



## **Supplemental Material to:**

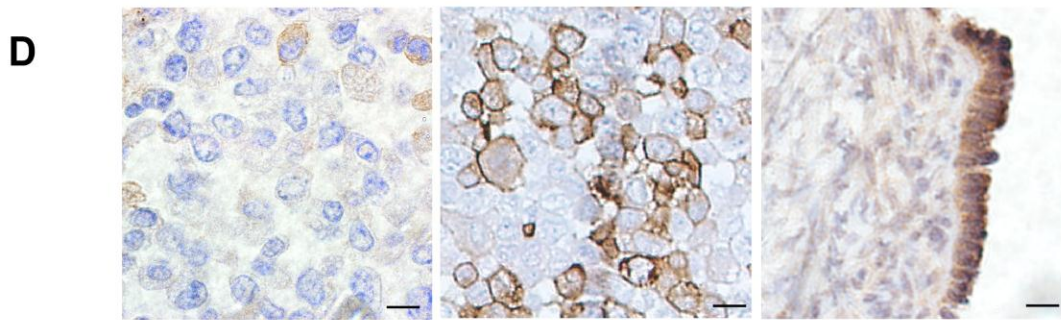
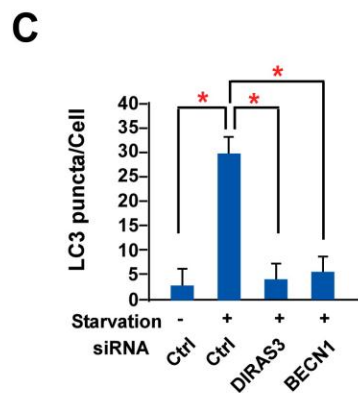
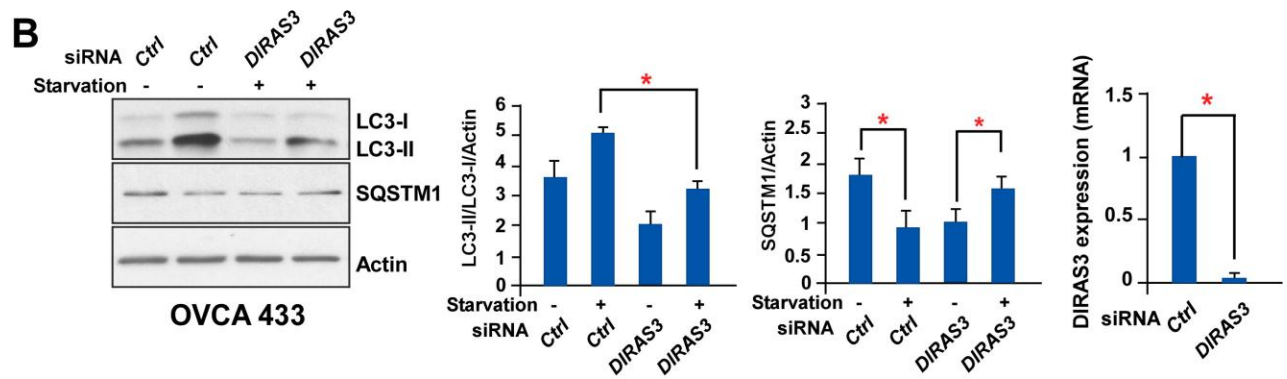
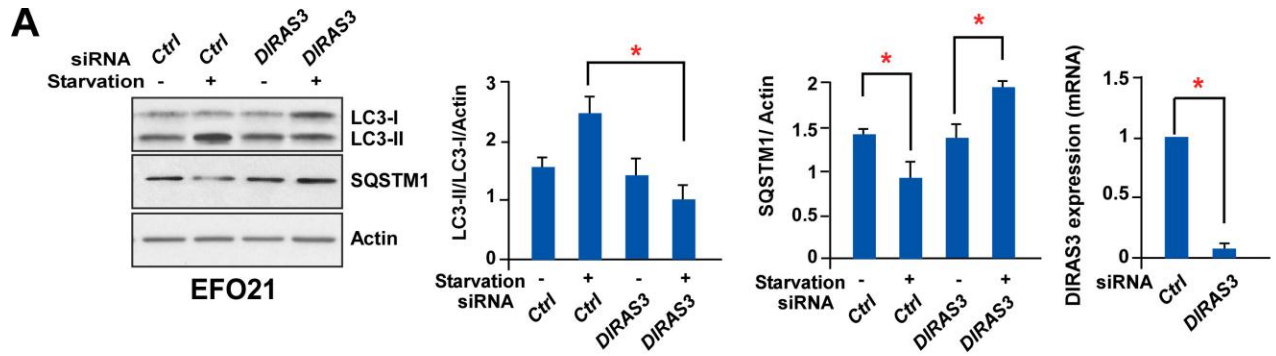
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**DIRAS3 regulates the autophagosome initiation complex  
in dormant ovarian cancer cells**

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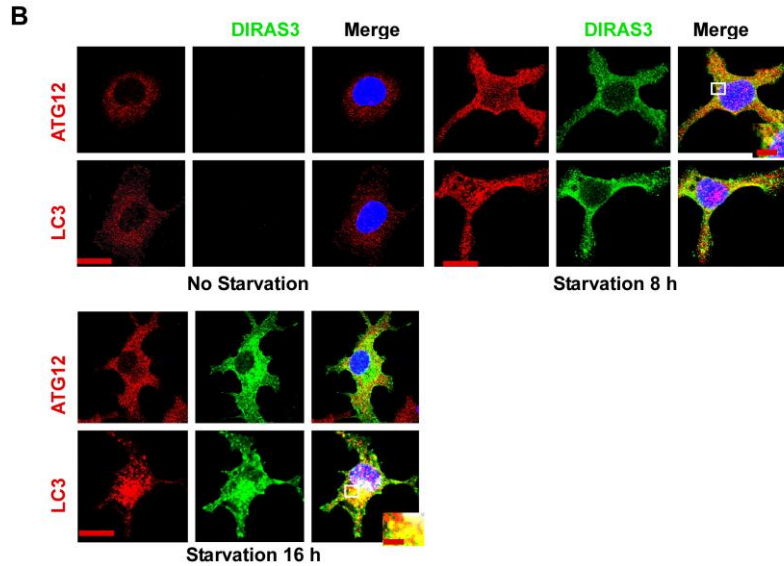
SKOV3-DIRAS3 -DOX SKOV3-DIRAS3 +DOX (24 hr) Normal ovarian epithelial cells

