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Supplementary Materials for

Congenital Zika syndrome is associated with maternal protein malnutrition

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/6/2/eaaw6284/DC1)

Table S5 (Microsoft Excel format). Differentially expressed genes between Co/ZIKV and LP/ZIKV.

Supplementary figures

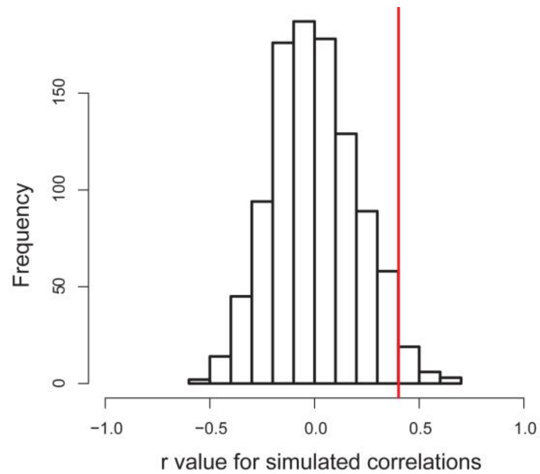

Fig. S1. Distribution of simulated *r* coefficients for the correlation between undernutrition and microcephaly cases in tropical states of Brazil obtained after permutation of the original dataset. A Monte Carlo simulation was performed by randomly correlation 1,000 times the original dataset (table S1). The resulting distribution of r coefficients has a large proportion of cases around 0, indicating no correlation, and a lower frequency of more extreme values in both tails (negative and positive) of the histogram, as expected. In red, the original r coefficient is indicated in the distribution of simulated r coefficients. The red r coefficient is in the positive extreme of the distribution and only 2.8% of simulated r coefficients are higher than the original, suggesting a p-adj value of 0.028.

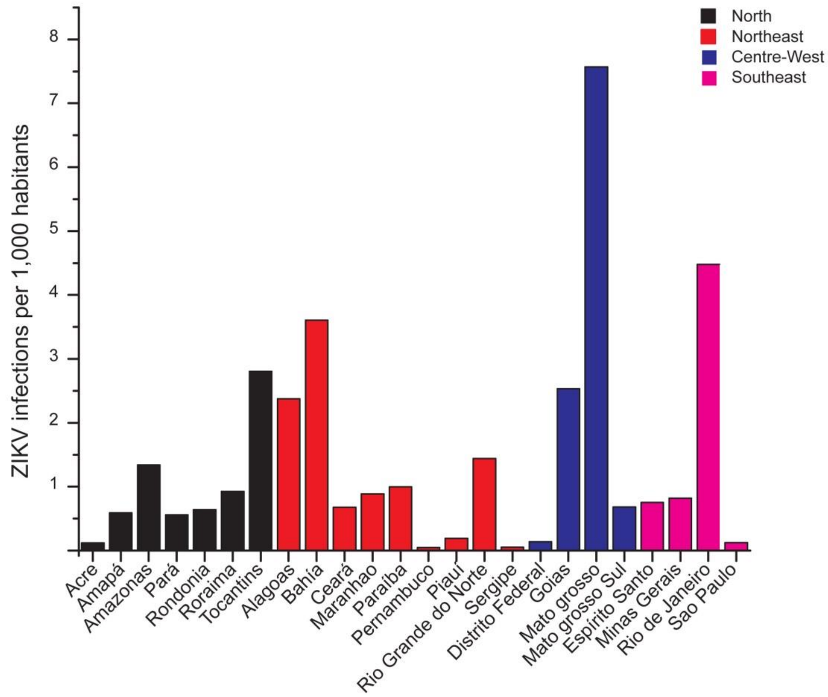

Fig. S2. Number of ZIKV infection confirmed cases in each Brazilian state. Total number of confirmed cases during 2016 and 2017 were summed in order to have an estimation of ZIKV prevalence in each state. Regions more affected by CZS (see Fig. 1) are not necessarily the only ones with high incidence ZIKV infections, as previously discussed in (*1*).

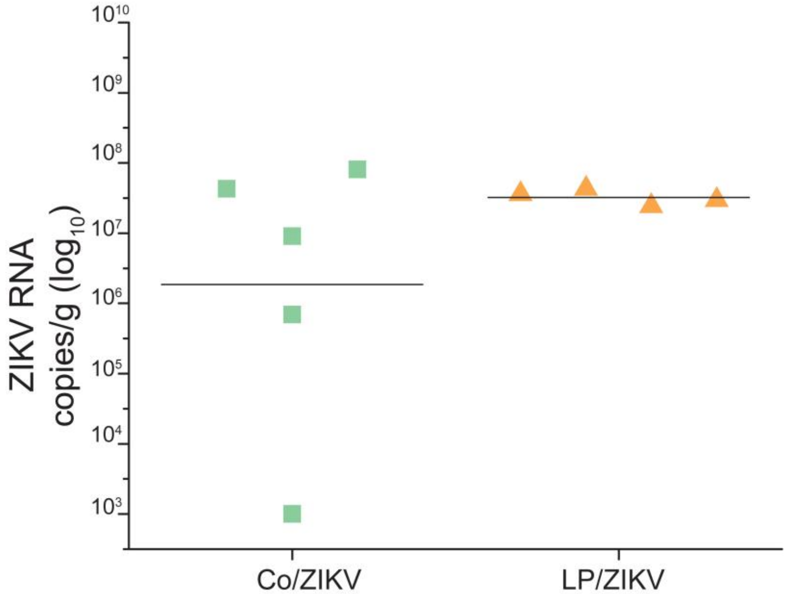

Fig. S3. Viral load in E18 placentas was quantified by RT-qPCR. Placentas derived from independent litters in each group (Co/ZIKV: 3 independent litter, number of samples from each litter=2, 2, 1. LP/ZIKV: 2 independent litters, number of samples from each litter= 2, 2). Lines indicate the geometric mean. U Mann-Whitney: Co/ZIKV vs LP/ZIKV: Z=-0.735, $p>0.05$.

