# Supporting Information for: Global Metabonomic and Proteomic Analysis of Human Conjunctival Epithelial Cells (IOBA-NHC) in Response to Hyperosmotic Stress

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# EXPERIMENTAL METHODS

## Cell culture

IOBA-NHC cells were maintained in a modified medium consisting of 1:1 mixture of Dulbecco's Modified Eagle Medium/Ham's F-12 culture media (Invitrogen) with 10% fetal bovine serum (Invitrogen), 2 ng/mL of recombinant human epidermal growth factor (Invitrogen), 1 µg/ml of bovine insulin (Sigma-Aldrich), 0.1 µg/mL of cholera toxin, (Sigma-Aldrich), 0.5 µg/mL of hydrocortisone and 1× strength of broad spectrum antibiotic-antimycotic (Invitrogen).

Cells for all experiments were treated using the same procedure. Each set consisted of three sub-cultures grown to 80% confluence and adapted to Keratinocyte serum-free medium (KSFM) (Invitrogen) overnight. Treatment media were applied to cells in each set and they were incubated in parallel for 24 h. The treatment conditions were as follows: control – unadjusted KFSM media at 277 mOsm/, T1 and T2 – KFSM media adjusted to 377 mOsm/L 477 and mOsm/L respectively.

Each hyperosmotic medium was prepared by the addition of 2 M NaCl prepared in PBS to commercial KFSM. Osmolarity measurements were performed using a vapour pressure osmometer (Vapro, Wescor Inc).

## Cell Viability Assay

Three sets of cells were grown and treated in black 96-well plates. The average cell viability under each condition was determined with CellTiter-Glo Luminescent Cell Viability Assay (Promega).

## Sample Preparation for Global Metabonomic Analysis

Five sets of cells were grown in 6-well plates and treated with control, T1 and T2 treatment media. Cells were washed twice with cold PBS (4°C) and lysed by the addition of 1.5 mL of methanol maintained at −80°C. Plates were scraped over a dry ice bath, and each lysate was transferred to a microcentrifuge tube. Lysates were vortex-mixed at 1200 rpm for 5 min and centrifuged for 10 min at 16,000 g (4°C). The supernatants were concentrated in a vacuum concentrator and freeze-dried subsequently.

## Sample Preparation for Targeted Metabolite Analysis

Three sets of cells were grown in 6-well plates and treated with control, T1 and T2 treatment media. Cells were dissociated from the plates with TrypLE express (Invitrogen), collected in microcentifuge tubes and re-suspended in 500 µL of PBS. The cell suspension of each sample was divided into two aliquots of 450 uL and 50 uL respectively, and cells in each aliquot were pelleted.

Cells from the 50 uL aliquot were lysed in 40 uL of RIPA buffer containing 1× protease inhibitor (Thermo Scientific). Samples were vortexed-mixed, placed in a cold ultrasonic bath for 30 s and mixed on an orbital shaker for 10 min. Lysates were centrifuged for 10 min at 15,000 g (4°C) and the protein concentration of each supernatant was quantified with a DC protein assay kit. (Bio-Rad Laboratories)

Cells from the 450 uL aliquot were lysed in 400 uL of 3:2 water/methanol containing 1 mM $K_2$EDTA and 10 mM ammonium bicarbonate. Samples were vortexed briefly, snap-frozen in liquid nitrogen, thawed and homogenized with a hand-held homogenizer for 1 min. 600 uL of methanol was added and lysates were centrifuged for 10 min at 15,000 g (4°C). The supernatants were dried and re-constituted in 30 uL of 9:1 methanol/water for targeted analysis.

## Sample Preparation for Global Proteomic Analysis

Three sets of cells were grown in 6-well plates and treated with control, T1 and T2 treatment media. Cells were washed twice with cold PBS (4°C) prior to harvesting. Flasks were scraped immediately after the addition of 1.5 mL of RIPA buffer with 1× protease inhibitor (Thermo Scientific). Cell suspensions were transferred to microcentrifuge tubes and gently agitated for 2 h at 4°C to complete lysis. Lysates were centrifuged for 10 min at 16,000 g (4°C) and the protein concentration of each supernatant was quantified using a Micro BCA protein assay kit (Thermo Scientific).

