Supporting Information

A novel transcriptional signalling pathway mediated by the trafficking protein Ambra1 via scaffolding Atf2 complexes

Christina Schoenherr[1], Adam Byron[1], Billie Griffith[1], Alexander Loftus[1], Jimi Wills[1], Alison F. Munro[1], Alex von Kriegsheim[1], and Margaret C. Frame[1*]
[1]Cancer Research UK Edinburgh Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh EH4 2XR, UK

Running title: The trafficking protein Ambra1 regulates transcription

[*]**Corresponding author:**
Prof Margaret Frame
Cancer Research UK Edinburgh Centre
Institute of Genetics and Molecular Medicine
University of Edinburgh
Crewe Road South
Edinburgh EH4 2XR
UK

Supporting Text

EXTENDED MATERIALS AND METHODS

Perinuclear fractionation

The protocol was adapted and optimized from Shaiken et al. (1). All buffers were supplemented with PhosSTOP and cOmplete Ultra phosphatase and protease inhibitor tablets (Roche, Welwyn Garden City, UK). Briefly, cells were washed twice in ice-cold PBS and then lysed in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.03% NP-40). Lysates were incubated for 5 minutes at 4 °C with rotation and then cleared by centrifugation at 800 g for 4 minutes. NP-40 was added to the supernatants (cytoplasmic fraction) to give a final concentration of 1% and cleared by centrifugation. Pellets were washed once with NP-40 containing buffer A and once with buffer A lacking NP-40. Pellets were resuspended in Buffer B (10 mM Tris-HCl, pH 7.4, 1 mM KCl, 2.5 mM $MgCl_2$, 0.2 M LiCl, 0.1% Triton X-100, 0.1% sodium deoxycholate) and incubated for 15 minutes at 4 °C with rotation and then cleared by centrifugation at 2000 g for 5 minutes. The supernatants (perinuclear fraction) were cleared by centrifugation. Pellets were washed once in buffer B, resuspended in RIPA buffer, sonicated and cleared by centrifugation (nuclear fraction).

Supporting Tables

Table S1. Details of all proteins identified by quantitative label-free MS analysis. *[Available online.]*

Table S2. Ambra1-interacting proteins identified by quantitative label-free MS. *[Available online.]*

Table S3. siRNAs used for knockdown experiments. All siRNAs were purchased from Horizondiscovery (formerly Dharmacon, Loughborough, UK).

siGENOME siRNA	Catalogue number
Ambra1 pool	M-059556-01
Akap8 pool	M-060714-01
Atf2 pool	M-042961-01
Cdk8 pool	M-053848-01
Cdk9 pool	M-040602-01
scrambled control	D-001206-13-20

Table S4. Primers used for qRT-PCR experiments.

Mouse target	Primers (5'–3')	
Angpt1	forward	AGCATCTGGAGCATGTGATG
	reverse	GTTGTATCTGGGCCATCTCC
Gapdh	forward	CGTCCCGTAGACAAAATGGT
	reverse	TTGATGGCAACAATCTCCAC
Itga8	forward	GCAGATACCGTTTGACACCA
	reverse	TTGTGAGCTCTCACTGTGGC
Itgb7	forward	GAAGGGCTGCTCCTCCTC
	reverse	AACTCACTCTGACCTCCGCC
Tgbf2	forward	ATAAAATCGACATGCCGTCC
	reverse	TTGTTGAGACATCAAAGCGG
Tgfb3	forward	ATTCGACATGATCCAGGGAC
	reverse	TCTCCACTGAGGACACATTGA

Supporting Figure Legends

Figure S1. Ambra1 is localised in the perinuclear and nuclear fractions in SCC FAK-WT and -/- cells. (A) Representative negative control immunofluorescence images of SCC FAK-WT and -/- cells, which were grown on glass coverslips for 24 h, fixed and stained with secondary fluorescent antibody Alexa Fluor anti-rabbit 488, Alexa Fluor anti-mouse 594 and DAPI. Scale bars, 20 μm. **(B, C)** Whole cell lysates, cytosolic, perinuclear and nuclear fractions of SCC FAK-WT and -/- cells **(B)** as well as of the human SCC cell lines Met4 **(C)** were analyzed by Western blot using anti-Ambra1 (2). Anti-Gm130, anti-Rcas1 (Golgi markers), anti-PDI (endoplasmic reticulum (ER) marker), anti-Lamin A/C, anti-H4 (nucleus markers), anti-GAPDH and anti-α-Tubulin (cytosol markers) were used as controls for the purity of the subcellular fractions as well as for loading. **(D)** Ambra1 is also in the nucleus in primary keratinocytes isolated from mouse tails. Whole cell and nuclear lysates of SCC FAK-WT and -/- cells as well as primary mouse keratinocytes were subjected to Western blot analysis with anti-Ambra1. Anti-GAPDH and anti-Lamin A/C served as controls for the purity of the nuclear extracts as well as loading controls.