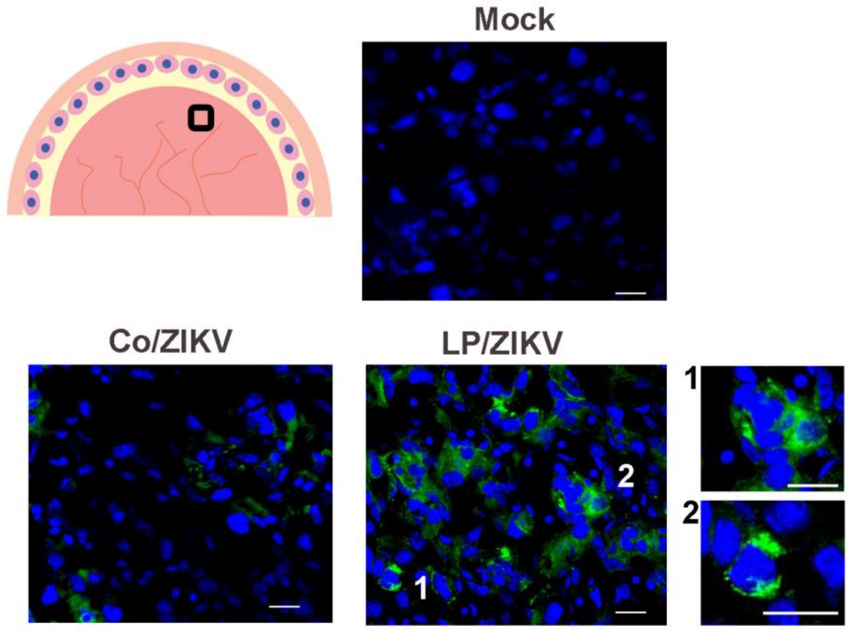

Fig. S4. Confirmation of ZIKV detection by immunohistochemistry in E15. Anti-NS1 antibody was used to detect ZIKV (green) counterstained with the nuclei marker DAPI (blue) in placental sections of both Co/ZIKV and LP/ZIKV. (Scale bar = 20µm).

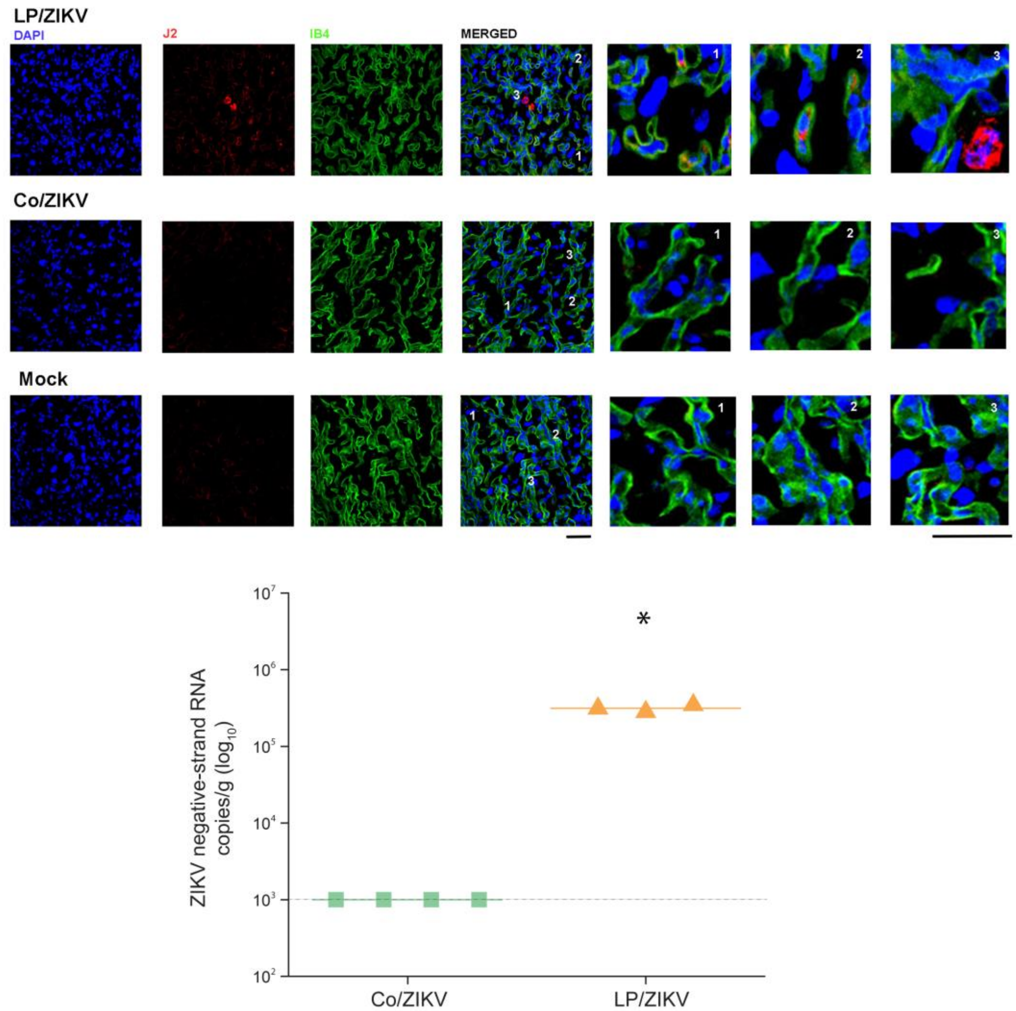

Fig. S5. Viral replication in LP/ZIKV placentas at E15. Top: Immunolabeling using anti-dsRNA (J2) of E15 placentas. E15 placenta sections stained with DAPI (blue), J2 (red) and IB4 (green) co-localize on the labyrinth of LP/ZIKV. (Scale bar = 50μm). Bottom: ZIKV negative-strand viral RNA quantification by specific RT-qPCR assay in E15 infected placentas. Placentas derived from 2 independent litters in each experimental group. U Mann-Whitney: Co/ZIKV vs LP/ZIKV: Z=-2.341, *p=0.019.

