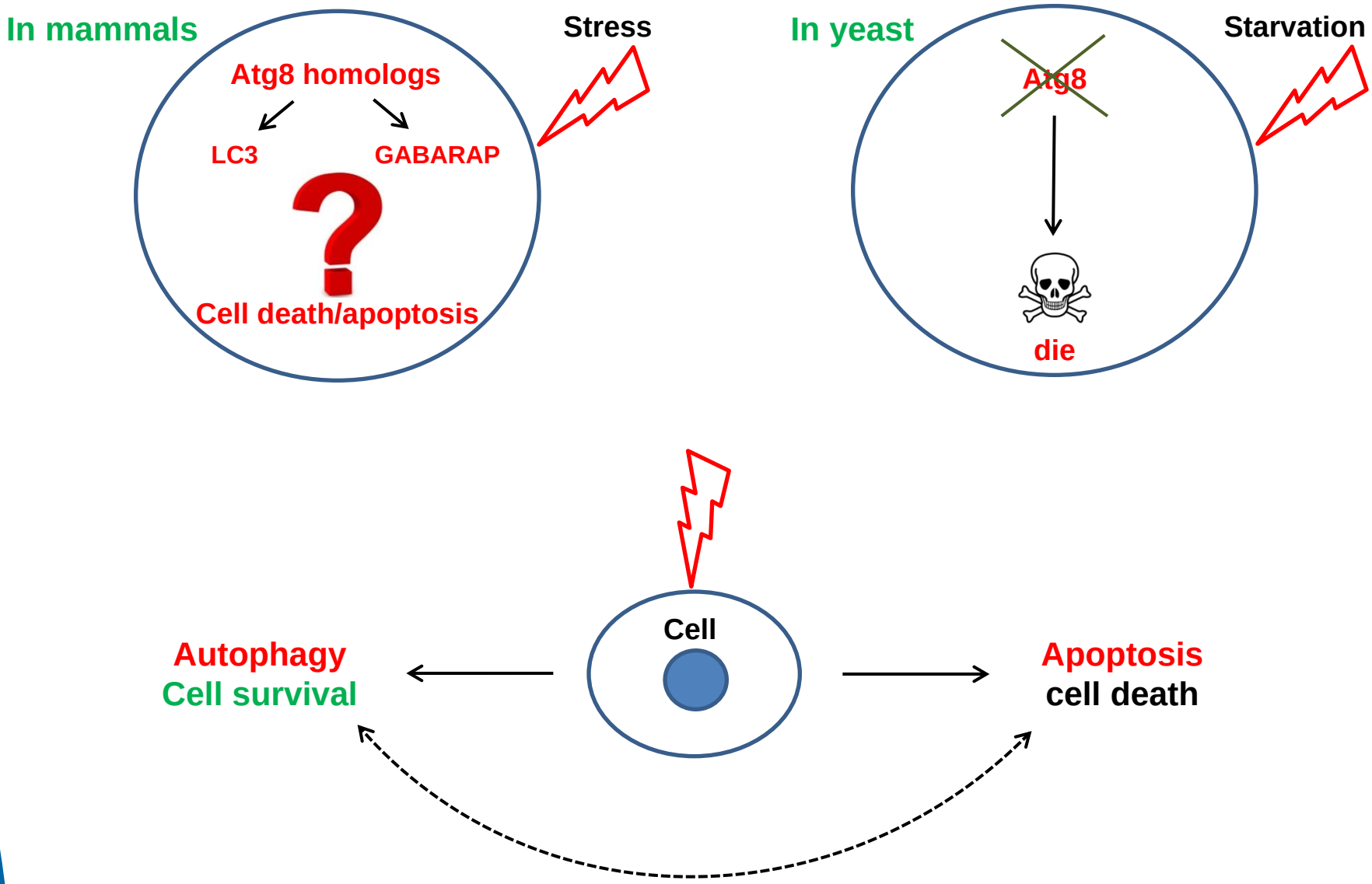
*In general, autophagy has been integrated into several **biological processes**:*




Processes that stimulate autophagy

Processes affected by autophagy

***Wang Y and Qin ZH, Acta Pharmacol Sin. 2013; 34(5): 585-94.***


In mammals
Stress
Atg8 homologs
LC3
GABARAP
Cell death/apoptosis
In yeast
Starvation
Atg8
die
Cell
Autophagy
Cell survival
Apoptosis
cell death

# Introduction: Autophagy and cell death

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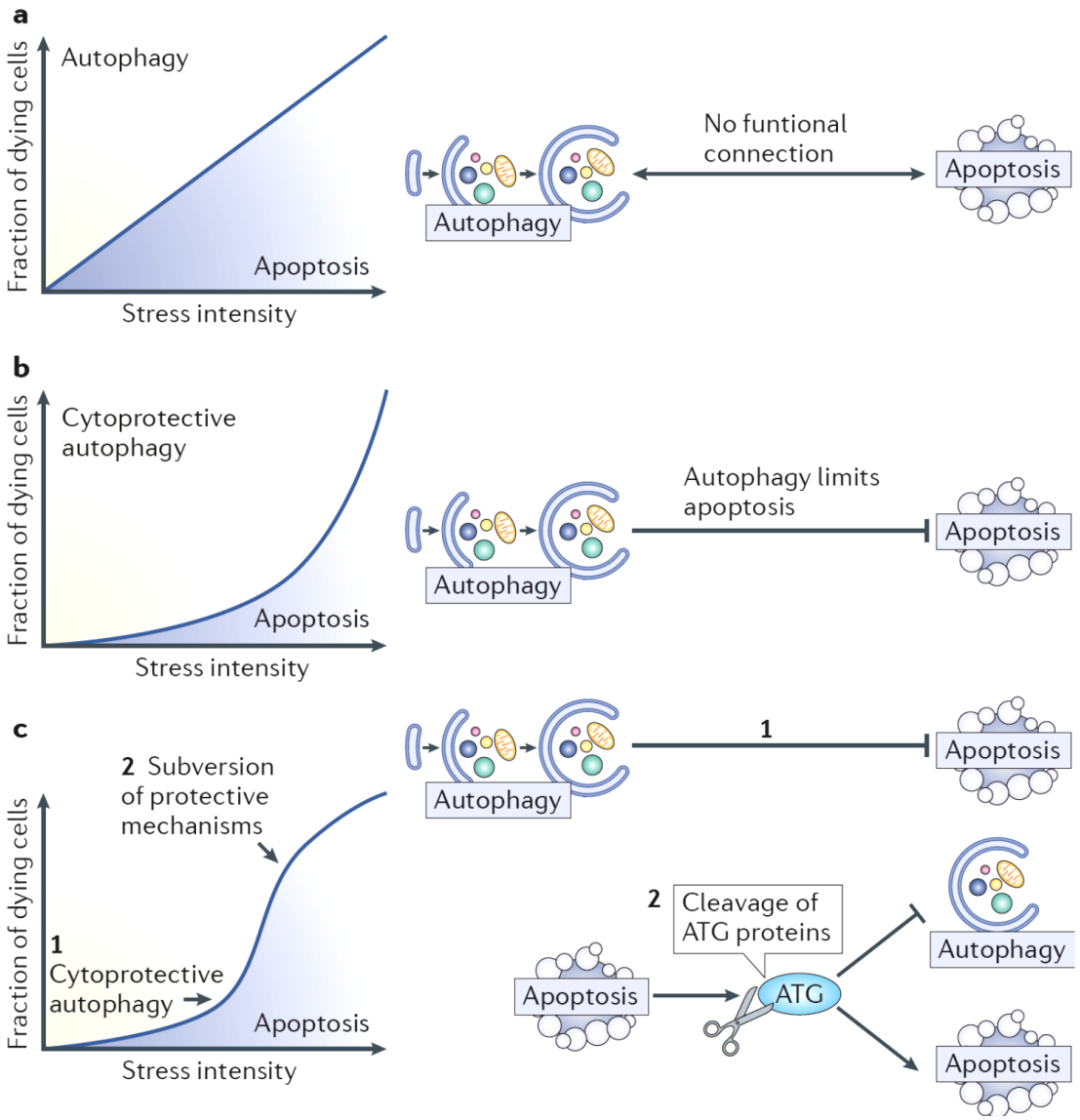