Figure S2. Ambra1 binds nuclear proteins involved in the regulation of transcription. (A) Nuclear Ambra1-binding proteins of SCC FAK-WT and -/- lysates identified by mass spectrometry were grouped using gene ontology enrichment analysis for biological processes. **(B)** Negative control-IP to show the specificity of Ambra1–nuclear protein interactions. Ambra1 was immunoprecipitated from nuclear lysates of SCC FAK-WT and -/- cells using anti-Ambra1, followed by Western blot analysis with anti-Ambra1, anti-PARP (negative control) and anti-Atf2 (positive control). Anti-GAPDH was used as a control for the purity of the nuclear lysates as well as a loading control. Unfortunately, in the Ambra1-IP with SCC FAK-WT lysates, the beads were lost, but as no difference in the binding of nuclear proteins to Ambra1 between SCC FAK-WT and -/- lysates could be identified, the absence of PARP binding to Ambra1 in SCC FAK -/- cell lysates shows the specificity of the identified nuclear Ambra1-binding proteins. **(C)** Annotated tandem mass spectrum identifying Cdk9 in nuclear Ambra1 immunoprecipitations analysed by label-free MS (*top*). Corresponding fragment ion coverage of peptide sequence is shown (*bottom*). Data obtained from

MS/MS analysis of biological replicate 3 of nuclear Ambra1 immunoprecipitation from SCC FAK-WT cells (scan 11,662; *m*/*z* 827.9).

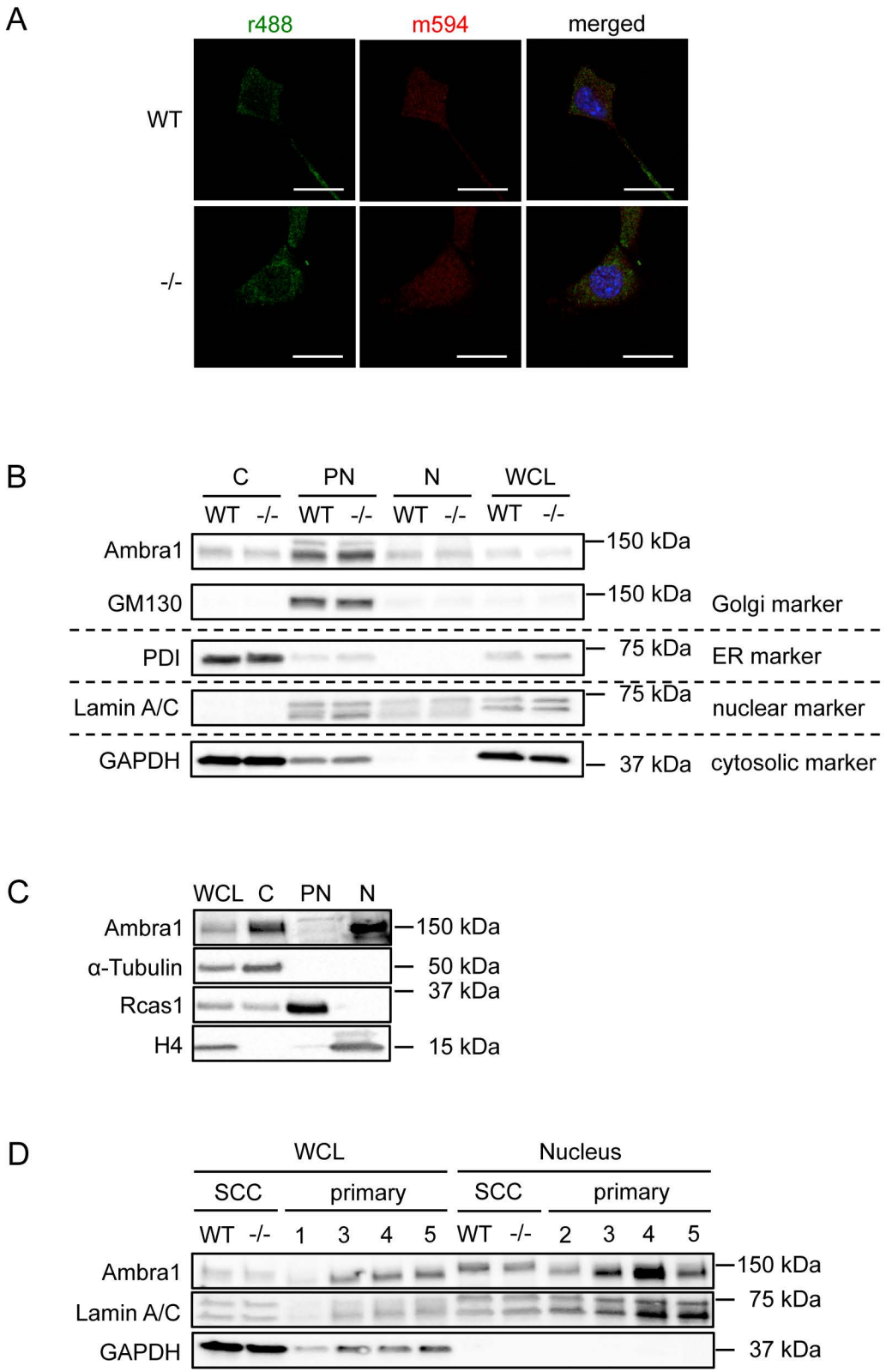
Figure S3. Ambra1, Akap8 and Atf2 regulate the transcription of many common genes. Relative gene expression of the genes regulated by Ambra1 and Akap8 **(A)**, Ambra1 and Atf2 **(B)**, Akap8 and Atf2 **(C)**, as well as Ambra1 **(D)**, Akap8 **(E)** and Atf2 alone **(F)** knockdown compared to siControl.