## Western Blot

Cells were grown in T25 flasks and harvested a similar manner as those for proteomic analysis.

# SUPPLEMENTARY TABLES

**Table S1 Data-processing parameters for peak finding and alignment of metabonomic data**

| *Data Processing Step* | | *Parameters* | |
|---|---|---|---|
| | | ***Positive ionization*** | ***Negative ionization*** |
| Peak detection | Subtraction offset/scans | 3 | |
| | Multiplication factor | 1.2 | |
| | Minimum spectral width/Da | 0.01 | |
| | Minimum chromatographic width/scans | 3 | |
| | RT window/min | 0.6 to 18 | |
| | Noise threshold/counts | 20 | 5 |
| Peak alignment | RT tolerance/min | 0.15 | 0.20 |
| | Mass tolerance/ppm | 10 | |
| | Minimum Number of Samples | 5 | |
| | Minimum Threshold | 20 | 5 |

**Table S2 Optimised MRM parameters for validation**

| ***Metabolite*** | ***Ionisation Mode*** | ***Q1 (m/z)*** | ***Q3 (m/z)*** | ***EP (V)*** | ***DP(V)*** | ***CE (V)*** |
|---|---|---|---|---|---|---|
| Carnitine | positive | 162.1 | 103.0 | 10 | 60 | 23 |
| Glycerophosphocholine | | 258.1 | 104.0 | | 70 | 22 |

**Table S3 Osmoprotecting metabolites from literature**

| *Metabolite* | *Dry Eye Model* | *Effect of Osmoprotecting Metabolite* | *Reference in Manuscript* |
|---|---|---|---|
| Carnitine | Primary human corneal epithelial cell culture (400 mOsm) | Reduced levels of activated JNK and p38 MAPK | Corrales *et al*. (2008) |
| | Primary human corneal epithelial cell culture (450 mOsm) | Suppressed expression of pro-inflammatory cytokines | Hua *et al.*(2015) |
| | Immortalised human corneal limbal epithelial cell culture (500 mOsm) | Reduced apoptosis | Khandekar *et al*. (2012) |
| | Mouse model, housed in desiccating environment | Suppressed expression of pro-inflammatory cytokines in conjunctiva, reduced apoptosis in cornea tissues | Chen *et al.* (2013) |
| Erythritol | Primary human corneal epithelial cell culture in hyperosmotic media (400 mOsm) | Reduced levels of activated JNK and p38 MAPK | Corrales *et al*. (2008) |
| | Primary human corneal epithelial cells (450 mOsm) | Suppressed expression of pro-inflammatory cytokines | Hua *et al.* (2015) |
| | Desiccating environment-housed mice | | Chen *et al.* (2013) |
| Betaine | Immortalised human corneal limbal epithelial cell culture (500 mOsm) | Reduced apoptosis | Garett *et al.* (2013) |
| | Primary human corneal epithelial cell culture (450 mOsm) | Suppressed expression of pro-inflammatory cytokines | Hua *et al.* (2015) |
| | Mouse model, housed in desiccating environment | Suppressed expression of pro-inflammatory cytokines in conjunctiva, reduced apoptosis in cornea tissues | Chen *et al.* (2013) |

**Table S4 Average carnitine and glycerophosphocholine levels in IOBA-NHC cells measured using MRM analysis from three replicates**

| *Metabolite* | *Amount (ng/mg protein in lysate)* | | |
|---|---|---|---|
| | *Control* | *T1* | *T2* |
| Carnitine | 0.050 | 0.072 | 0.11 |
| Glycerophosphocholine | 33 | 88 | 191 |