*Mariño et al., Nat Rev Mol Cell Biol 2014; 15: 81-94.*

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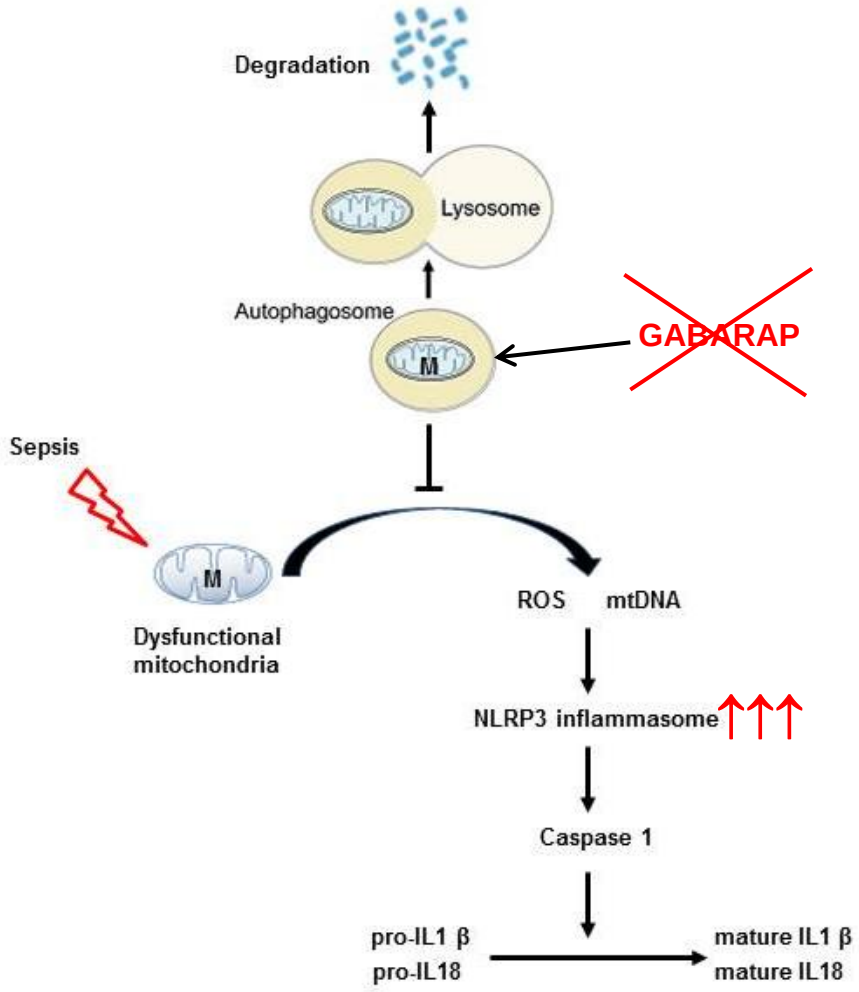
*Inactivation of GABARAP and other autophagy genes activates the inflammasome*




**NLRP3**; NOD-like receptor family, pyrin domain containing 3

***Adapted from Zhang et al., J Immunol. 2013; 190: 3517-24.***

**Explore the role of GABARAP in tumorigenesis by using a knockout mouse model**

- **Treatment of GABARAP knockout mice with carcinogens affect tumorigenesis?**
- **GABARAP knockout mice influence the growth of inoculated tumor cells?**
- **What are the cellular mechanisms being affected by GABARAP deficiency?**
  - **Apoptosis**
  - **Immunity**

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Wild-type (C57BL/6) mice

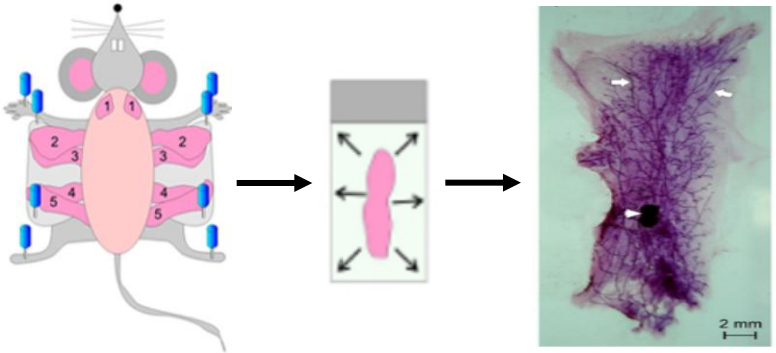
GABARAP knockout mice (C57BL/6 background)

Primary culture (MEFs)
Protein expression (WB)

Tumor inoculation
B16 melanoma cell

Carcinogen treatment (DMBA)

- Whole mount analysis of mammary glands

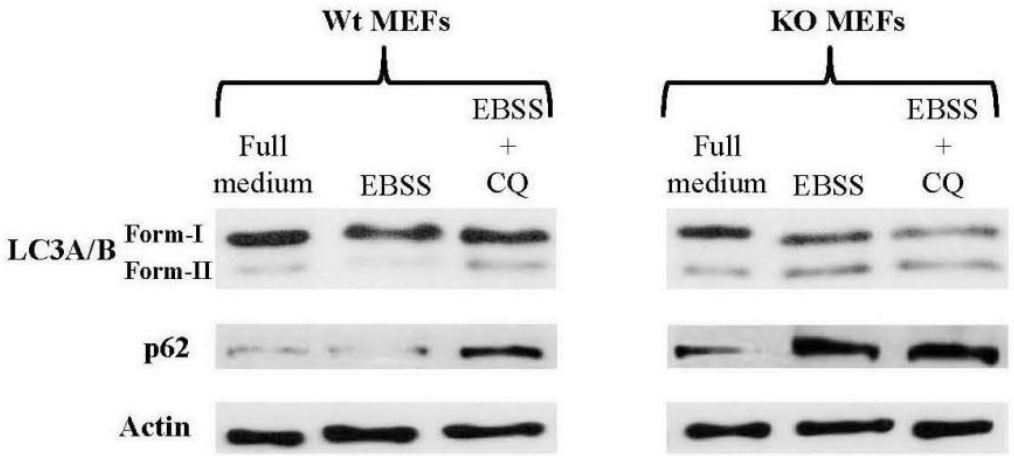



- Proliferation assay (IHC) by using Ki-67
- Apoptosis assay (TUNEL)
- cDNA microarray gene expression profiling
- qRT-PCR

Immunologic experiments:

- Cell surface marker analysis (FACS)
- Macrophages isolation
- Lymphocytes isolation
- Cytokine analysis (ELISA)

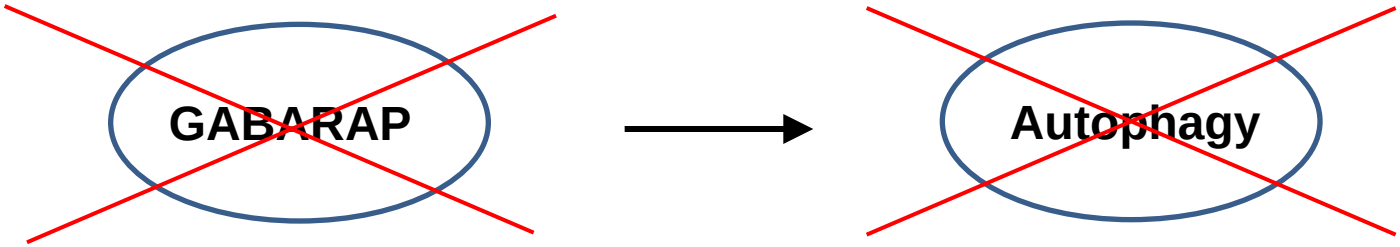
# Results: Involvement of GABARAP in autophagic process




**EBSS**; starvation media activate autophagy

**CQ**; chloroquine autophagy inhibitor

**GABARAP gene has an essential role in the autophagic process in our cell model system**



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## GABARAP KO inhibits tumor formation after DMBA treatment

| Tumor type | C57BL/6 (Wt) | GABARAP $^{-/-}$ (KO) |
|---|---|---|
| DMBA treatment | n = 29 | n = 33 |
| Mammary | 3 | - |
| Skin | 7 | 2 |
| Lymphoma | 2 | - |
| Liver | - | 1 |
| Undifferentiated tumors | 2 | 1 |
| Total | 14 (48.3%) | 4 (12.1%)** |