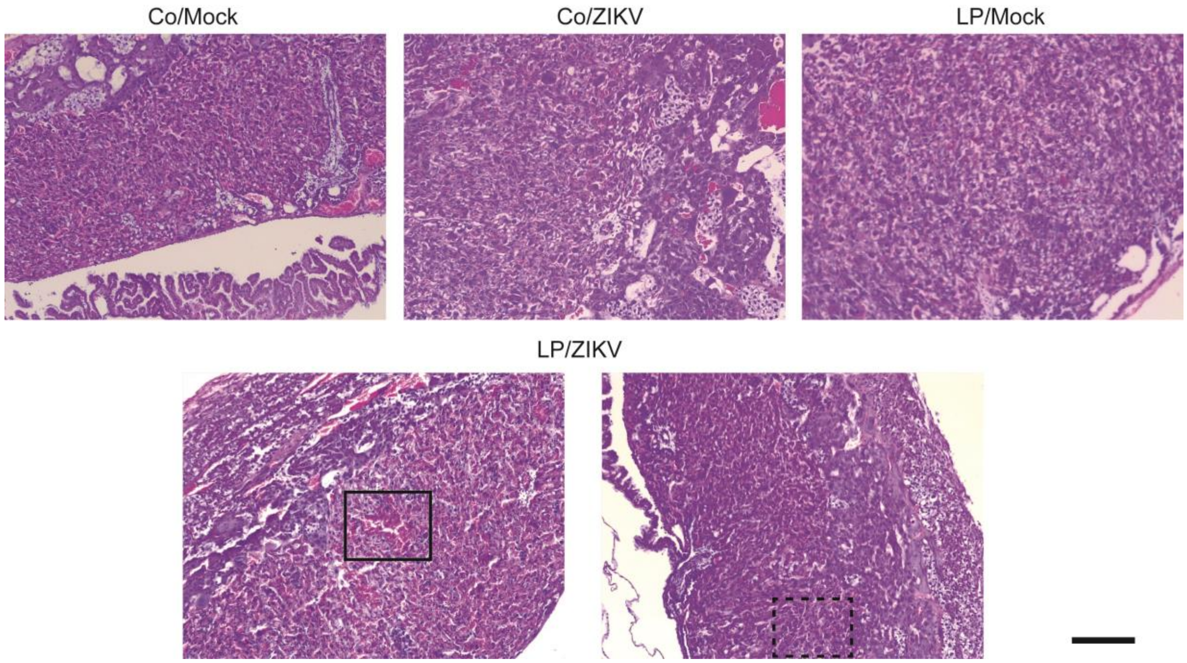

Fig. S6. H&E staining in E15 placentas. Low-magnification of placental sections show haemorrhage (left, black solid frame) and necrosis (right, dotted frame) areas in LP/ZIKV sections. (Scale bar = 500μm).

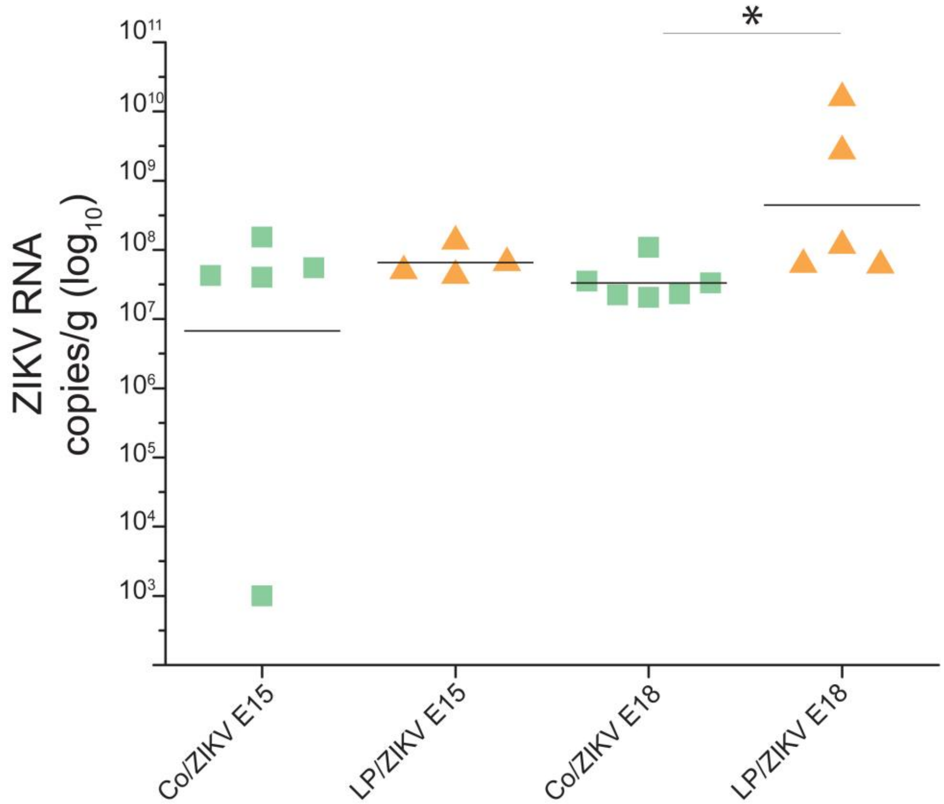

Fig. S7. Viral load in E15 and E18 embryonic brains quantified by RT-qPCR. Lines indicate the geometric mean. A significant difference between Co/ZIKV and LP/ZIKV was found in the E18 brains (u Mann-Whitney:-2.373*). Brains were derived from 2 independent litters in each group for E15 brains (number of embryos: Co/ZIKV= 3, 2; LP/ZIKV=2, 2) and 3 independent litters for E18 brains (number of embryos: Co/ZIKV= 2, 2, 2; LP/ZIKV=2, 2, 1). *$p < 0.05$, $p>0.05$.