**Figure S1.** DIRAS3 expression is required and sufficient for induction of autophagy. **(A)** Knockdown of DIRAS3 in EFO21 cells impairs starvation-induced LC3 puncta formation and proteolysis of SQSTM1. EFO21 cells in growth medium were transfected with siControl or siDIRAS3 48 hrs before incubation in growth medium or amino acid starvation medium for 4 h. Cell lysates were then collected for western blot analysis. Results from 3 independent experiments are shown. Band intensity was quantified using ImageJ. The columns indicate the ratio of mean or relative expression, and the bars indicate the s.e. (\* $p < 0.05$ ). **(B)** Knockdown of DIRAS3 in OVCA433 cells impairs starvation-induced LC3 puncta formation and proteolysis of SQSTM1. OVCA433 cells in growth medium were transfected with siControl or siDIRAS3 48 h before incubation in growth medium or amino acid starvation medium for 4 h. Cell lysates were collected for western analysis. Results from 3 independent experiments are shown. Band intensity was quantified using ImageJ. The columns indicate the ratio of mean or relative expression, and the bars indicate the s.e. (\* $p < 0.05$ ). **(C)** Knockdown of DIRAS3 in SKOV3-DIRAS3 significantly impaired the starvation-induced LC3 puncta formation and proteolysis of SQSTM1. The quantification of green LC3 puncta is shown. Results from 3 independent experiments are shown. The columns indicate the mean, and the bars indicate the s.e. (\* $p < 0.05$ ). **(D)** Comparable DIRAS3 expression in DOX-induced SKOV3-DIRAS3 cells and the normal ovarian epithelial (NOE) cells. SKOV3 cells were incubated with or without DOX for 24 h, pelleted by centrifugation, washed, fixed in 10% formalin, embedded in paraffin, sectioned (5  $\mu\text{m}$ ) and stained immunohistochemically with anti-DIRAS3 antibody. Sections (5  $\mu\text{m}$ ) of normal ovary were similarly processed and stained as a control.

**A**

**Colocalization analysis with ImageJ**

	Pearson's correlation	Overlap coefficient
DIRAS3_ATG12	0.716988	0.97027
DIRAS3_LC3	0.773758	0.961876

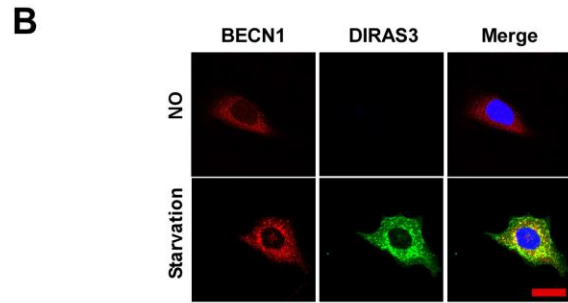


	Pearson's correlation	Overlap coefficient
DIRAS3_ATG12	0.682365 (8 hrs) 0.588931 (16 hrs)	0.87012 (8 hrs) 0.77117 (16 hrs)
DIRAS3_LC3	0.428170 (8 hrs) 0.893197 (16 hrs)	0.67825 (8 hrs) 0.99828 (16 hrs)

**Figure S2.** DIRAS3 colocalizes with autophagy protein markers at different intervals. **(A)** DIRAS3 colocalizes with ATG12 and LC3. Figure 2A images were analyzed with ImageJ “Colocalization\_Finder” plugin (Christophe Laummonerie; 2006/08/29: Version 1.2). **(B)** DIRAS3 colocalizes with ATG12 and LC3 in additional confocal studies. SKOV3-DIRAS3 cells were incubated in growth medium or amino acid starvation medium for the times indicated. Immunofluorescence staining of DIRAS3 and endogenous ATG12 and LC3 were analyzed by confocal microscopy. Scale bars: 10  $\mu$ m. Higher magnifications of indicated regions are shown in the insets; scale bars: 1  $\mu$ m. The colocalization DIRAS3 with ATG12 and LC3 was analyzed using ImageJ.

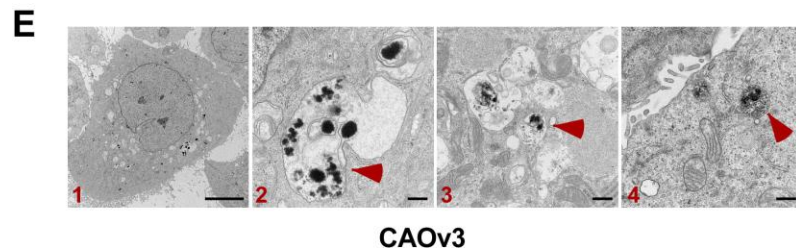
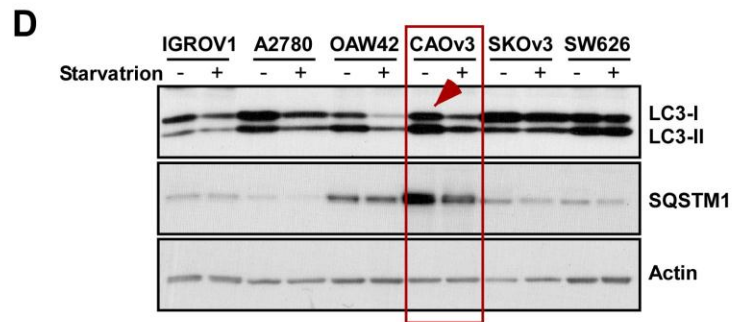
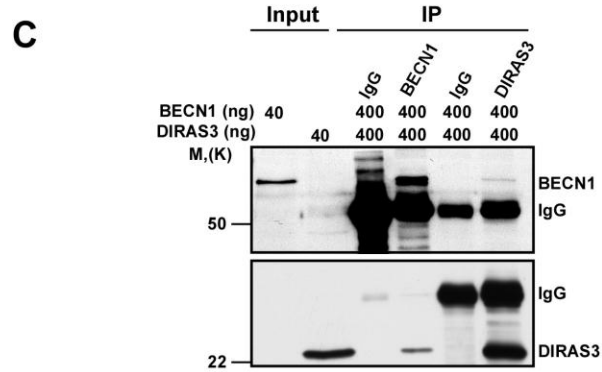
**A** Colocalization analysis with ImageJ