## SUPPLEMENTARY FIGURES

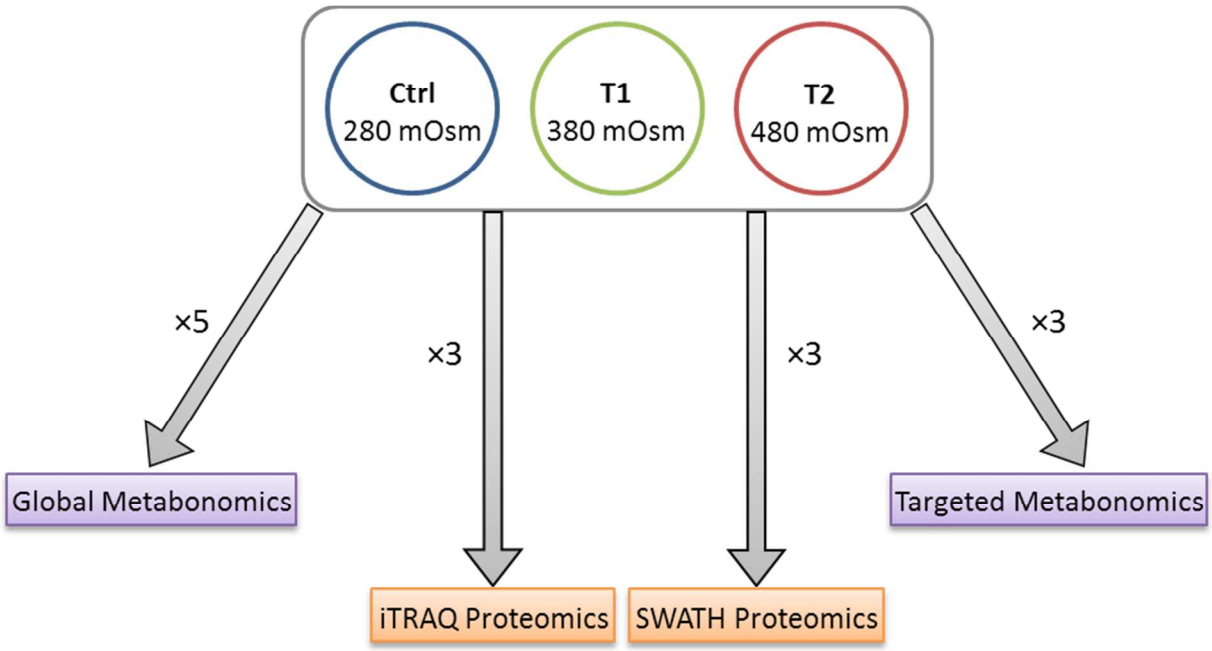


**Figure S1 Schematic of experiments performed to investigate changes in expression of metabolites and proteins in IOBA-NHC cells under different degrees of hyperosmotic stress.**