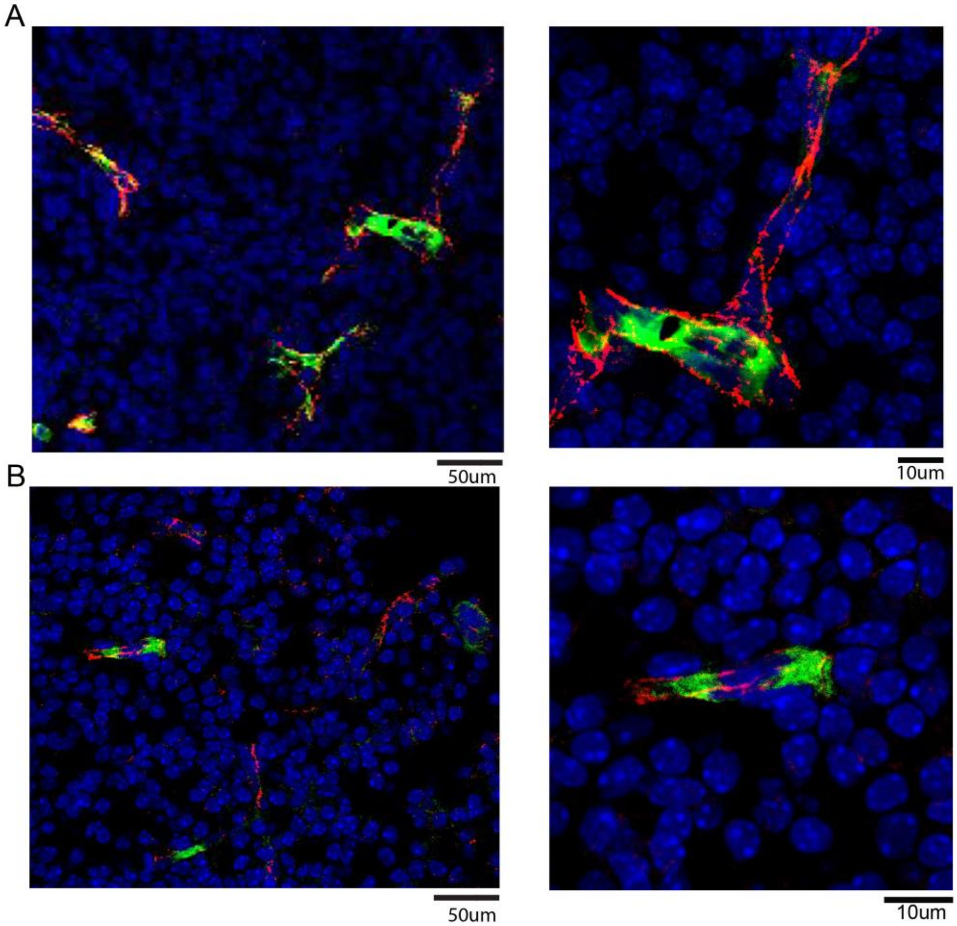

Fig. S8. ZIKV-infected cells colocalize with endothelial cells in E15 brains. Anti-NS1 antibody was used to detect ZIKV (green) and anti-CD31 antibody to detect endothelial cells (red) counterstained with the nuclei marker DAPI (blue) both in LP/ZIKV (A) and Co/ZIKV (B).

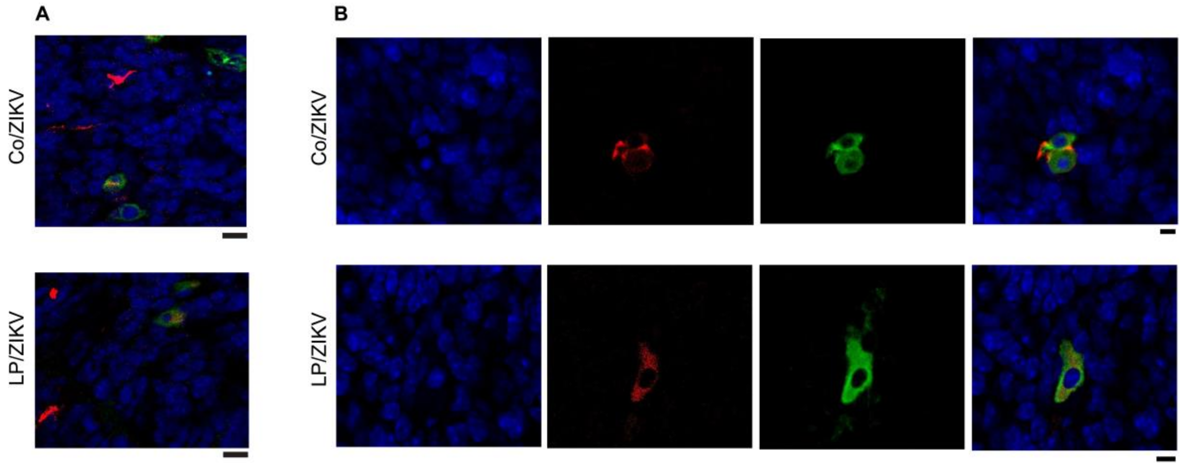

Fig. S9. ZIKV-infected cells colocalize with microglial cells in E15 brains. Anti-NS1 antibody was used to detect ZIKV (green) and anti-Iba1 antibody to detect microglial cells (red) counterstained with the nuclei marker DAPI (blue). In (A), lower magnitude images of E15 cerebral cortex. Both in Co/ZIKV and LP/ZIKV some Iba1+ cells are found co-localized with NS1+ (Scale bar = 10µm). In (B) higher magnification shows co-localization of NS1+ and Iba1+ cells (Scale bar = 5µm).