** *p-value < 0.01, Fisher's exact test.*

# Results: Morphologic and histologic features of tumors

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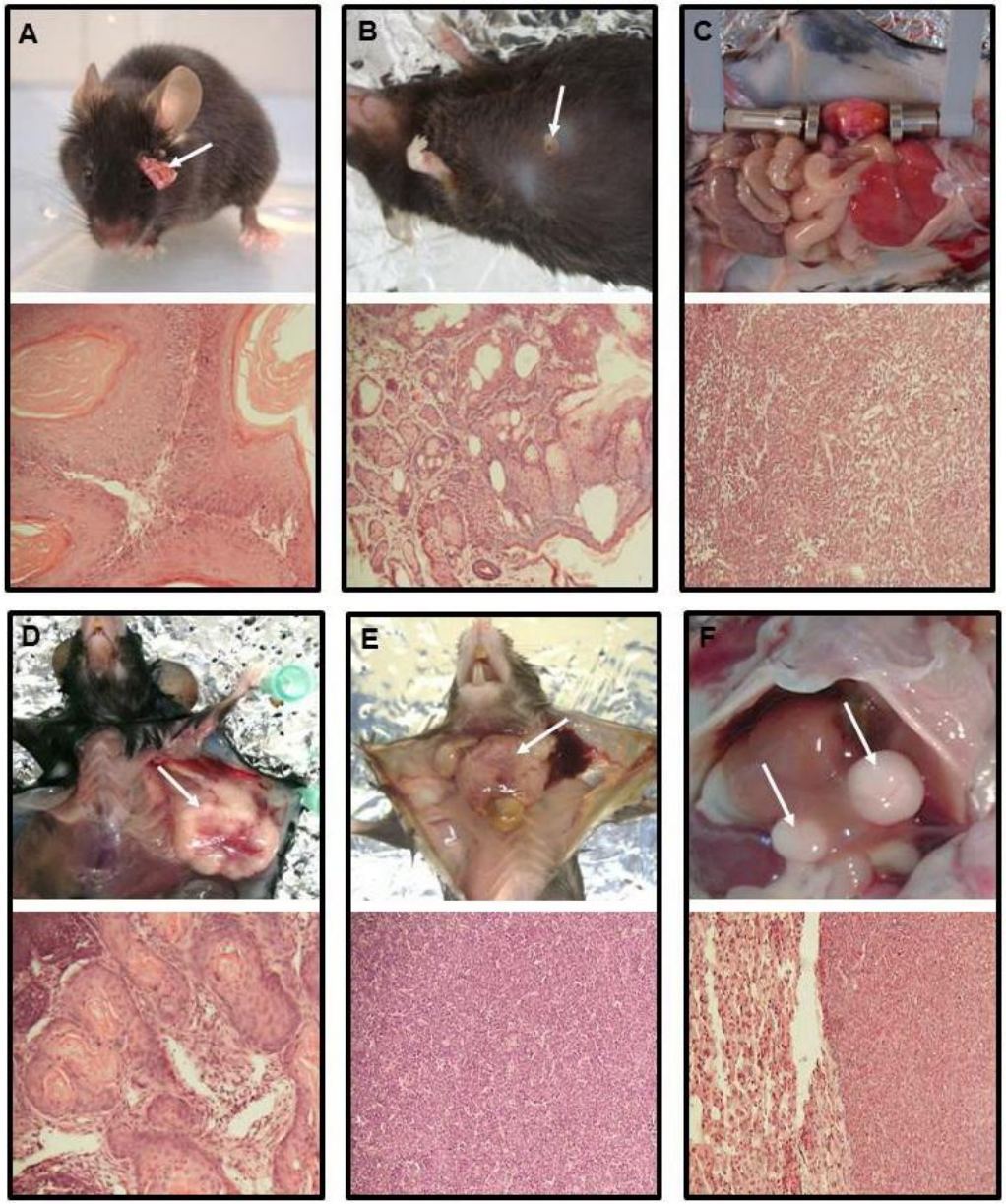

# Results: Influnce of GABARAP KO on tumor latency and size

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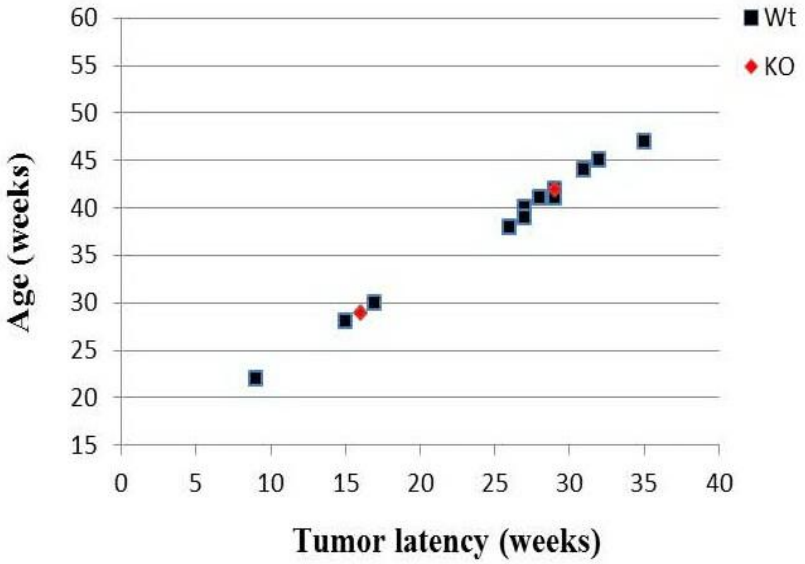
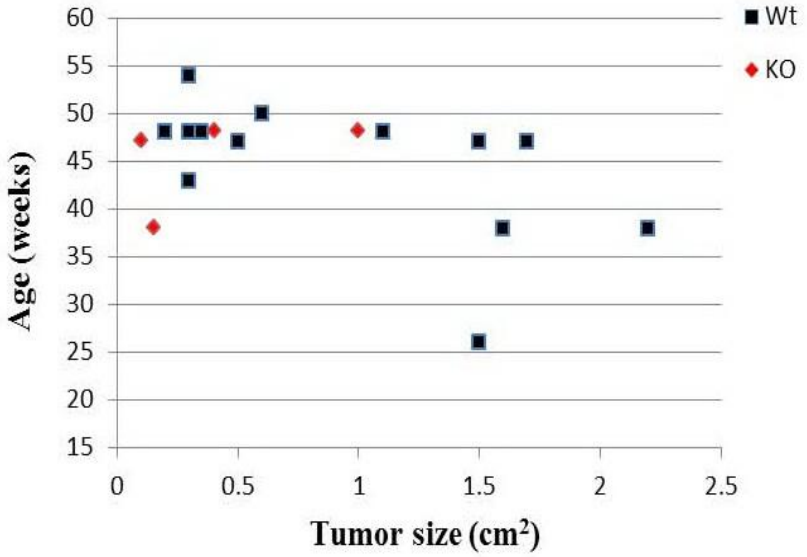
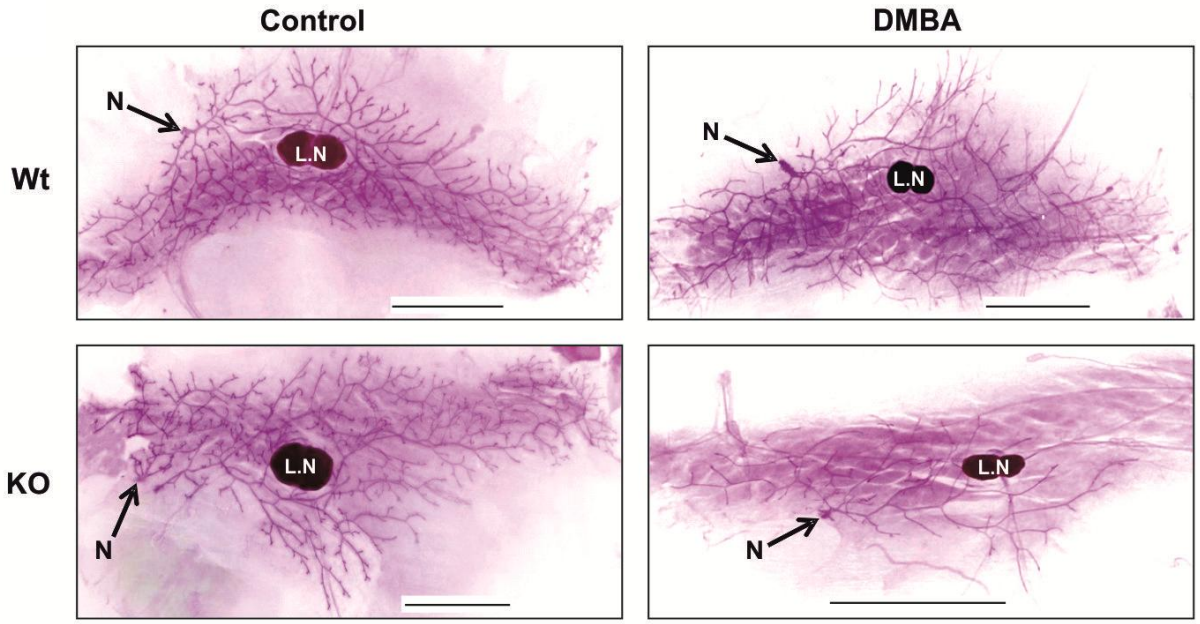


