Supplemental Material to:

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Hypoxia-induced *MIR155* is a potent autophagy inducer by targeting multiple players in the MTOR pathway

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# Supplemental Materials

## Table S1. Primers used for the present study.

| Gene amplified | Forward primer sequence | Reverse primer sequence |
|----------------|-------------------------|-------------------------|
| **Primers for plasmids** |
| RHEB 3’UTR WT | TGCTCTAGATTCTGCTGCAAAGCC | CCGCTCGAGGCAAGCAAAACTATAT |
| RHEB 3’UTR MUT | AAGAATTTTATCGCCGTGAATTTT | ATTCACGGCGATAAAAAATCTTTGGTCAT |
| RICTOR 3’UTR WT | ATTTAAATTTTCCCATTATAG | TAAATTTTTTTTAATATGGTATG |
| RICTOR 3’UTR MUT | TAGGAAACCGTGAA | GTATTATTTCACGG |
| RPS6KB2 3’ UTR WT | TGGCACTACCATCCACAC | CCGCATATCTGAAGTCTATG |
| RPS6KB2 3’ UTR MUT | AAAAAACCGTGAA | GTCAATTTACGGG |
| Pre-MIR155 | CCGGATATCTCTCTTGCAGGT | CCGGATATCTGTCTACAGGT |
| pMSCV-GFP-LC3B | ACGCGTCGACTAGTTATTAATAGT | CCCAGCTTTTACACTGACAATTTCAT |

## Primers for QPCR

| Gene amplified | Forward primer sequence | Reverse primer sequence |
|----------------|-------------------------|-------------------------|
| RHEB | TGCTCTAGATTCTGCTGCAAAGCC | CATCACCGAGCATGAAGACTT |
| RICTOR | TGAGG | CATCACCGAGCATGAAGACTT |
| RPS6KB2 | ATTTAAATTTTCCCATTATAG | TAAATTTTTTTTAATATGGTATG |
| MTOR | TAGGAAACCGTGAA | GTATTATTTCACGG |
| ATG3 | TGGCACTACCATCCACAC | CCGCATATCTGAAGTCTATG |
| BCL2 | ACGCGTCGACTAGTTATTAATAGT | CCCAGCTTTTACACTGACAATTTCAT |
| GAPDH | ACGCGTCGACTAGTTATTAATAGT | CCCAGCTTTTACACTGACAATTTCAT |
Figure S1
**Figure S2**

| Protein          | CNE Normoxia | CNE Hypoxia | HeLa Normoxia | HeLa Hypoxia |
|------------------|--------------|-------------|---------------|--------------|
| HIF1A            |              |             |               |              |
| P-MTOR S2448     | 100 kDa      |             |               |              |
| P-MTOR/GAPDH     | 1            | 0.55        | 1             | 0.42         |
| MTOR             | 170 kDa      |             |               |              |
| MTOR/GAPDH       | 1            | 0.77        | 1             | 0.46         |
| RHEB             | 15 kDa       |             |               |              |
| RHEB/GAPDH       | 1            | 0.18        | 1             | 0.40         |
| RPS6KB2          | 70 kDa       |             |               |              |
| RPS6KB2/GAPDH    | 1            | 0.45        | 1             | 0.39         |
| RICTOR           | 170 kDa      |             |               |              |
| RICTOR/GAPDH     | 1            | 0.59        | 1             | 0.68         |
| SQSTM1           | 55 kDa       |             |               |              |
| SQSTM1/GAPDH     | 1            | 0.13        | 1             | 0.13         |
| GAPDH            | 35 kDa       |             |               |              |

**Notes:**
- The experiment was conducted on CNE and HeLa cell lines.
- The images show protein expression levels under normoxia and hypoxia conditions.
- The relative protein expression is quantified and compared hierarchically.
Figure S3
Figure S4
Figure S5
Figure S6
**Figure S1.** Stable transfection of MIR155 targets the expression of RHEB, RICTOR and RPS6KB2. (A) qRT-PCR measurement of RHEB, RPS6KB2 and RICTOR mRNA levels in CNE or HeLa cells stably expressing MIR155. (means ± s.d. of independent experiments, n = 4, *p<0.05, **p<0.01, ***p<0.001, Student’s two-tailed t-test). (B) Western blot analysis of indicated proteins in CNE or HeLa cells stably expressing MIR155. Protein ratios were calculated following ImageJ densitometric analysis. (3 independent experiments gave similar results).

**Figure S2.** Hypoxia induced downregulation of RHEB, RICTOR and RPS6KB2 proteins. CNE or HeLa cells were cultured in normal conditions or exposed to 1% oxygen to induce hypoxia. The cellular amounts of MTOR, phosphor-MTOR (Ser2448), RHEB, RPS6KB2, RICTOR, SQSTM1 and GAPDH were determined by western blots. Protein ratios were calculated following Image J densitometric analysis. (3 independent experiments gave similar results).

**Figure S3.** RHEB siRNA induces autophagy. (A) CNE or HeLa cells stably expressing GFP-LC3 were transfected with miRNA control (NC), MIR155, control siRNA and RHEB-siRNA. GFP-LC3 dots per cell were quantified as described previously. Data are shown as the mean± s.d. of four independent experiments. (B) Western blot analysis 48 h after transfection with NC, MIR155, control-siRNA and RHEB-siRNA.

**Figure S4.** MIR155 regulates RHEB, RICTOR and RPS6KB2 3’UTR reporters. Luciferase reporter vectors containing 3’UTR fragments of RHEB, RICTOR and RPS6KB2 were cotransfected with MIR155 or control antigomirs. Normalized luciferase activity was determined at 24 h after transfection. Data show are mean ± s.d. of three independent experiments, *p<0.05, **p<0.01, Student’s two tailed t-test.

**Figure S5.** Blockage of endogenous MIR155 increases the protein expression levels of RHEB, RICTOR and RPS6KB2. CNE or HeLa cells transfected with NC, MIR155, LNA-NC or LNA-155 were cultured in hypoxia conditions for 24 h. The samples were harvested for western blot analysis to detect the protein levels of RHEB, RICTOR, RPS6KB2 and GAPDH.
**Figure S6.** Knockdown of *ATG5* inhibits autophagy. HeLa cells were transfected with NC, *MIR155, MIR885-5P* or cotransfected with *MIR155* and *ATG5* siRNA. Cells were subjected to western blot analysis to determine the cellular amount of ATG5, LC3 and GAPDH.