	Pearson's correlation	Overlap coefficient
DIRAS3_BECN1	0.762936	0.978953

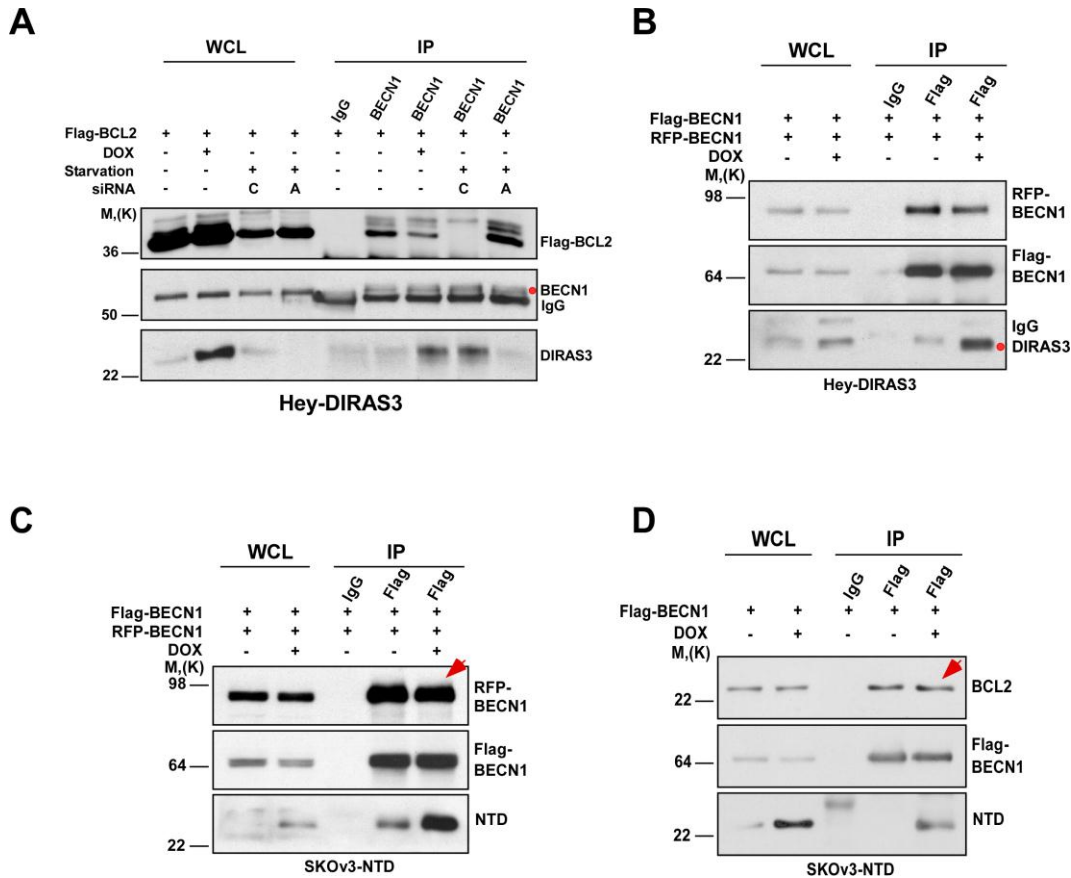


Colocalization analysis with ImageJ

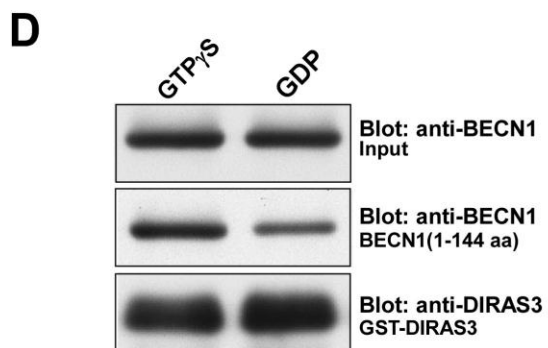
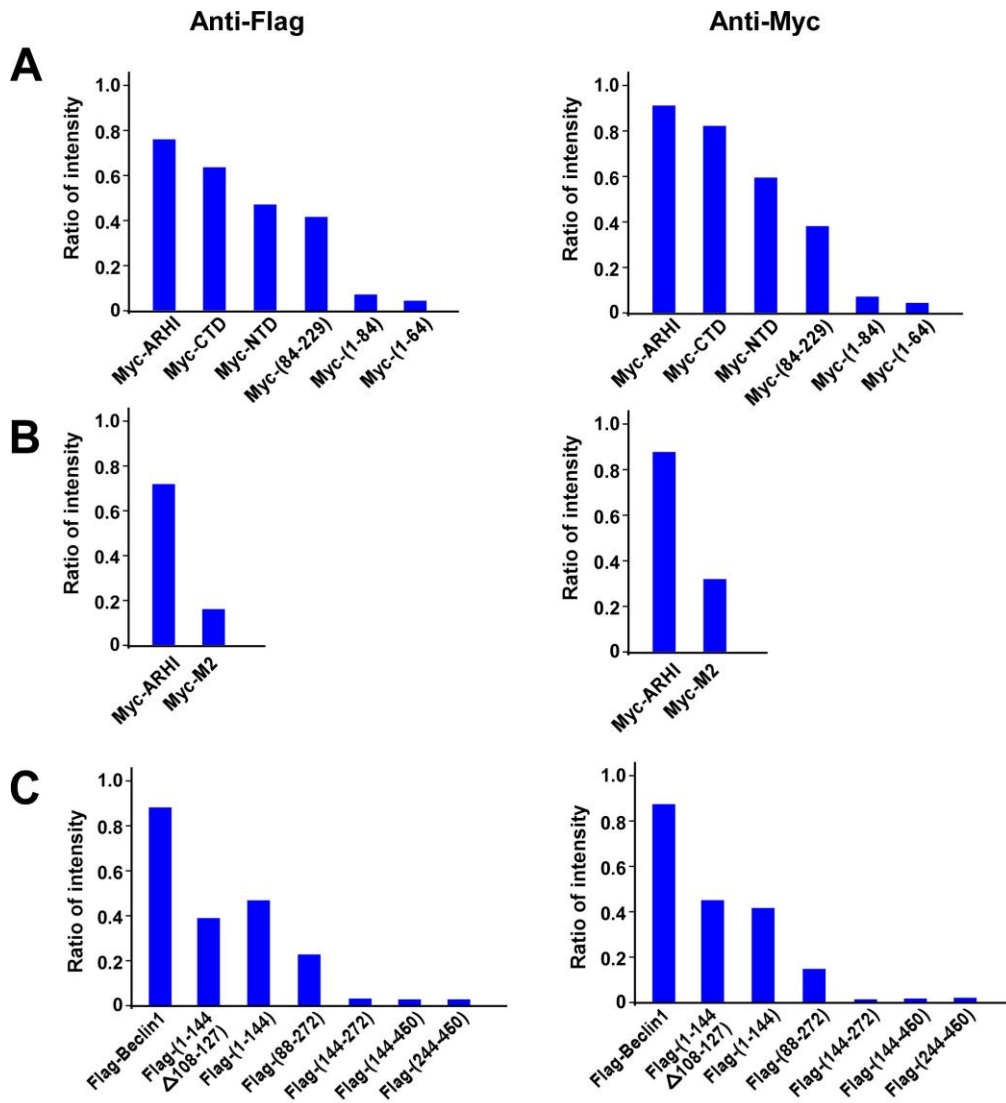
	Pearson's correlation	Overlap coefficient
DIRAS3_BECN1	0.702286	0.971363



**Figure S3.** DIRAS3 colocalizes and interacts with BECN1. Caov3 cells have high basal autophagy. **(A)** DIRAS3 colocalizes with BECN1. Figure 3A images were analyzed with ImageJ “Colocalization\_Finder” plugin (Christophe Laummonerie; 2006/08/29: Version 1.2). **(B)** DIRAS3 colocalizes with BECN1 in additional confocal analysis. SKOV3-DIRAS3 cells were treated with amino acid starvation medium for 16 h. Immunofluorescent staining of expressed DIRAS3 and endogenous BECN1 were analyzed by confocal microscopy. Scale bars: 10  $\mu\text{m}$ . The colocalization of DIRAS3 with BECN1 was analyzed using ImageJ. **(C)** DIRAS3 interacts with BECN1 *in vitro*. Purified recombinant DIRAS3 and BECN1 proteins were incubated together to allow complex formation. The DIRAS3-BECN1 complexes that formed were immunoprecipitated with anti-BECN1 or anti-DIRAS3 antibodies and analyzed by western blotting. **(D)** CAOV3 cells have high basal autophagosome formation with western blot analysis as indicated. **(E)** CAOV3 cells have high basal autophagosome formation with EM analysis. 1 Low magnification scale bar: 10  $\mu\text{m}$ ; 2, 3, and 4 high magnification scale bars: 500 nm.