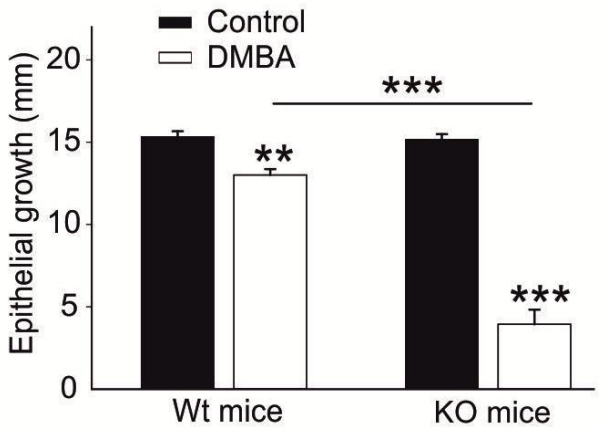




**GABARAP deletion increases the latency of tumor development and reduces the tumor size**

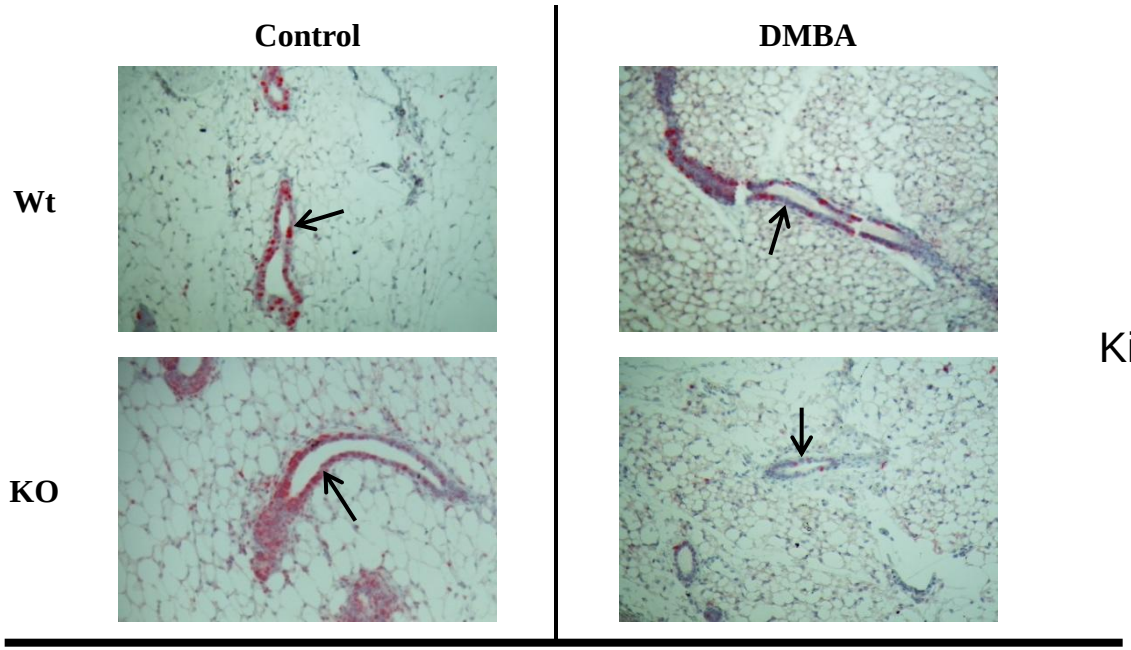
# Results: Impact of GABARAP KO on mammary glands upon DMBA




N = nipple
L.N = lymph node

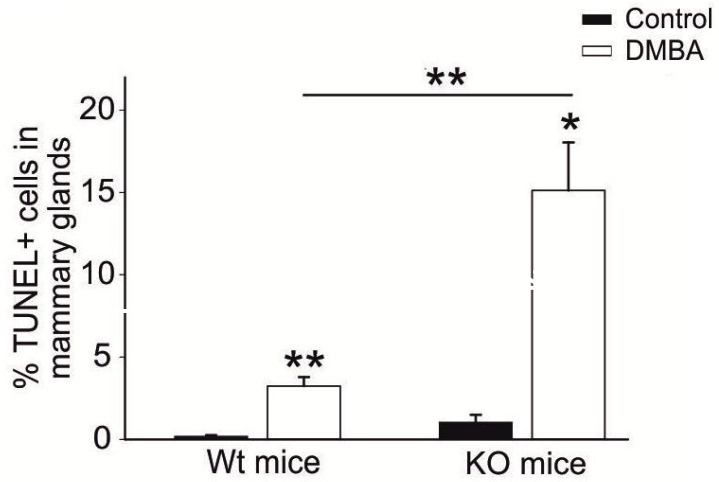


# Results: Proliferation (Ki-67) and TUNEL assay for mammary glands




Ki67 analysis

GABARAP deletion reduces mammary epithelial cell growth and tumor formation by enhancing apoptosis




TUNEL assay

- **GABARAP-dependent alteration in gene expression**
- **Alteration of gene expression in DMBA-treated mice**