Figure S4. Ambra1-, Akap8- and Atf2-regulated genes occupy the same signalling pathway gene sets. Table of the enriched signalling pathway gene sets according to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of Ambra1-, Akap8- and Atf2-regulated genes.

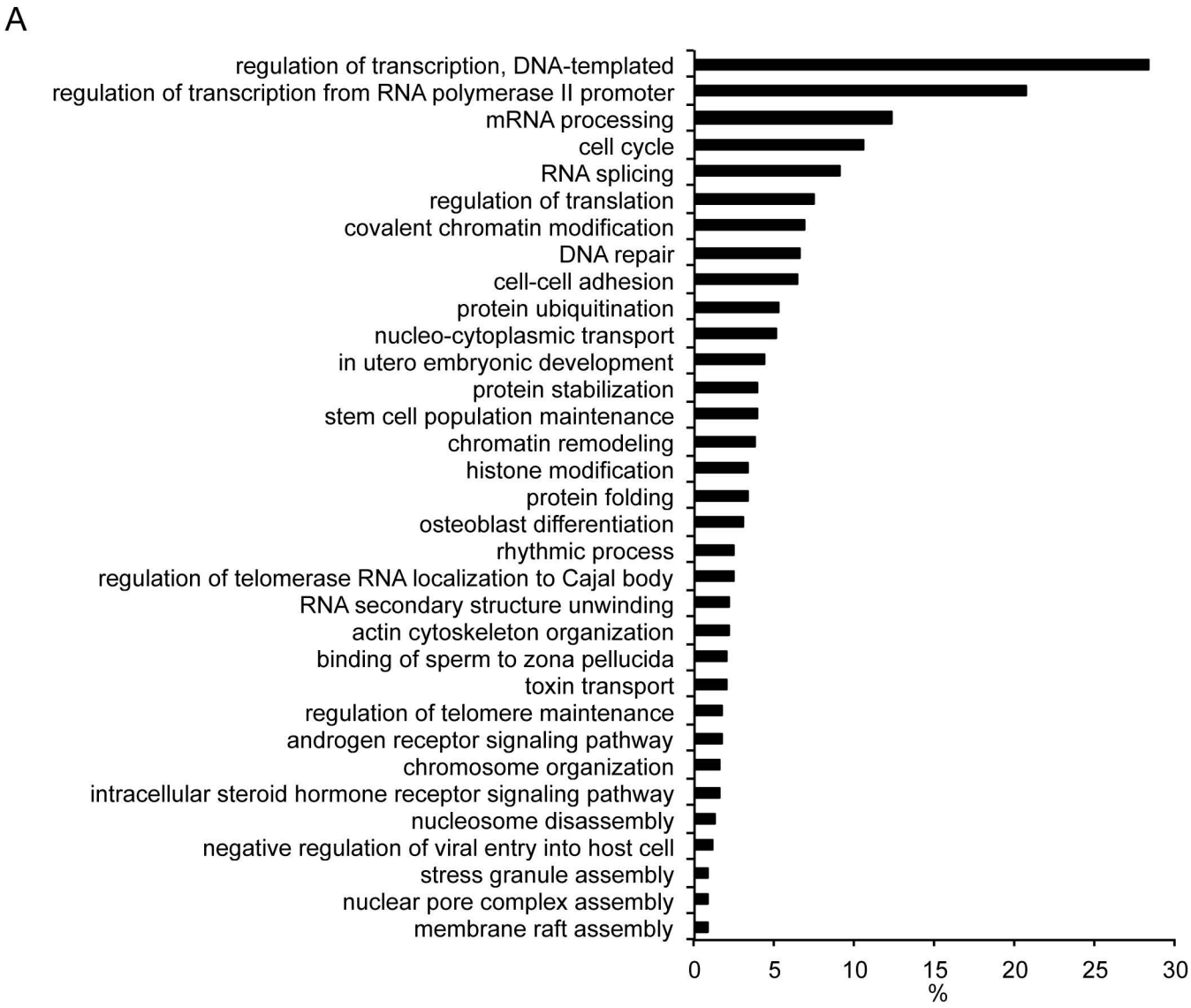
Supporting References

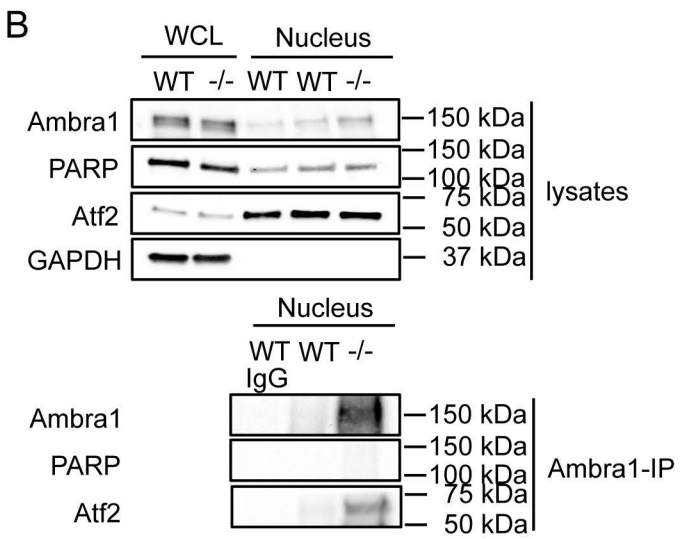
1. Shaiken, T. E., and Opekun, A. R. (2014) Dissecting the cell to nucleus, perinucleus and cytosol. *Sci. Rep.* **4**, 4923
2. Proby, C. M., Purdie, K. J., Sexton, C. J., Purkis, P., Navsaria, H. A., Stables, J. N., and Leigh, I. M. (2000) Spontaneous keratinocyte cell lines representing early and advanced stages of malignant transformation of the epidermis. *Exp. Dermatol.* **9**, 104–117