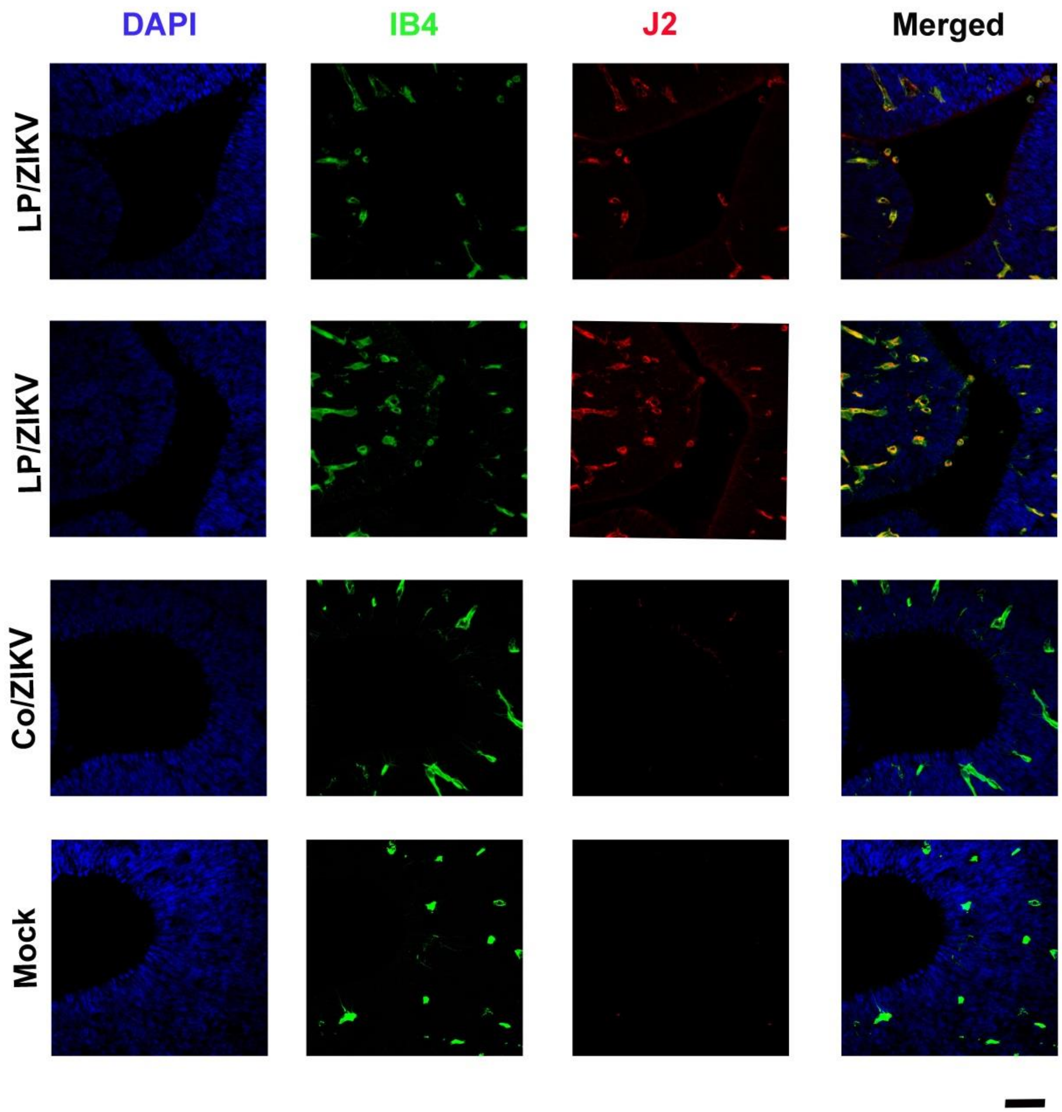

Fig. S10. Staining of J2 on E15 embryonic brains colocalized with IB4. J2 antibody is used to mark dsRNA and detect the localization of ZIKV in brain tissue. Since dsRNA is an intermediate structure of viral genome replication, its localization here indicates the presence of ZIKV in the tissue. In the same sections, IB4 is used to mark vessels as well as macrophage and microglia. Double-labelled IB4/J2 positive cells are found in the infected ventricular zone of LP/ZIKV in contrast to other experimental groups. (Scale bar = 50µm).

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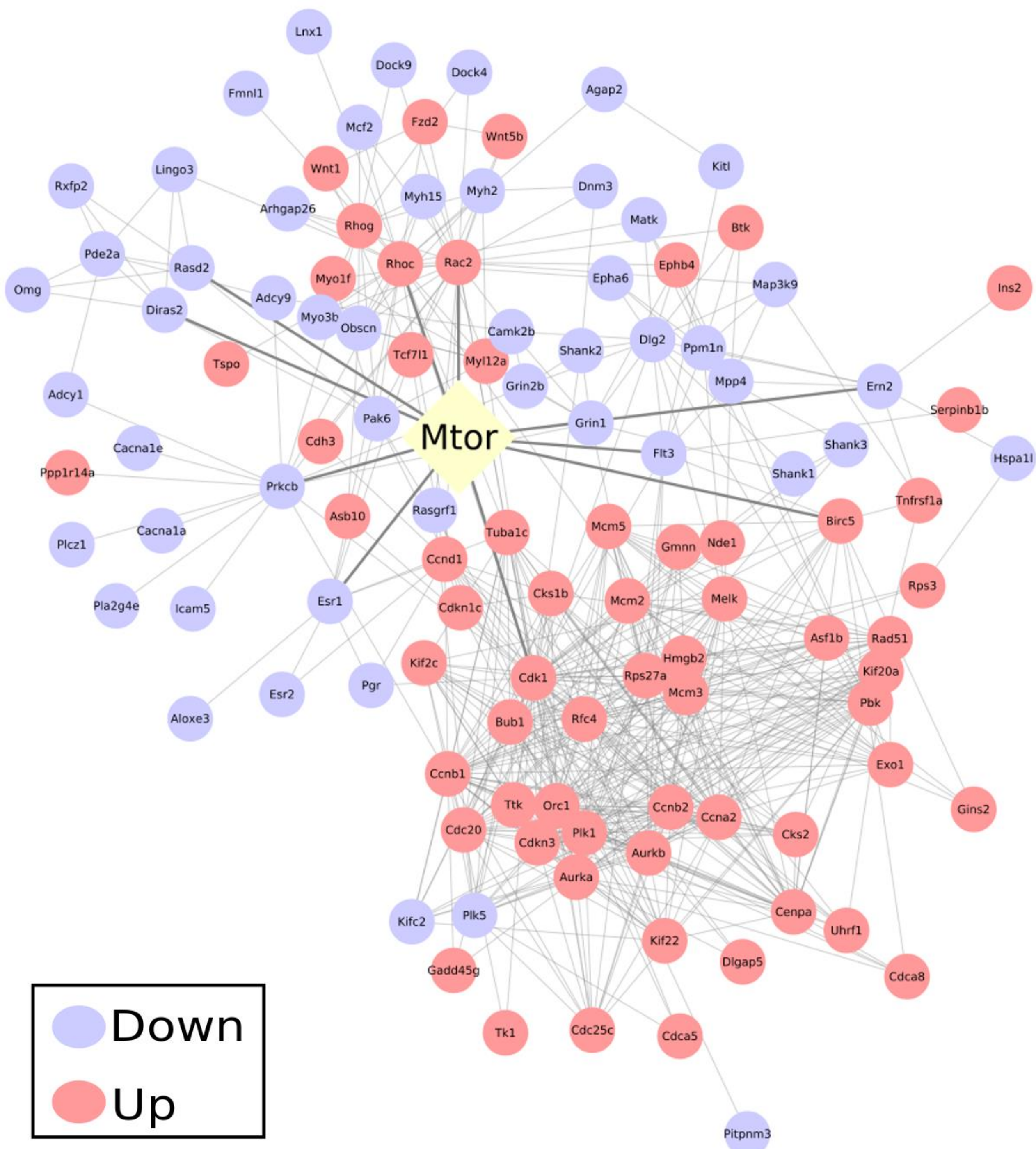

Fig. S11. Network describing altered genes involved in the mTOR pathway. *Mtor* (in yellow) is in the center of the network, while genes in red are upregulated and those in blue are downregulated in LP/ZIKV in comparison to Co/ZIKV.