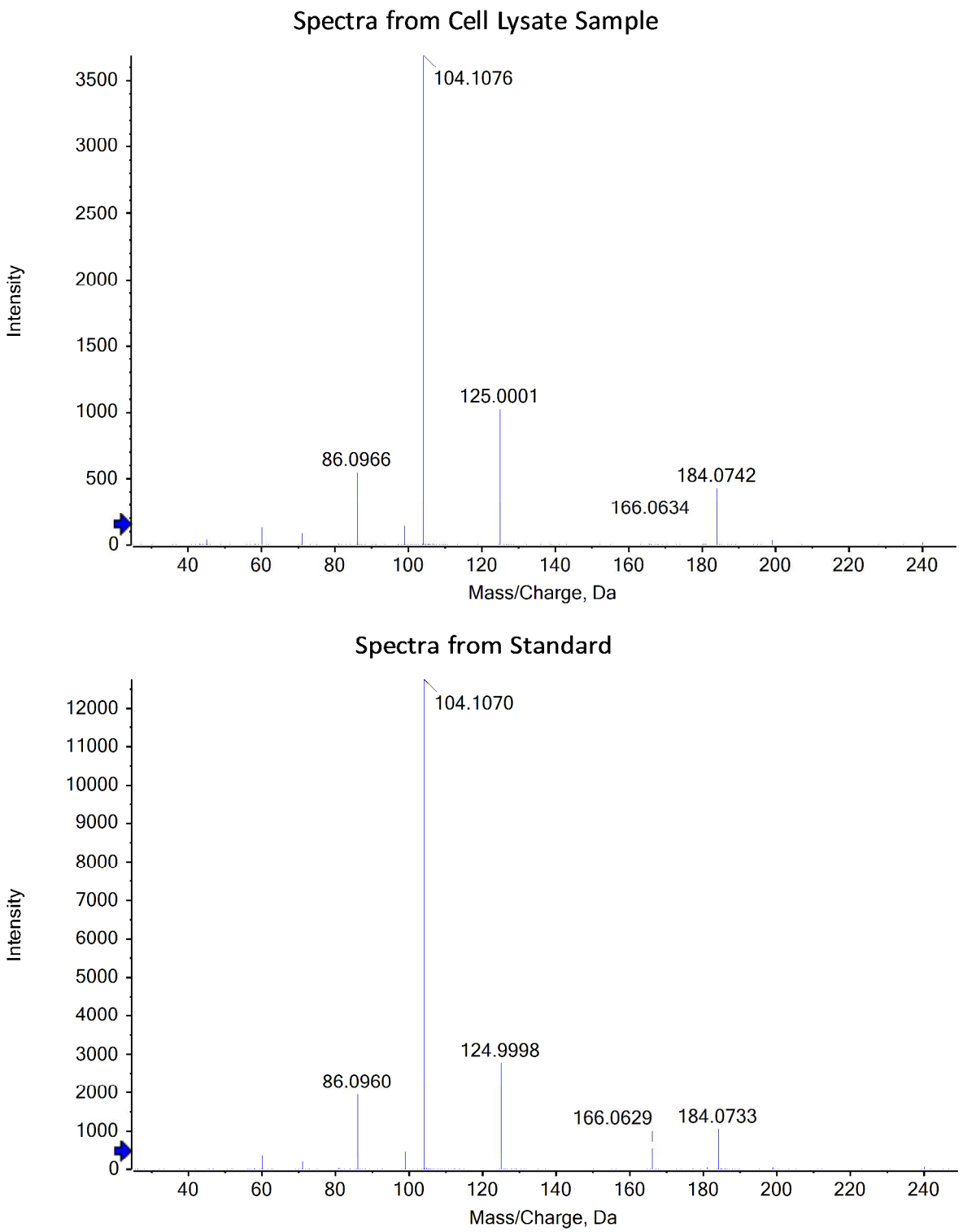


**Figure S2 MS/MS of glycerophosphocholine from metabonomic analysis (top) verified with standard (bottom).