# Results: GABARAP-dependent alteration in gene expression

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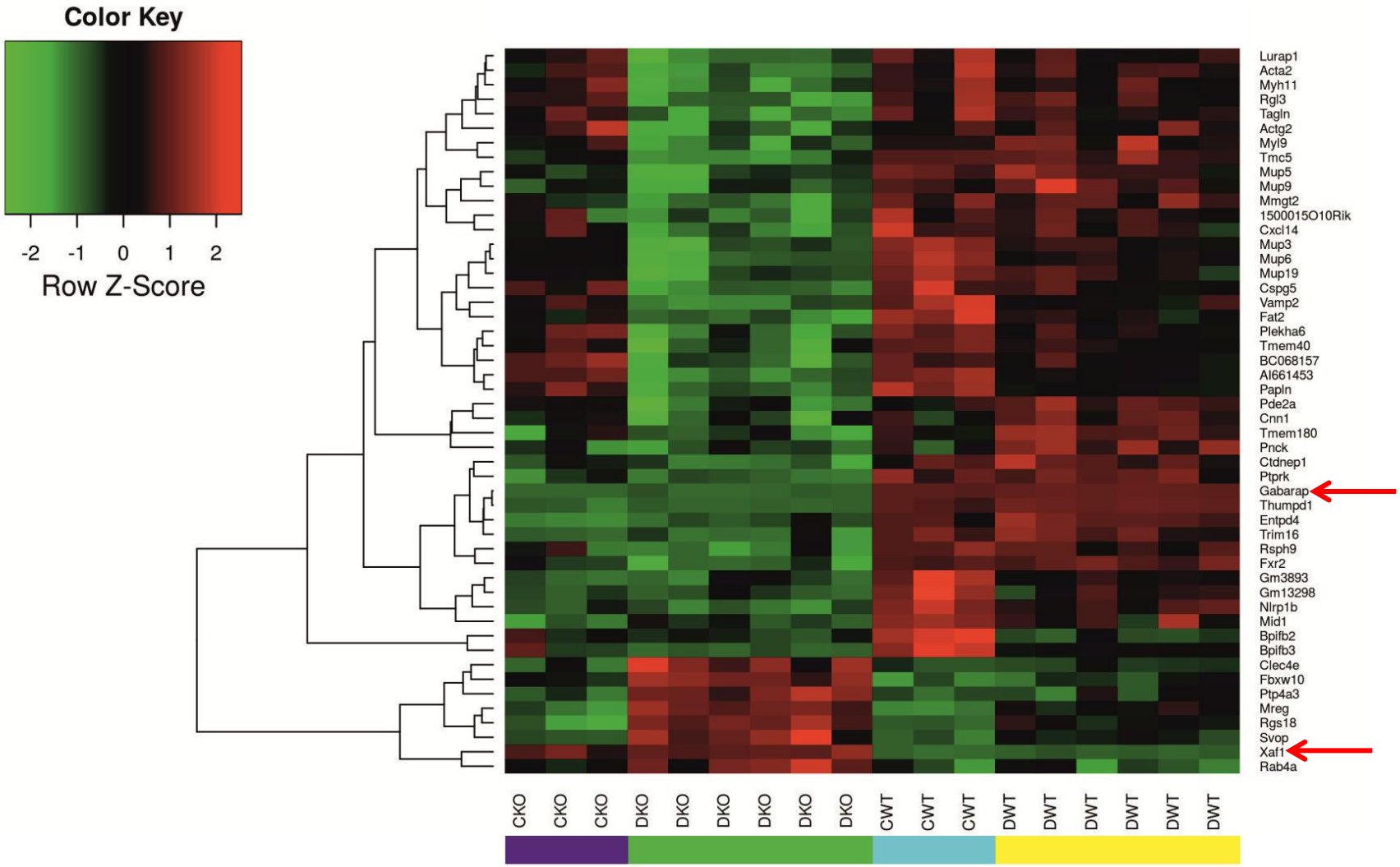
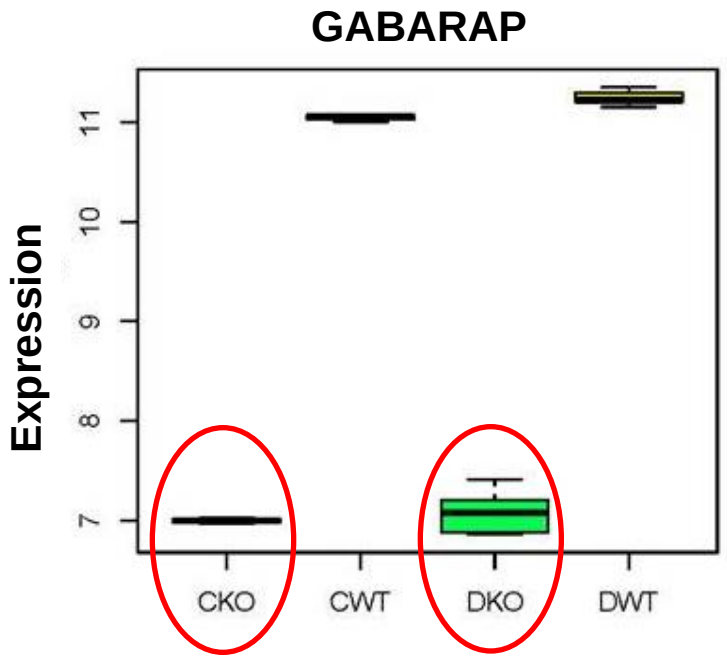
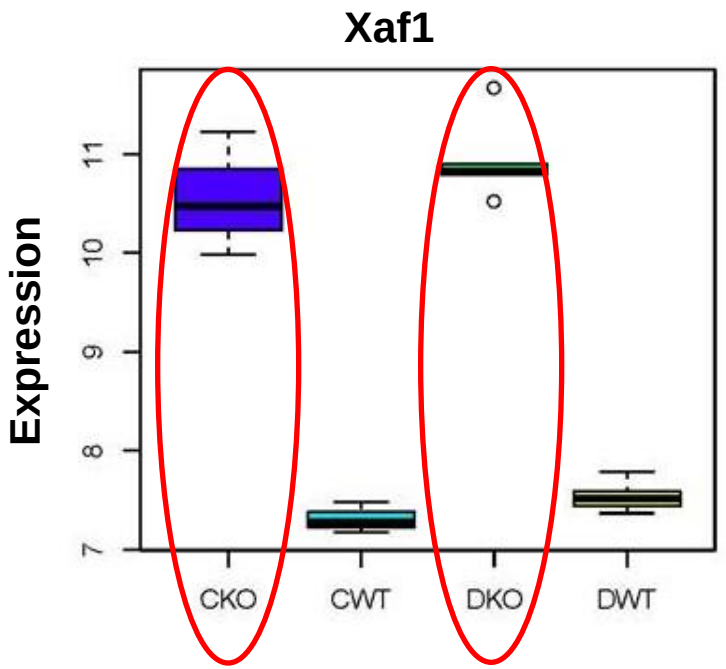



**CKO** = control (vehicle-treated) GABARAP KO, **DKO** = DMBA-treated GABARAP KO, **CWT** = control (vehicle-treated) wild-type, **DWT** = DMBA-treated wild-type.

# Results: GABARAP-dependent altered gene expression




- Xaf1 is differentially expressed in GABARAP KO mice.
- It is an apoptosis inducer and tumor suppressor gene.

**CKO** = control (vehicle-treated) GABARAP KO, **DKO** = DMBA-treated GABARAP KO, **CWT** = control (vehicle-treated) wild-type, **DWT** = DMBA-treated wild-type.

- **Alteration of gene expression in DMBA-treated mice**

# Results: Alteration of gene expression upon DMBA treatment

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| Gene | Full name | CWT vs DWT | CKO vs DKO | Function |
|---|---|---|---|---|
| GABARAP | gamma-aminobutyric acid receptor associated protein | 0.2 | 0.09 | autophagy |
| Xaf1 | XIAP associated factor 1 | 0.22 | 0.36 | apoptosis |
| Xiap | X-linked inhibitor of apoptosis | **0.86*** | 0.05 | apoptosis |
| Bid | BH3 interacting domain death agonist | 0.53 | **0.76*** | apoptosis |
| Apaf1 | apoptotic peptidase activating factor 1 | 0.08 | **0.59*** | apoptosis |
| Bax | BCL2-associated X protein | **1.79*** | **2.03*** | apoptosis |
| Tnfrsf10b | tumor necrosis factor receptor superfamily, member 10b | 0.62 | **0.89*** | cell death |
| Ripk1 | receptor (TNFRSF)-interacting serine-threonine kinase1 | 0.29 | **0.53*** | cell death |
| Siva1 | apoptosis-inducing factor | **1.3*** | **1.79*** | apoptosis |
| Stmn4 | stathmin-like 4 | -0.51 | **-1.14*** | microtubule destabilizer |
| Il1r1 | interleukin 1 receptor, type I | 0.08 | **0.95*** | cytokine receptor |
| Cdkn1a | cyclin-dependent kinase inhibitor 1A (**p21**) | **3.08*** | **3.73*** | cell cycle control |
| Cdkn2c | cyclin-dependent kinase inhibitor 2C (**p18**) | 0.58 | **0.86*** | cell cycle control |
| Rbx1 | ring-box 1 | **0.49*** | 0.3 | cell cycle control |
| Cdc7 | cell division cycle 7 | **0.5*** | 0.23 | cell cycle control |
| Cdk1 | cyclin-dependent kinase 1 | **1.06*** | 0.35 | cell cycle control |
| Tgfb3 | transforming growth factor, beta 3 | -0.51 | **-0.86*** | DNA replication |
| NF-κB1 | nuclear factor of kappa light polypeptide gene enhancer in B-cell 1 | **0.71*** | 0.6 | transcription factor |
| Smad2 | SMAD family member 2 | **0.67*** | 0.38 | transcription factor |
| E2f1 | E2F transcription factor 1 | **-0.9*** | **-0.62*** | transcription factor |
| E2f4 | E2F transcription factor 4 | **1.24*** | 0.76 | transcription factor |
| Tfdp2 | transcription factor Dp 2 | **0.68*** | 0.47 | transcription factor |
| GABARAPL2 | GABARAP-like 2 | **0.65*** | **0.62*** | autophagy |
| Atg12 | autophagy related 12 | **0.53*** | **0.62*** | autophagy |
| Prkaa1 | protein kinase, AMP-activated, alpha 1 catalytic subunit | **0.79*** | **0.7*** | autophagy |
| Atg3 | autophagy related 3 | **0.79*** | **1.08*** | autophagy |
| Atg5 | autophagy related 5 | **0.5*** | 0.28 | autophagy |

* Gene expression with significant differences between the compared groups

# Results: Confirmation of gene expression data by qRT–PCR

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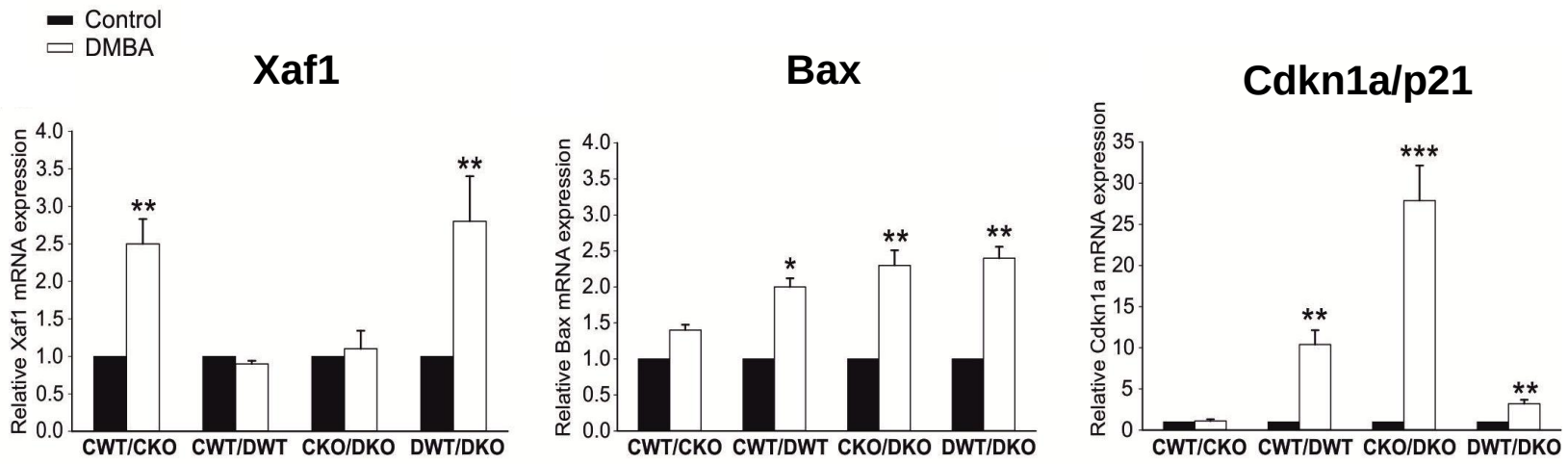