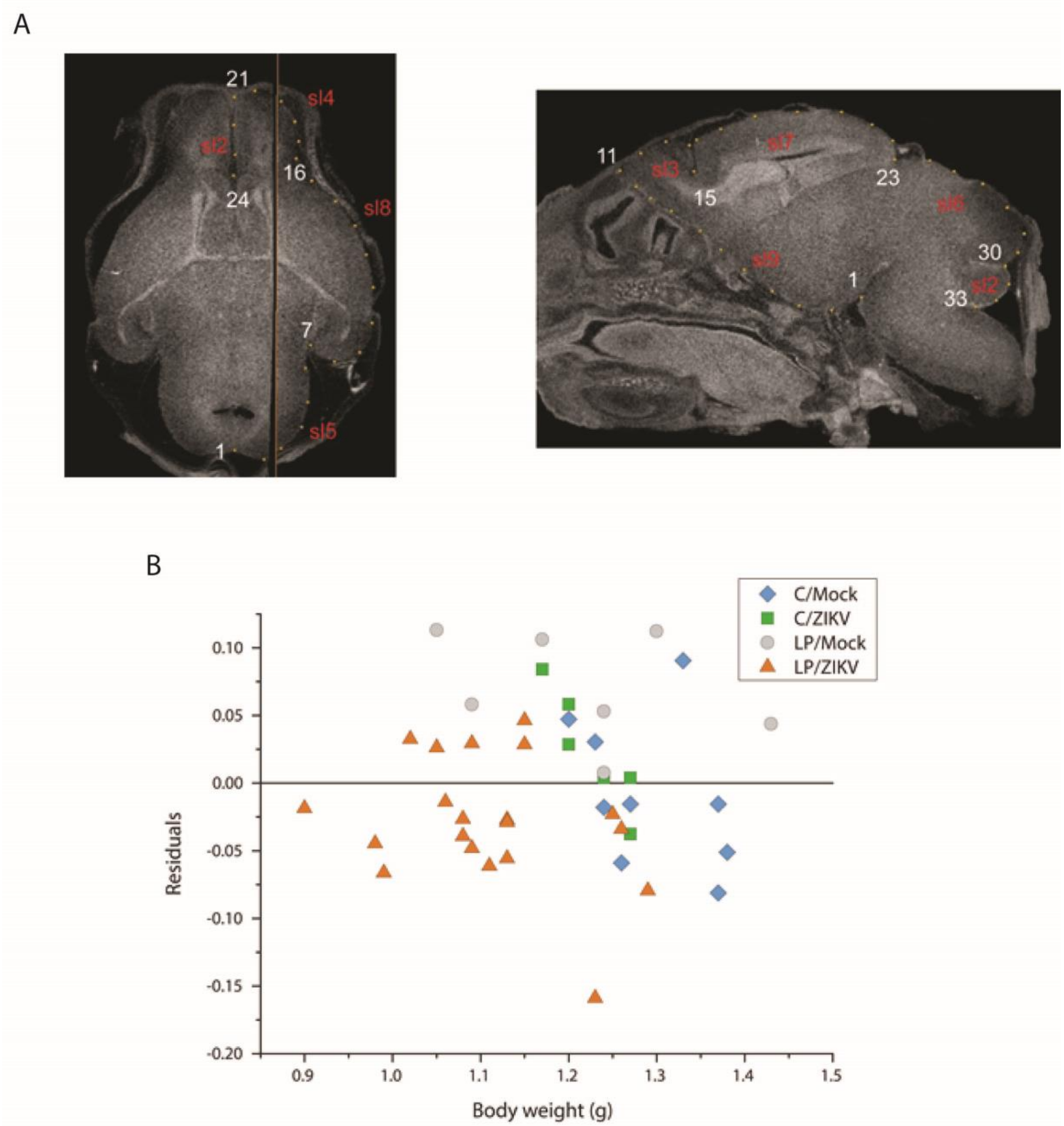

Fig. S12. Morphometric analysis of brain images obtained through μCT. (**A**) Landmarks and semilandmarks digitized on brain images. (**B**) Relationship between brain size and body weight. Residuals derive from a linear regression between brain size and body weight.