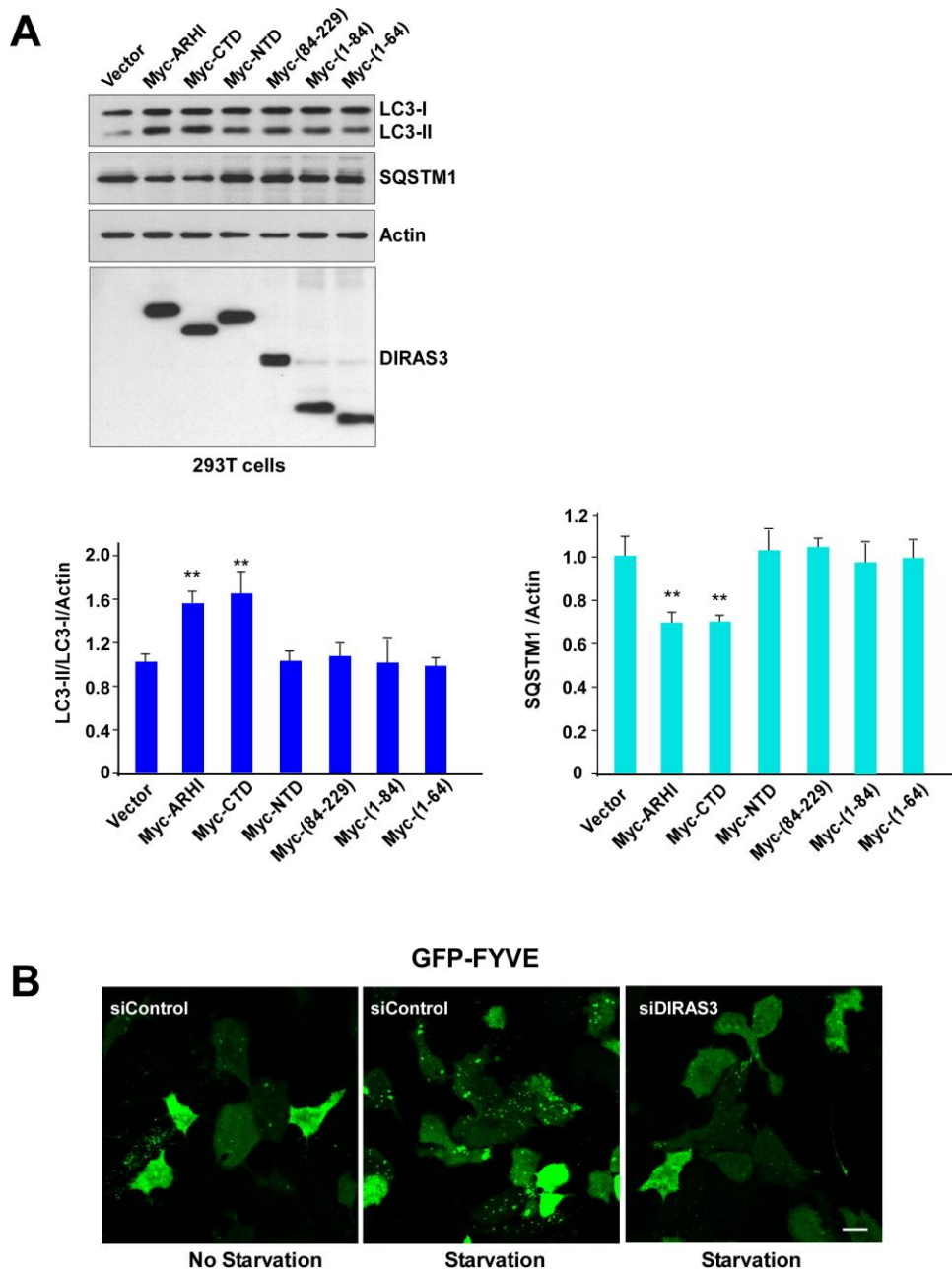


**Figure S4.** DIRAS3 inhibits BECN1 and BCL2 interaction and DIRAS3 inhibits BECN1 homodimerization. **(A)** DIRAS3 is required for amino acid starvation-induced dissociation of BECN1 and BCL2. Hey-DIRAS3 cells in growth medium were treated with DIRAS3 siRNA for 48 h before incubation in growth medium or in amino-acid starvation medium for 16 h with or without DOX treatment. The BECN1-BCL2 and BECN1-DIRAS3 complexes were immunoprecipitated with anti-BECN1 antibody or anti-BCL2 antibody and analyzed for both proteins by western blot analysis. **(B)** DIRAS3 inhibits Flag-BECN1 and RFP-BECN1 dimerization. Hey-DIRAS3 cells were treated with DOX for 24 h and then transfected with Flag-BECN1 and RFP-BECN1 for 24 h. Flag-BECN1/RFP-BECN1 complexes were immunoprecipitated with anti-Flag or anti-RFP antibodies and analyzed for Flag-BECN1 and RFP-BECN1 by western blot analysis. **(C)** NTD did not inhibit Flag-BECN1 and RFP-BECN1 dimerization. SKOv3-NTD cells were treated with DOX for 24 h and then transfected with Flag-BECN1 and RFP-BECN1 or RFP-Vector. Flag-BECN1-RFP-BECN1 or Flag-BECN1-RFP-Vector was immunoprecipitated with anti-Flag and analyzed by western blotting. **(D)** NTD does not inhibit Flag-BECN1 and endogenous BCL2 interaction. SKOv3-DIRAS3 cells were treated with DOX for 24 h, then transfected with Flag-BECN1. Flag-BECN1-BCL2 complexes were immunoprecipitated with anti-Flag and analyzed by western blotting.

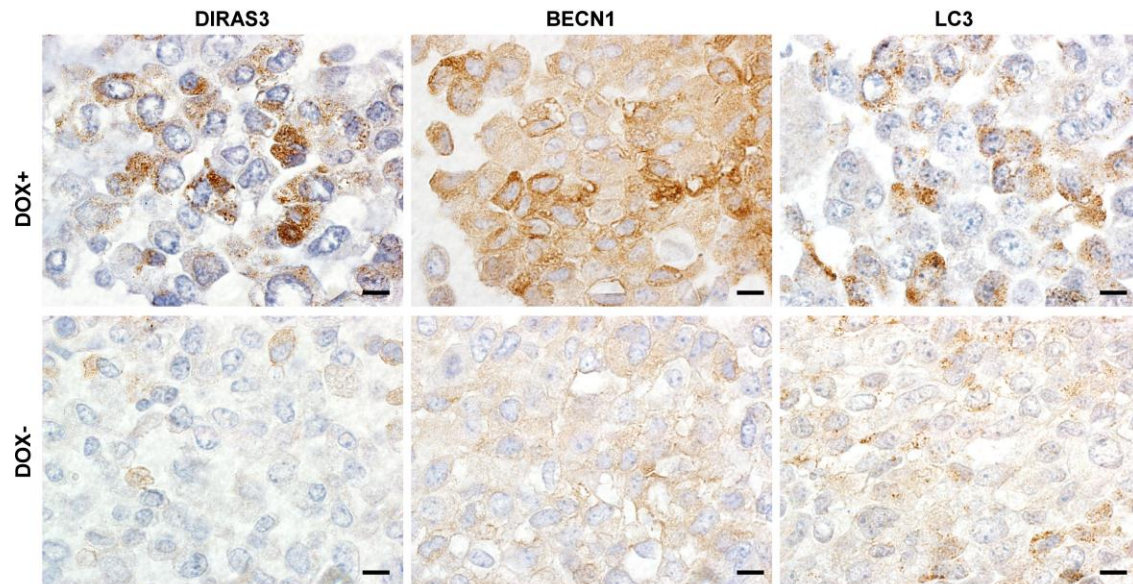
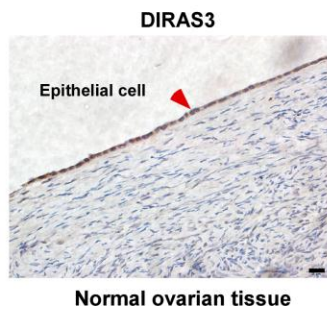