These finding indicated that Xaf1 upregulation in mammary glands of GABARAP KO mice may contribute to the inhibition of tumor formation and may enhance apoptosis induction upon DMBA treatment

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# Immunotoxicity and immune response upon genotoxic stress mediated by DMBA

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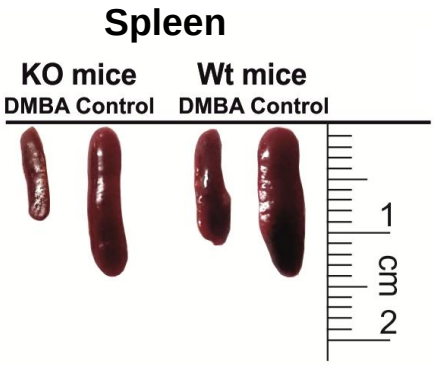
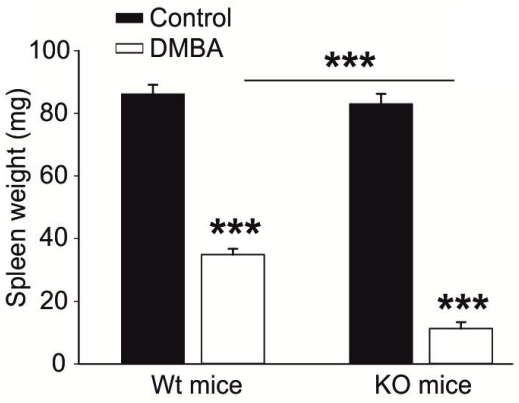
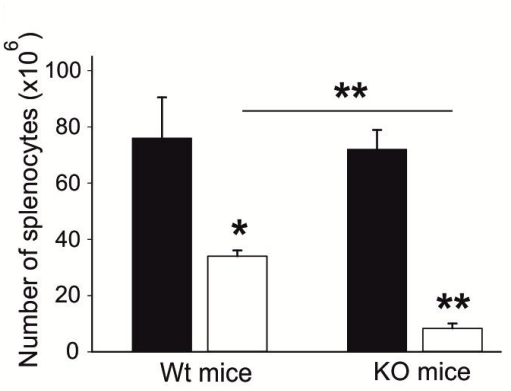
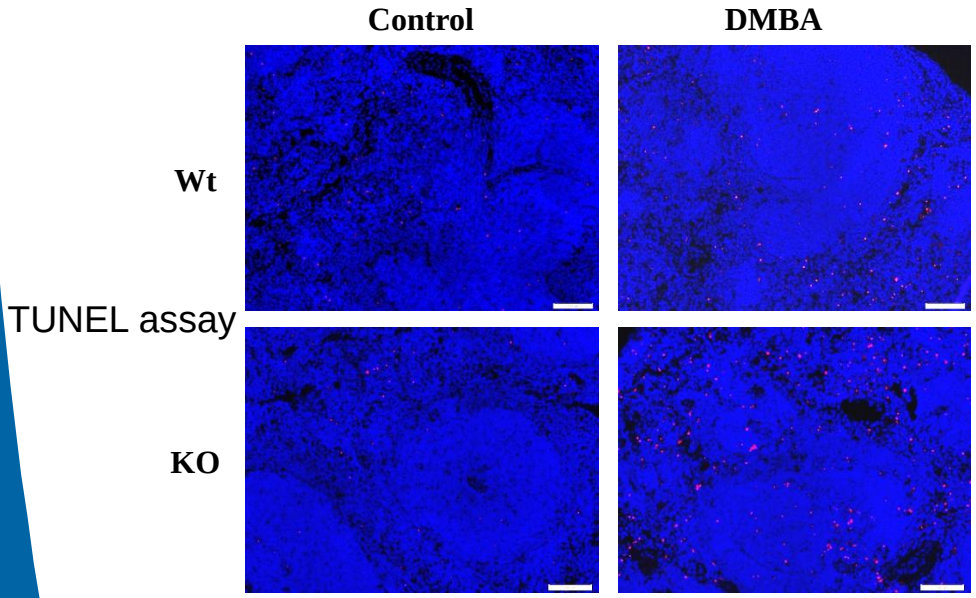
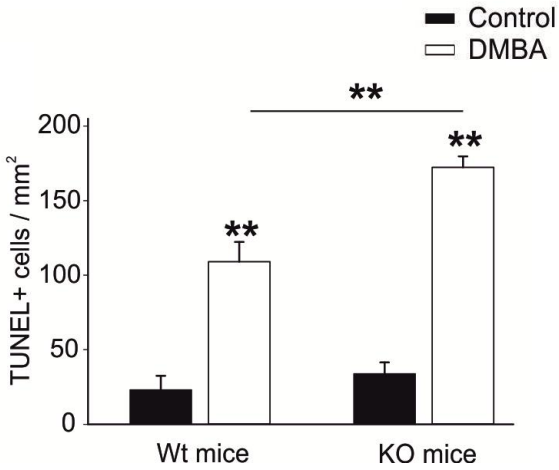
GABARAP KO enhanced the genotoxic effect of DMBA in the spleen



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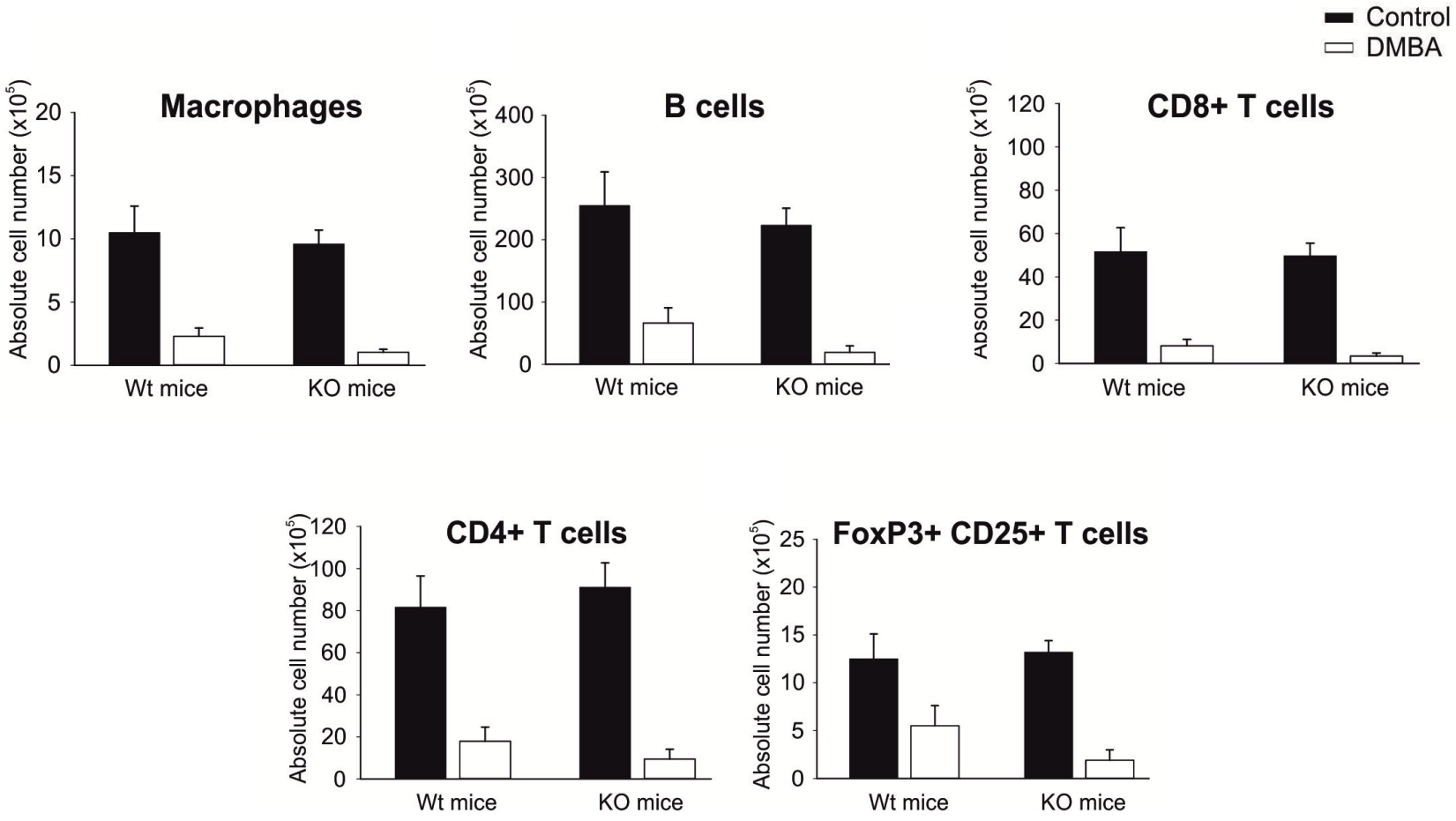
- FACS analysis