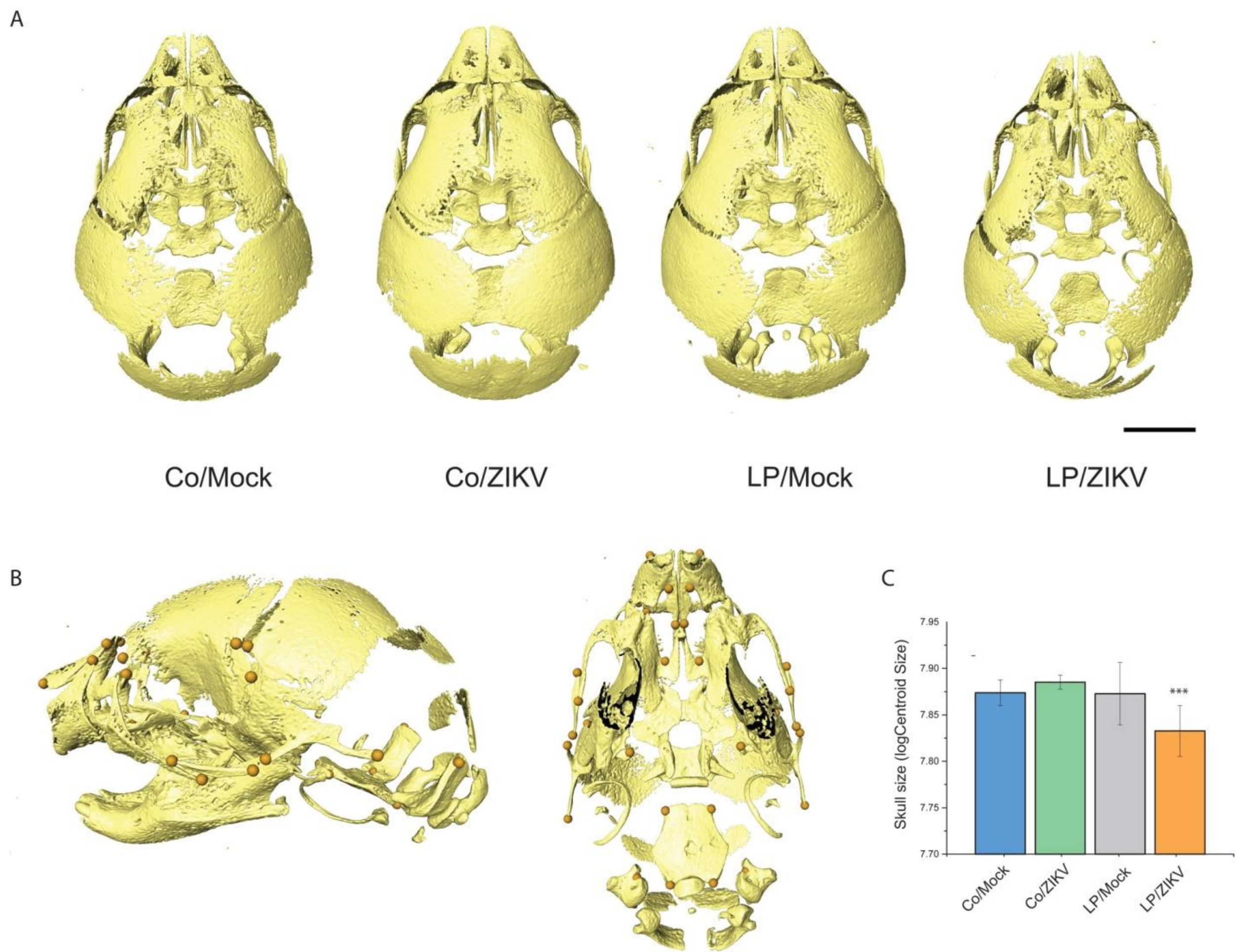

Fig. S13. Skull size of LP/ZIKV newborns is reduced. Skull reconstructions were obtained from µCT images. (**A**) A set of 42 bilateral landmarks were digitized in each specimen skull and their three-dimensional positions were registered. (**B**) Size differences are evident from representative skulls of each group (Scale bar = 2 mm). (**C**) Raw coordinates of landmarks were subjected to generalized Procrustes analysis and the centroid size, a comprehensive measure of scale for each skull, was obtained. Centroid size of the skull is highly significant affected in LP/ZIKV (***$p<0.001$).

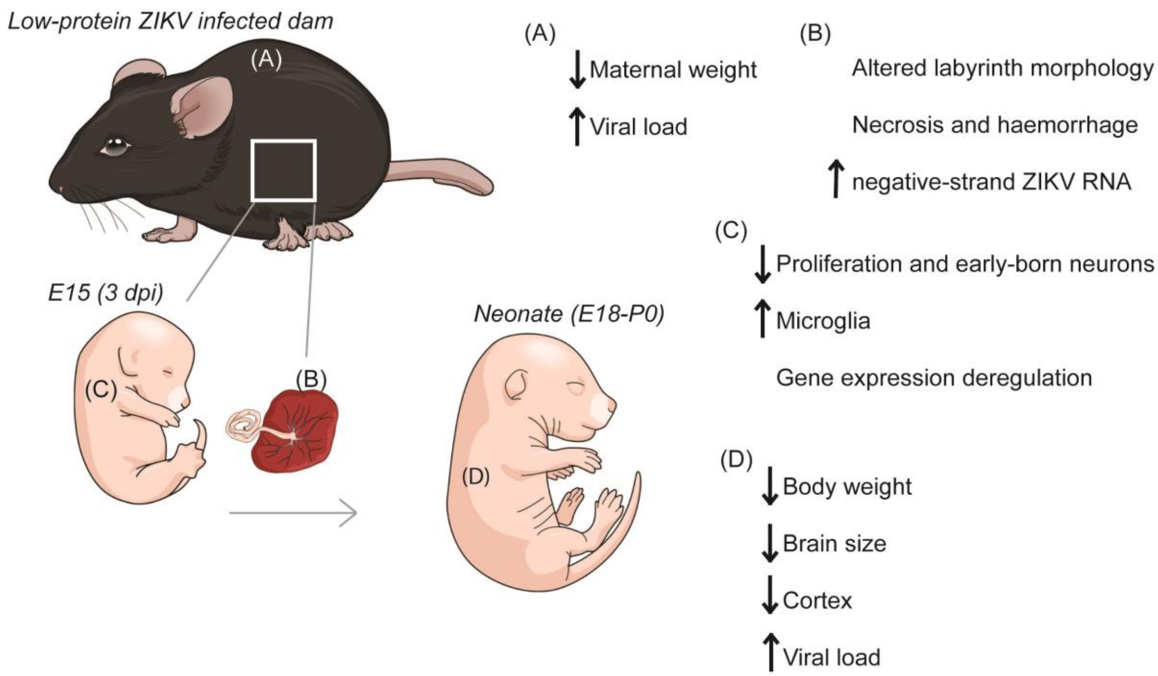

Fig. S14. Summary of the results obtained with the experimental model. Arrows indicate patterns in the LP/ZIKV group in comparison to the Co/ZIKV group.