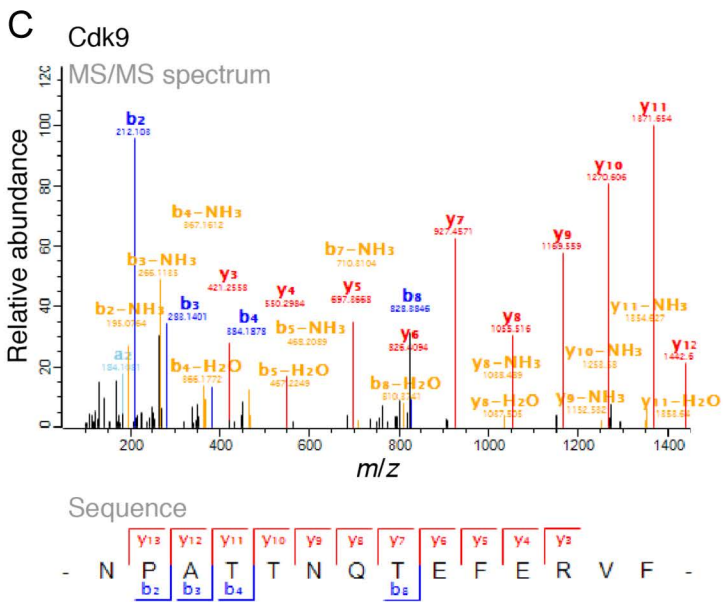
Supplementary Figure 1 Ambra1 is localised to the perinuclear and nuclear fractions in SCC FAK-WT and -/- cells



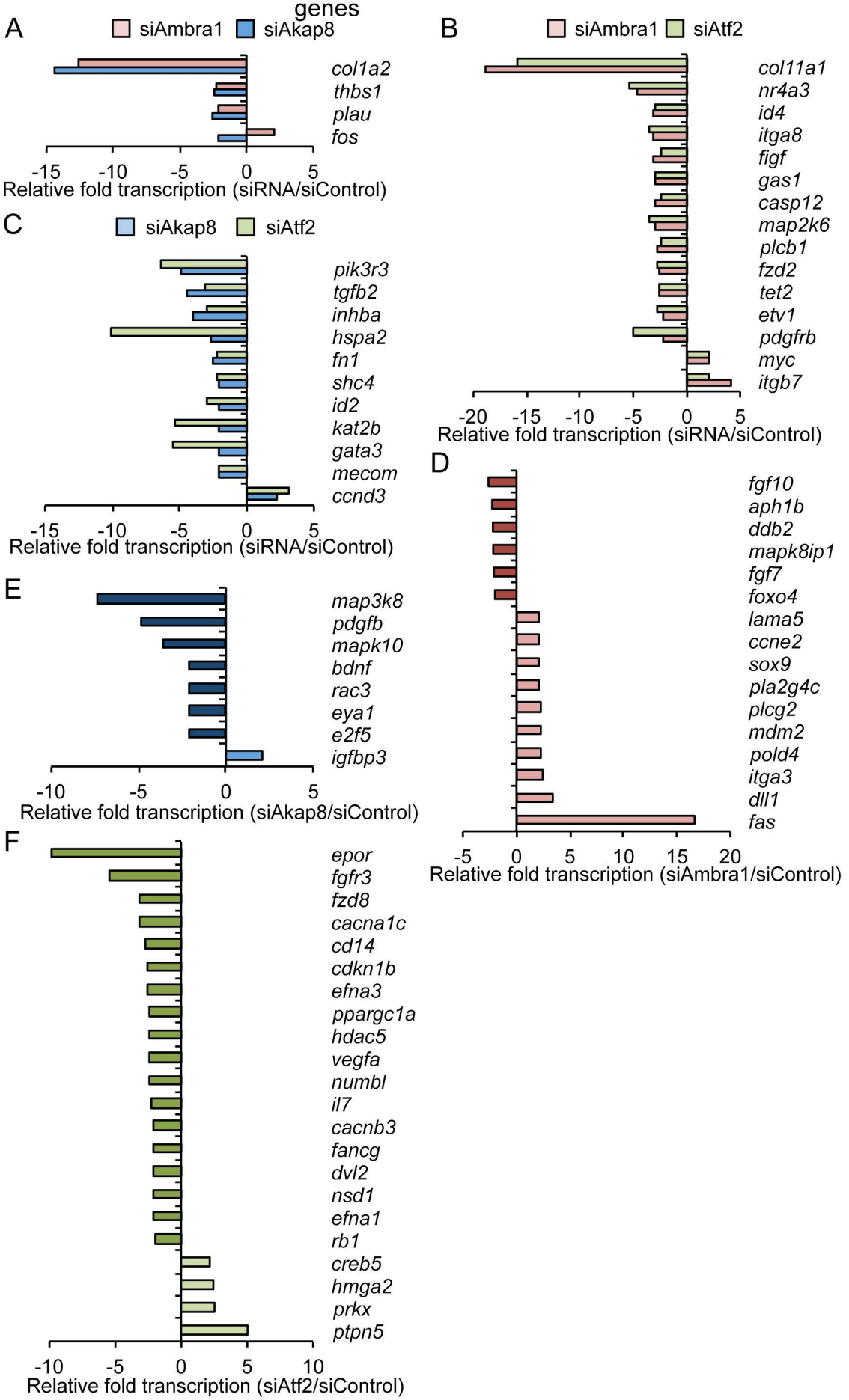
Supplementary Figure 2 Ambra1 binds nuclear proteins involved in the regulation of transcription