There was no discrimination for the genotoxic/immunotoxic effect of DMBA on particular cell types of splenocytes in both GABARAP KO and wild-type mice

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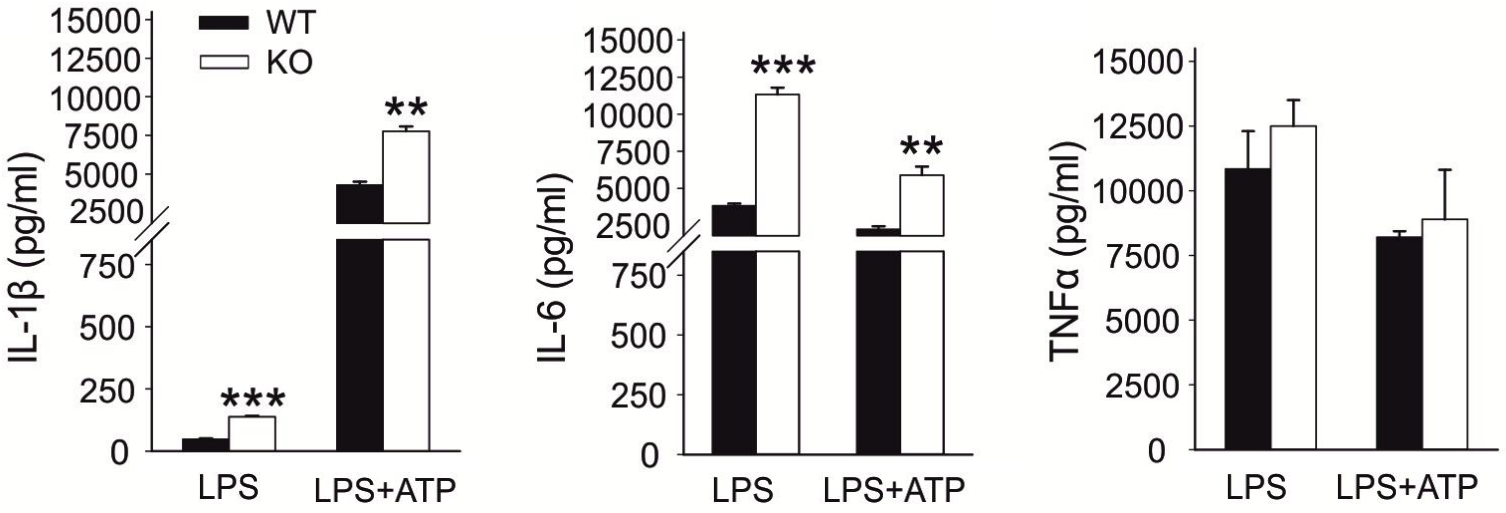
- Peritoneal macrophages from DMBA-treated mice (ELISA)




Inflammatory cytokine secretion (IL-1β, IL-6) is significantly enhanced in GABARAP KO cells, i.e. knockout may activate the inflammasome.

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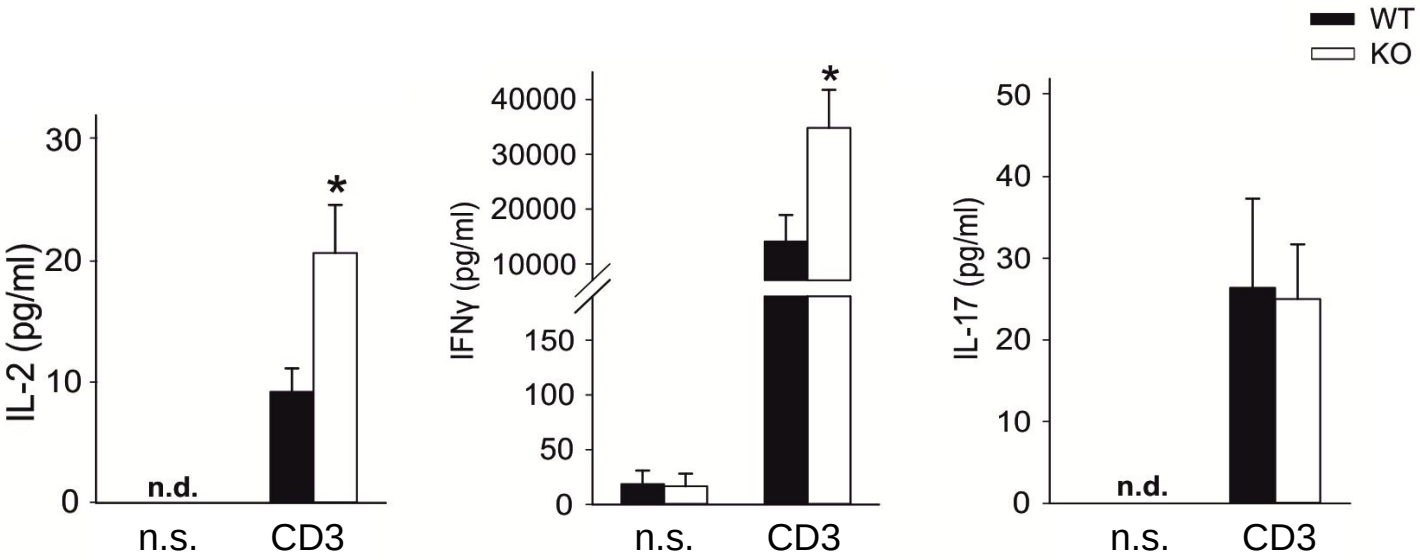
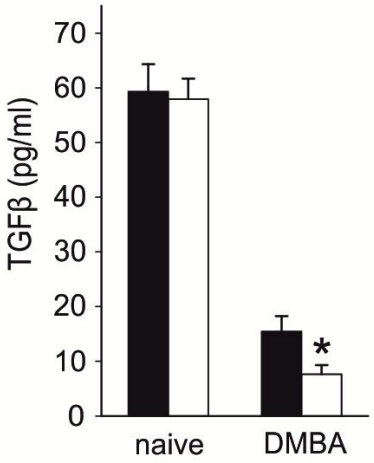
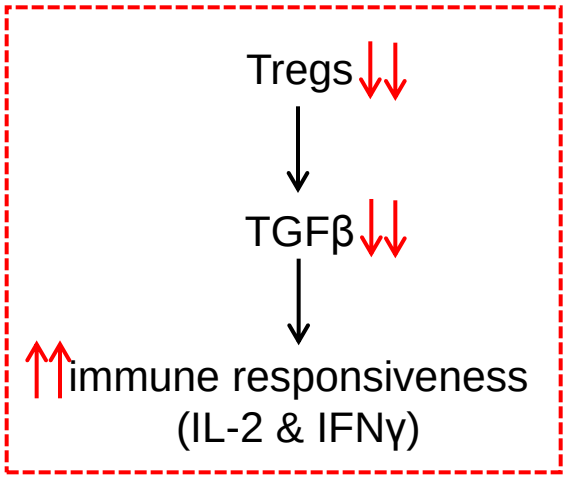
- Splenic lymphocytes from DMBA-treated mice (ELISA)