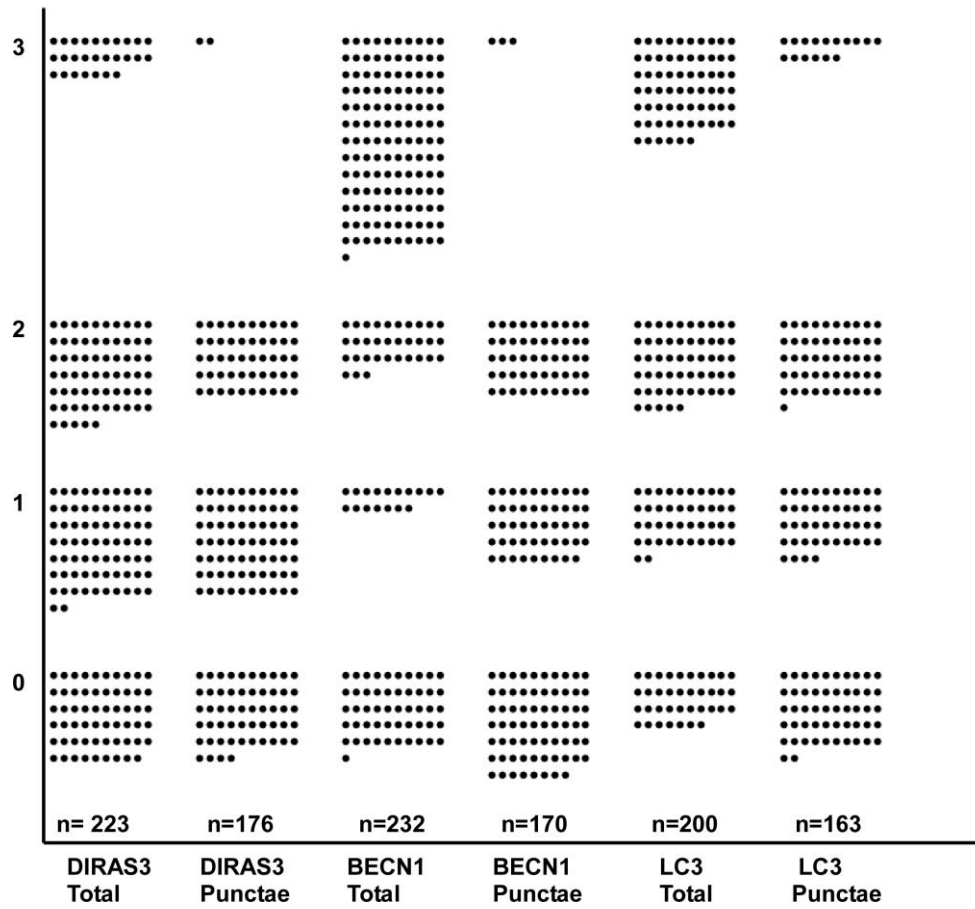
**Figure S5.** DIRAS3 and BECN1 interaction. **(A, B and C)** Analysis of DIRAS3-BECN1 interaction presented in Figure 5. The ratio of intensity was determined based on the anti-Myc (DIRAS3) ratios relative to the amount of Flag-BECN1 that was affinity isolated with the anti-Flag antibody, and the anti-Flag (BECN1) ratios relative to the amount of Myc-DIRAS3 that was affinity isolated with the anti-Myc antibody. **(D)** GTP-dependent interaction between DIRAS3 and BECN1. GST affinity isolation assay was performed with purified GST-DIRAS3 protein and purified BECN1 protein (1-144 aa) loaded with GTP $\gamma$ S or GDP. BECN1-DIRAS3 complexes bound to beads were analyzed by western blots with anti-BECN1 and anti-DIRAS3 antibodies. Input loaded 1/40 volume of the reaction mixture used for the GST affinity isolation.



**Figure S6.** Analysis of DIRAS3 mutants to induce autophagy in 293T cells and detection of PIK3C3 kinase activity in SKOv3-DIRAS3 cells. **(A)** Induction of autophagy by DIRAS3 and DIRAS3 mutants. DIRAS3 and DIRAS3 deletion mutants were transfected into 293T cells for 24 h. Induction of autophagy was examined by western blotting of LC3 and SQSTM1. Three independent experiments were carried out. Band intensity was quantified using ImageJ and is shown at the bottom. The columns indicate the mean, and the bars indicate the s.e. (\*\* $p < 0.01$ ). **(B)** Knockdown of DIRAS3 decreases PIK3C3 kinase activity. SKOv3-DIRAS3 cells were transfected with siDIRAS3 for 48 h and then treated with amino acid starvation medium for 16 h. GFP-2x-FYVE puncta were quantified using ImageJ. Scale bars: 10  $\mu\text{m}$ . Data were obtained from 3 independent experiments. Values are the means  $\pm$  S.D. (\* $p < 0.05$ ).

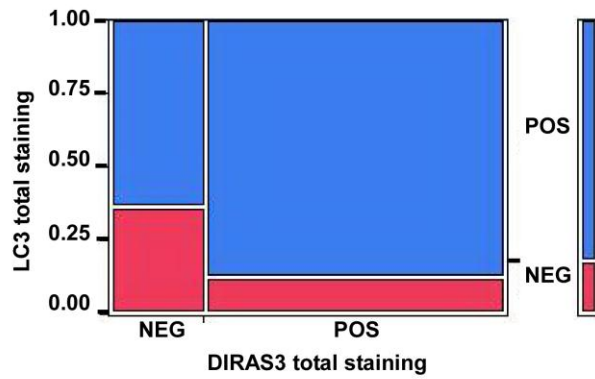
**A****B**

**Figure S7.** DIRAS3 and BECN1 expression positively correlated with punctate LC3B punctate staining in autophagosomes within ovarian cancers. **(A)** Representative images from paraffin-embedded cells. Induced and uninduced SKOV3-DIRAS3 cells were stained with the indicated antibodies to the controls of the tissue array. Scale bars: 10  $\mu$ m. **(B)** Representative tissue microarray image. Normal ovarian epithelial cells were stained with DIRAS3 antibody as a control for the tissue array. Scale bar: 2  $\mu$ m.



**Figure S8.** Sample distribution. A dot plot shows the distribution of cases from the tissue array for total or puncta staining for DIRAS3, LC3, and BECN1 on an intensity scale of 0-3.