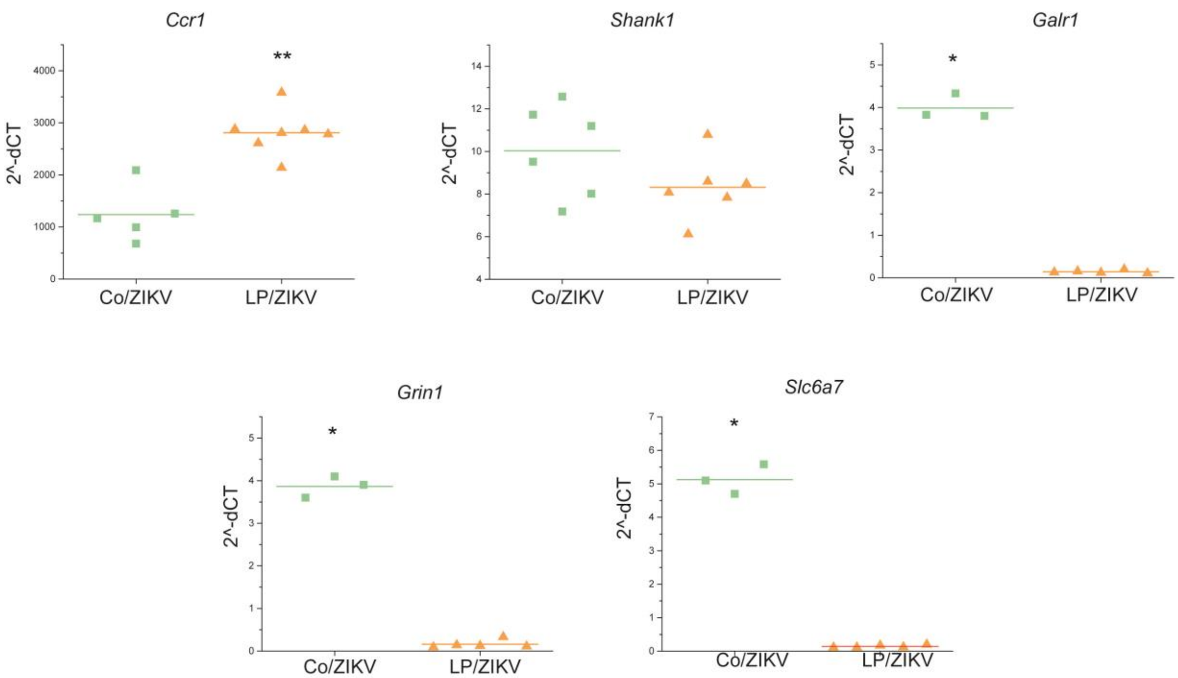

Fig. S15. Validation of differentially expressed genes by RT-qPCR in E15 brains of Co/ZIKV and LP/ZIKV. Selected genes included in Fig. 4C-D were validated (*Ccr1*and *Galr1*). Genes involved in mTOR pathway (fig. S11) were also validated (*Grin1* and *Galr1*). Some of the most differentially expressed genes in RNA-Seq analyses such as *Grin1* and *Slc6a7* were also validated. Relative quantity is expressed in 2^-dCT. Using u Mann-Whitney test significant differences were found for: *Ccr1* (-2.373, p=0.004), *Galr1* (-2.236, *p=0.025), *Grin1* (-2.236, *p=0.025) and *Slc6a7* (-2.249, *p=0.024).

Supplementary tables

Table S1. Relationship between cases of undernutrition reported in hospitals and medical services and cases of microcephaly between 2015 and 2018. Values are standardized by 1,000 inhabitants per state.

State	Undernutrition cases between 2009 and 2018 per 1,000 hab.	Microcephaly cases confirmed and under investigation between 2015 and 2018 per 1,000 hab.
Acre	0.95271166	0.030473942
Alagoas	3.489278731	0.042450412
Amapá	0.39849575	0.027235321
Amazonas	0.729358194	0.020076602
Bahía	3.675983536	0.073734764
Ceará	0.900894783	0.029953673
Distrito Federal	0.645540881	0.0192798
Espírito Santo	2.418605366	0.049788959
Goias	1.318239811	0.024540724
Maranhao	1.173866957	0.02794046
Mato grosso	1.544062848	0.072617844
Mato grosso Sul	1.918164601	0.012581075
Minas Gerais	2.866514839	0.019386264
Pará	2.066276674	0.014815681
Paraíba	5.137965014	0.099989743
Pernambuco	1.972799155	0.075986658
Piauí	1.679465757	0.042477793
Rio de Janeiro	2.37756639	0.036759859
Rio Grande do Norte	1.567247701	0.080075337
Rondonia	1.625059007	0.044085491
Roraima	1.106180053	0.050280912
Sao Paulo	1.058625878	0.008085313
Sergipe	1.305927316	0.073266158
Tocantins	1.511084576	0.119664659

Table S2. The estimated amount of daily protein (g) intake for each of the 83 interviewed mothers who have children with CZS.

Municipality	Amount of protein (g) consumed per day
Fortaleza	67.82143
Fortaleza	51.29286
Fortaleza	65.40714
Fortaleza	63.75714
Fortaleza	57.37143
Fotaleza	52.99286
Fortaleza	64.16429
Fortaleza	67.32857
Fortaleza	60.75714
Fortaleza	58.83571
Fortaleza	66.07857
Fortaleza	74.7
Fortaleza	77.7
Fortaleza	53.76429
Fortaleza	74.78571
Fortaleza	82.65
Fortaleza	84.13571
Fortaleza	68.25
Fortaleza	60.53571
Fortaleza	70.27143
Fortaleza	57.49286
Fortaleza	57.66429
Juazeiro do Norte	70.27143
Jardim	44.43571
Juazeiro do Norte	59.15
Juazeiro do Norte	68.37143
Crateus	59.12143
Juazeiro do Norte	54.22857
Crateus	63.15
Crato	50.17857
Barbalha	59.12143
Várzea Alegre	51.07857
Caririaçu	75.82857
Juazeiro do Norte	54.84286
Juazeiro do Norte	73.07857
Juazeiro do Norte	63.33571
Juazeiro do Norte	70.97857
Barbalha	68.98571
Jamacaru	67.58571
Juazeiro do Norte	41.6

Crato	72.82143
Barbalha	54.46429
Fortaleza	72
Fortaleza	72.92143
Fortaleza	51.01429
Fortaleza	62.26429
Irauçuba	70.18571
Irauçuba	54.75
Catunda	66.00714
Sobral	73.61429
Coreaú	75.73571
Varjota	67.95714
Coreaú	67.25
Caucaia	63.68571
Quixeré	81.65
Limoeiro do Norte	76.15
Quixeré	57.01429
Sobral	81.04286
Santa Quitéria	52.42143
Forquilha	66.59286
Moraújo	78.75
Sobral	66.22857
Varjota	77.19286
Quixadá	64.23571
Quizeramobim	68.13571
Senador Pompeu	74.56429
Quixeramobim	62.52143
Quixeramobim	46.35
Quixadá	68.76429
Ibaretama	72.90714
Marco	59.67857
Apuiarés	73.09286
Caucaia	65.85
Santana do Cariri	52.39286
Pacoti	61.93571
Icó	53.24286
Icó	55.96429
Altaneiro	39.50714
Capistrano	60.22143
Icó	61.49286
Aracoiaba	61.56429
Ipaumirim	48.28571
Capistrano	78.13571

Table S3. Relative risk of CZS in relation to protein intake. Odd ratio was calculated as follows: (37.35/28.43)/(62.65/71.57), resulting in a value of 1.5, then CZS is more probable to occur in the protein restricted group than in the well-nourished mothers.

	Under 60 g/d protein	Above 60 g/d protein
CZS interviewed mothers	37.35%	62.65%
Ceará population	28.43%	71.57%

Table S4. Maternal body weight (mean and SD) at the different pregnancy stages of the mouse model.

Co/Mock		Co/ZIKV		LP/Mock		LP/ZIKV		
Mean	SD	Mean	SD	Mean	SD	Mean	SD	Age
20.338	2.090	22.329	1.929	20.225	2.859	20.964	1.714	E0
20.733	2.306	23.014	2.309	21.075	2.149	22.129	1.845	E7
24.429	1.151	25.586	2.064	23.457	2.379	23.882	1.710	E12
27.600	1.796	28.414	2.450	25.350	2.867	24.955	2.132	E15