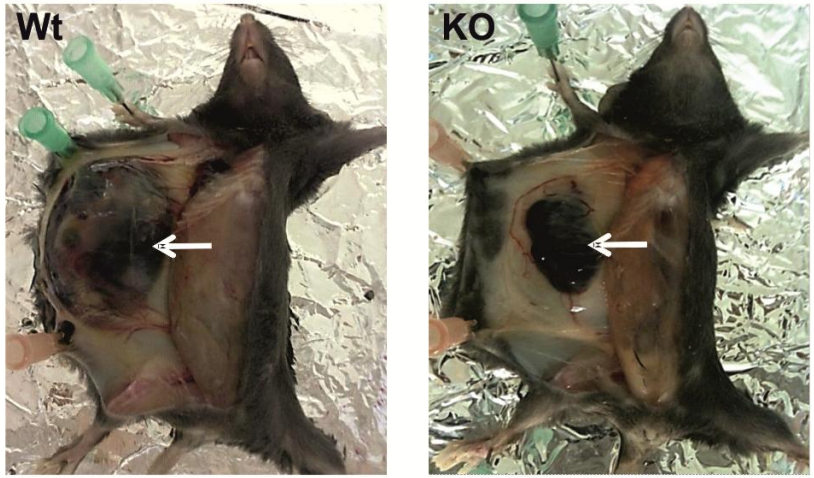


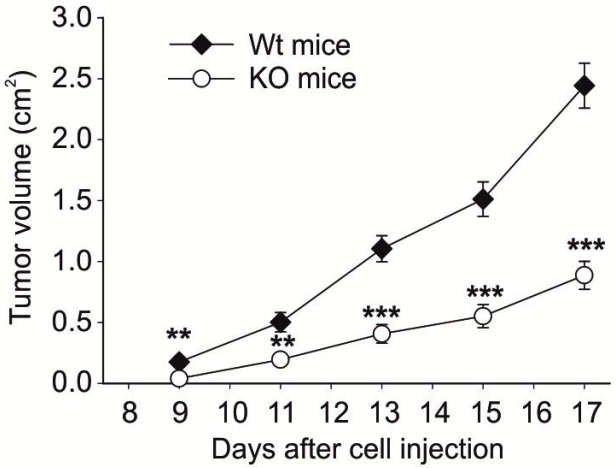

- TGFβ level in serum (ELISA)



# Results: Tumor cells inoculation




This result suggests that intact GABARAP function in the host animal is advantageous for the growth of the inoculated syngenic tumor cells

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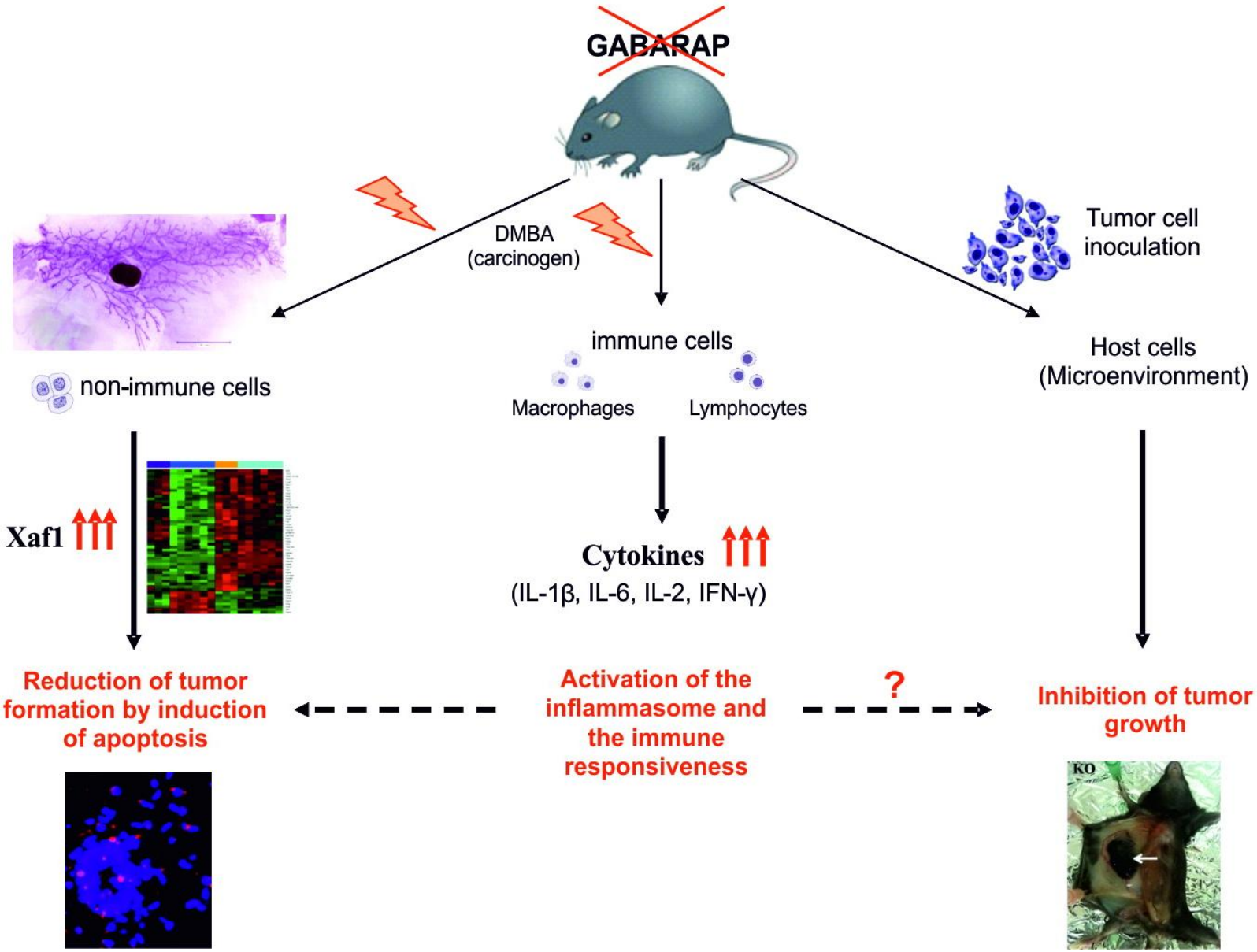

OPEN

Citation: Cell Death and Disease (2016) 7, e2205; doi:10.1038/cddis.2016.93

www.nature.com/cddis

# Tumor suppression in mice lacking GABARAP, an Atg8/LC3 family member implicated in autophagy, is associated with alterations in cytokine secretion and cell death

FS Salah[1,2], M Ebbinghaus[3], VY Muley[4,5], Z Zhou[6], KRD Al-Saadi[8], M Pacyna-Gengelbach[7], GA O'Sullivan[8], H Betz[8,9], R König[4,5], Z-Q Wang[6,10], R Bräuer[1] and I Petersen*[,1]

GABARAP belongs to an evolutionary highly conserved gene family that has a fundamental role in autophagy. There is ample evidence for a crosstalk between autophagy and apoptosis as well as the immune response. However, the molecular details for these interactions are not fully characterized. Here, we report that the ablation of murine GABARAP, a member of the Atg8/LC3 family that is central to autophagosome formation, suppresses the incidence of tumor formation mediated by the carcinogen DMBA and results in an enhancement of the immune response through increased secretion of IL-1$\beta$, IL-6, IL-2 and IFN-$\gamma$ from stimulated macrophages and lymphocytes. In contrast, TGF-$\beta$1 was significantly reduced in the serum of these knockout mice. Further, DMBA treatment of these GABARAP knockout mice reduced the cellularity of the spleen and the growth of mammary glands through the induction of apoptosis. Gene expression profiling of mammary glands revealed significantly elevated levels of Xaf1, an apoptotic inducer and tumor-suppressor gene, in knockout mice. Furthermore, DMBA treatment triggered the upregulation of pro-apoptotic (Bid, Apaf1, Bax), cell death (Tnfrsf10b, Ripk1) and cell cycle inhibitor (Cdkn1a, Cdkn2c) genes in the mammary glands. Finally, tumor growth of B16 melanoma cells after subcutaneous inoculation was inhibited in GABARAP-deficient mice. Together, these data provide strong evidence for the involvement of GABARAP in tumorigenesis *in vivo* by delaying cell death and its associated immune-related response.
*Cell Death and Disease* (2016) 7, e2205; doi:10.1038/cddis.2016.93; published online 28 April 2016

## Acknowledgment


- **Prof. Dr. med.** Iver Petersen
- **Prof. Dr.** Rolf Bräuer
- **Dr.** Matthias Ebbinghaus **(**Institute of Physiology 1, Jena**)**
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Thank you for attention