### Mosaic Plot



### Contingency Table

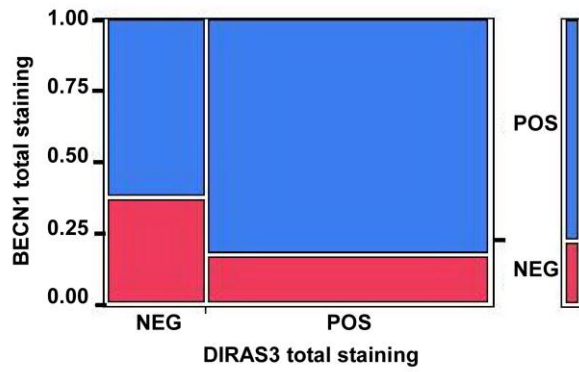
		LC3 total staining		
		NEG	POS	
DIRAS3 total staining	Count			
	Total %			
	Col %			
Row%				
NEG	17 8.81 48.57 38.17	30 15.54 18.99 63.83	47 24.35	
POS	18 9.33 51.43 12.33	128 80.66 78.53 82.58	146 75.85	
	35 18.13	158 81.87	193	

### Tests

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	12.181	0.0005
Likelihood Ratio	13.613	0.0002

**Figure S9.** Chi-squared analysis correlates DIRAS3 total expression with LC3B total expression.

**Mosaic Plot**



**Contingency Table**

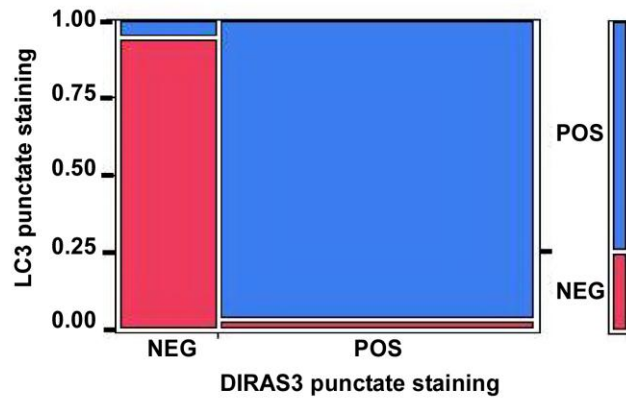
		BECN1 total staining		
		NEG	POS	
DIRAS3 total staining	Count	21	16	56
	Total %	9.95	16.59	26.54
	Col %	43.75	21.47	
	Row%	37.50	82.50	
	POS	27	128	155
	Total %	12.80	80.66	73.48
	Col %	56.25	78.53	
	Row%	14.42	82.58	
	NEG	48	163	211
	Total %	22.75	77.25	

**Tests**

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	8.824	0.0030
Likelihood Ratio	9.439	0.0021

**Figure S10.** Chi-squared analysis correlates DIRAS3 total expression with BECN1 total expression.

### Mosaic Plot



### Contingency Table

LC3 punctate staining

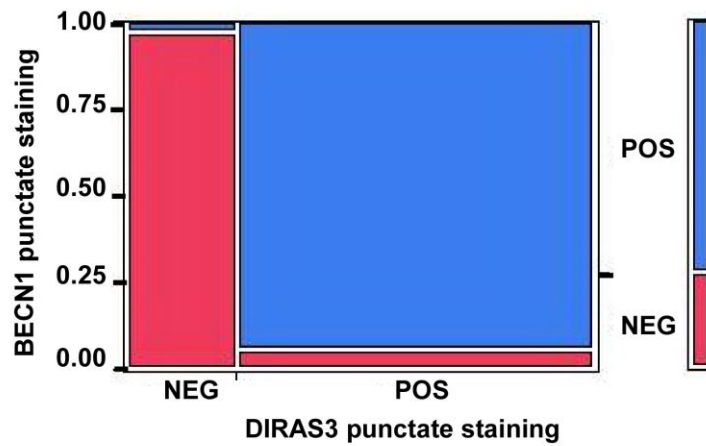
		LC3 punctate staining		
		NEG	POS	
DIRAS3 punctate staining	Count			
	Total %			
	Col %			
	Row%			
NEG	32 23.02 88.89 94.12	2 1.44 1.94 5.88	34 24.46	
POS	4 2.88 11.11 3.81	101 72.88 98.08 96.16	105 75.54	
	38 25.90	103 74.10	139	

### Tests

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	109.816	<0.001
Likelihood Ratio	109.143	<0.001

**Figure S11.** Chi-squared analysis correlates DIRAS3 puncta expression with LC3 puncta expression.

### Mosaic Plot



### Contingency Table

		BECN1 punctate staining		
		NEG	POS	
DIRAS3 punctate staining	Count			
	Total %			
	Col %			
	Row%			
NEG	32 23.36 84.21 96.97	1 0.73 1.01 3.03	33 24.09	
POS	6 4.38 15.79 5.77	98 71.53 98.99 94.23	146 75.85	
	38 27.74	99 72.26	137	

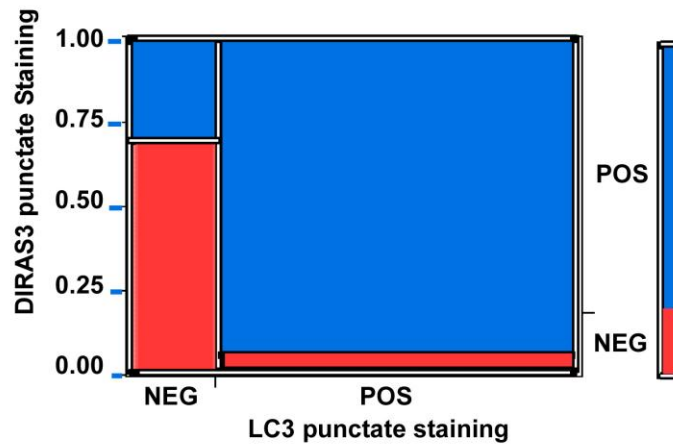
### Tests

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	106.944	<0.0001
Likelihood Ratio	103.954	<0.0001