Supplementary Figure 3 Ambra1, Akap8 and Atf2 regulate the transcription of many common genes



Supplementary Figure 4 Ambra1 Akap8 and Atf2 regulated genes group in the same KEGG signalling pathway gene sets

siAmbra1		siAkap8		siAtf2	
Term	%	Term	%	Term	%
PI3K-Akt signaling pathway	40.4	Pathways in cancer	36.6	PI3K-Akt signaling pathway	36.9
Pathways in cancer	38.5	Focal adhesion	34.1	Pathways in cancer	32.3
Focal adhesion	25.0	PI3K-Akt signaling pathway	34.1	Focal adhesion	21.5
MAPK signaling pathway	21.2	MAPK signaling pathway	26.8	MAPK signaling pathway	20.0
MicroRNAs in cancer	21.2	MicroRNAs in cancer	22.0	MicroRNAs in cancer	18.5
ECM-receptor interaction	19.2	Ras signaling pathway	19.5	Rap1 signaling pathway	16.9
Proteoglycans in cancer	17.3	TGF-beta signaling pathway	17.1	Signaling pathways regulating pluripotency of stem cells	15.4
Rap1 signaling pathway	17.3	Choline metabolism in cancer	17.1	Hippo signaling pathway	15.4
Regulation of actin cytoskeleton	17.3	Proteoglycans in cancer	17.1	Ras signaling pathway	15.4
Ras signaling pathway	17.3	Rap1 signaling pathway	17.1	Transcriptional misregulation in cancer	13.8
Transcriptional misregulation in cancer	13.5	Regulation of actin cytoskeleton	17.1	Regulation of actin cytoskeleton	13.8
p53 signaling pathway	11.5	ECM-receptor interaction	14.6	Cell cycle	12.3
Cell cycle	11.5	Toll-like receptor signaling pathway	14.6	Proteoglycans in cancer	12.3
Platelet activation	11.5	TNF signaling pathway	14.6	TGF-beta signaling pathway	10.8
TGF-beta signaling pathway	9.6	Neurotrophin signaling pathway	14.6	ECM-receptor interaction	10.8
Choline metabolism in cancer	9.6	Cell cycle	14.6	Wnt signaling pathway	10.8
TNF signaling pathway	9.6	FoxO signaling pathway	14.6	Jak-STAT signaling pathway	10.8
Thyroid hormone signaling pathway	9.6	Hippo signaling pathway	14.6	Central carbon metabolism in cancer	9.2
Inflammatory mediator regulation of TRP channels	9.6	cAMP signaling pathway	14.6	Estrogen signaling pathway	9.2
FoxO signaling pathway	9.6	Prolactin signaling pathway	12.2	HIF-1 signaling pathway	9.2
Central carbon metabolism in cancer	7.7	Estrogen signaling pathway	12.2	FoxO signaling pathway	9.2
Fc epsilon RI signaling pathway	7.7	Osteoclast differentiation	12.2	ErbB signaling pathway	7.7
		Transcriptional misregulation in cancer	12.2	Toll-like receptor signaling pathway	7.7
		p53 signaling pathway	9.8	TNF signaling pathway	7.7
		Fc epsilon RI signaling pathway	9.8	Thyroid hormone signaling pathway	7.7
		B cell receptor signaling pathway	9.8	Notch signaling pathway	6.2
		ErbB signaling pathway	9.8		
		T cell receptor signaling pathway	9.8		
		HIF-1 signaling pathway	9.8		