**

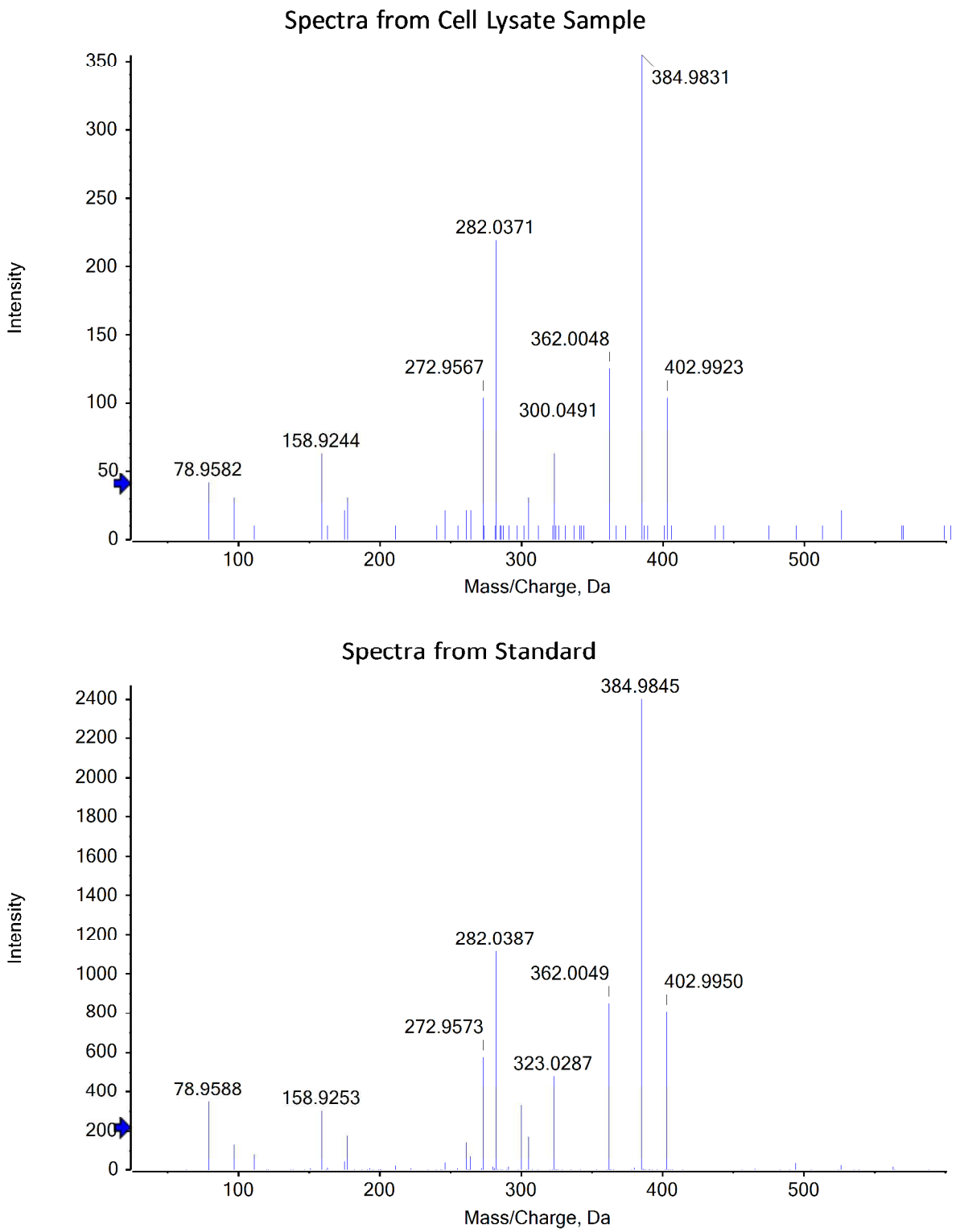


**Figure S3 MS/MS spectra of UDP-*N*-acetylglucosamine from metabonomic analysis (top) verified with standard (bottom).**