**Figure S12.** Chi-squared analysis correlates DIRAS3 puncta expression with BECN1 puncta expression.



### Mosaic Plot



### Contingency Table

DIRAS3 punctate staining

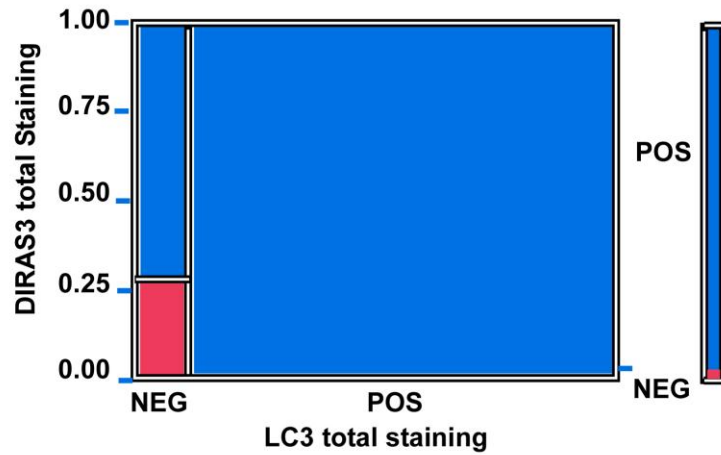
		DIRAS3 punctate staining		
		NEG	POS	
LC3 punctate staining	Count	9	4	13
	Total %	13.85	6.15	20.00
	Col %	75.00	7.55	
	Row%	69.23	30.77	
	POS	3	55	52
	Total %	4.62	75.38	80.00
	Col %	25.00	92.45	
	Row%	5.77	94.23	
	NEG	12	53	65
	Total %	18.46	81.54	

### Tests

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	23.194	<.0001
Likelihood Ratio	27.024	<.0001

**Figure S13.** Chi-squared analysis correlates DIRAS3 puncta staining with LC3 puncta staining.

### Mosaic Plot



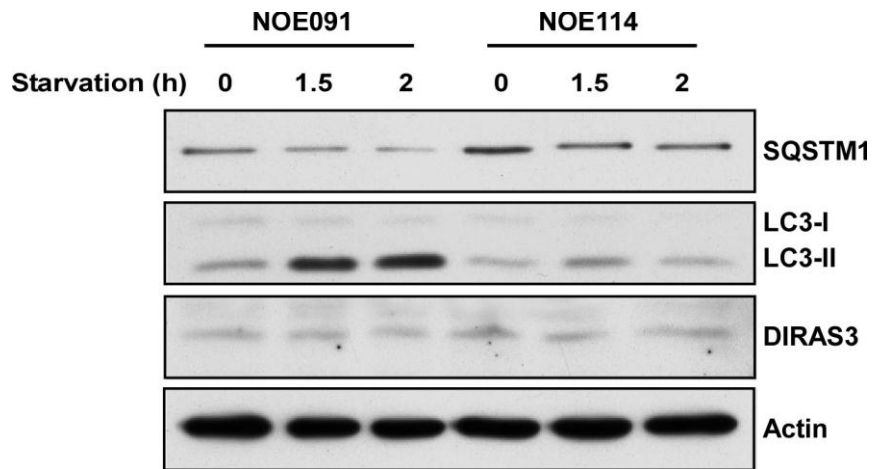
### Contingency Table

		DIRAS3 total staining		
		NEG	POS	
LC3 total staining	NEG	2 3.23 100.00 28.57	5 8.06 8.33 71.43	7 11.29
	POS	0 0.00 0.00 0.00	55 88.71 91.67 100.00	55 88.71
		2 3.23	60 96.77	62

### Tests

Test	ChiSquare	Prob>ChiSq
Likelihood Ratio	9.295	0.0023
Likelihood Ratio	16.238	<.0001

**Figure S14.** Chi-squared analysis correlates DIRAS3 total expression with LC3 total expression.



**Figure S15.** Amino acid-mediated autophagy in normal ovarian epithelial cells (NOE cells) is DIRAS3 independent. Normal ovarian epithelial cells were incubated in amino acid starvation medium for 1.5 and 2 h, then cells were lysed and total cell lysate was subjected to western blot analysis.

**Table S1.** Yale University ovarian cohort characteristics.

<b>Variable</b>	<b>Number (%) (n= 301)</b>
<b>Age at Diagnosis</b>	290 (96.3)
Mean age	60
Median age	63
<b>Stage</b>	203
3	184 (90.6)
4	19 (9.4)
<b>Censor</b>	260
Censored (20 years)	168 (64.6)
Uncensored (20 years)	92 (35.4)
<b>Follow-up time (months)</b>	290 (96.3)
Mean	35.4
Median	20.2

**Table S2.** Memorial Sloan Kettering Cancer Center ovarian cohort characteristics.

<b>Variable</b>	<b>Number (%) (n= 34 pairs)</b>
<b>Stage</b>	34
3	30 (88.2)
4	4 (11.8)
<b>Histology</b>	34
Serous adenocarcinoma	31 (91.2)
Mixed carcinoma	3 (8.8)

**Table S3.** DIRAS3 positively correlates with BECN1, and the autophagy marker, LC3 in primary human ovarian cancers (Spearman Rho Correlations).

	DIRAS3 total (n=223)	DIRAS3 puncta (n=176)	BECN1 total (n=232)	BECN1 puncta (n=170)	LC3 total (n=200)	LC3 puncta (n=163)
		Rho ( <i>P</i> *)	Rho ( <i>P</i> *)	Rho ( <i>P</i> *)	Rho ( <i>P</i> *)	Rho ( <i>P</i> *)
DIRAS3 total			0.442 (0.0001)		0.322 (0.0001)	
DIRAS3 puncta				0.797 (0.0001)		0.847 (0.0001)
BECN1 total					0.499 (0.0001)	
BECN1 puncta						0.568 (0.0001)

*P*\* is given for correspondence analysis based on Spearman rho.