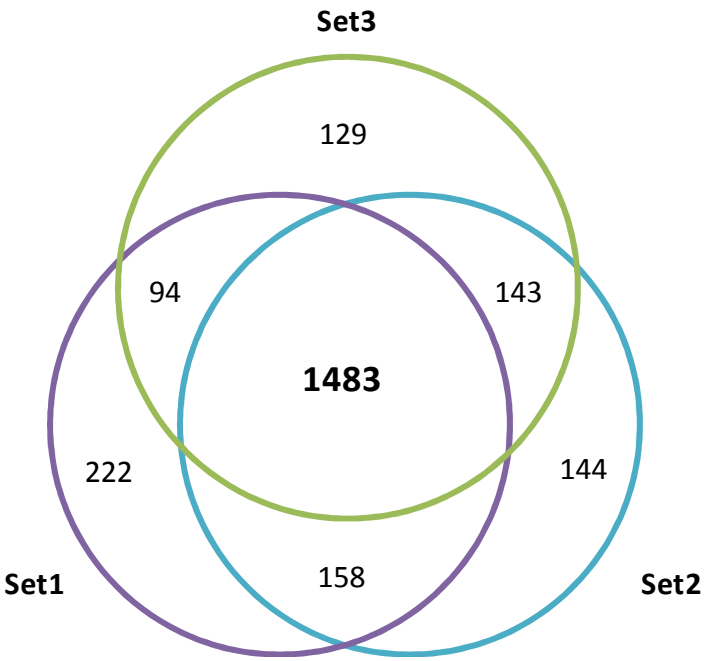


**Figure S4 Proteins detected in three iTRAQ-based analyses of IOBA-NHC cell lysates.**

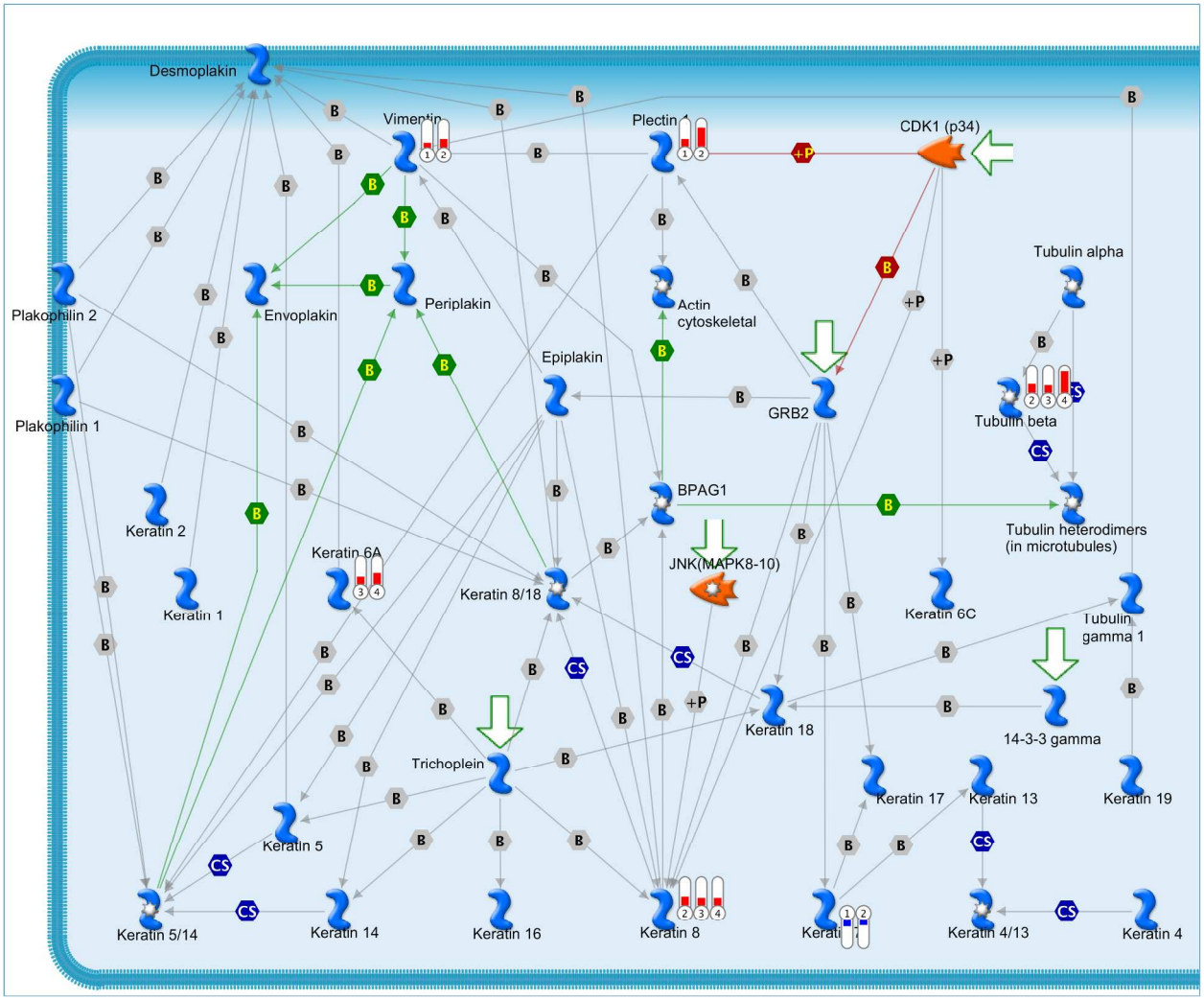


**Figure S5 Up-regulation of keratin 6A, keratin 8, plectin, tubulin beta and vimentin in IOBA-NHC cells under osmotic stress demonstrates the involvement of cytoskeleton remodeling. Vertical bars denote fold-changes versus control with regards to the following sets of treatment and analysis method: 1) T1 analysed with iTRAQ, 2) T2 analysed with iTRAQ, 3) T1 analysed with SWATH and 4) T2 analysed with SWATH.**

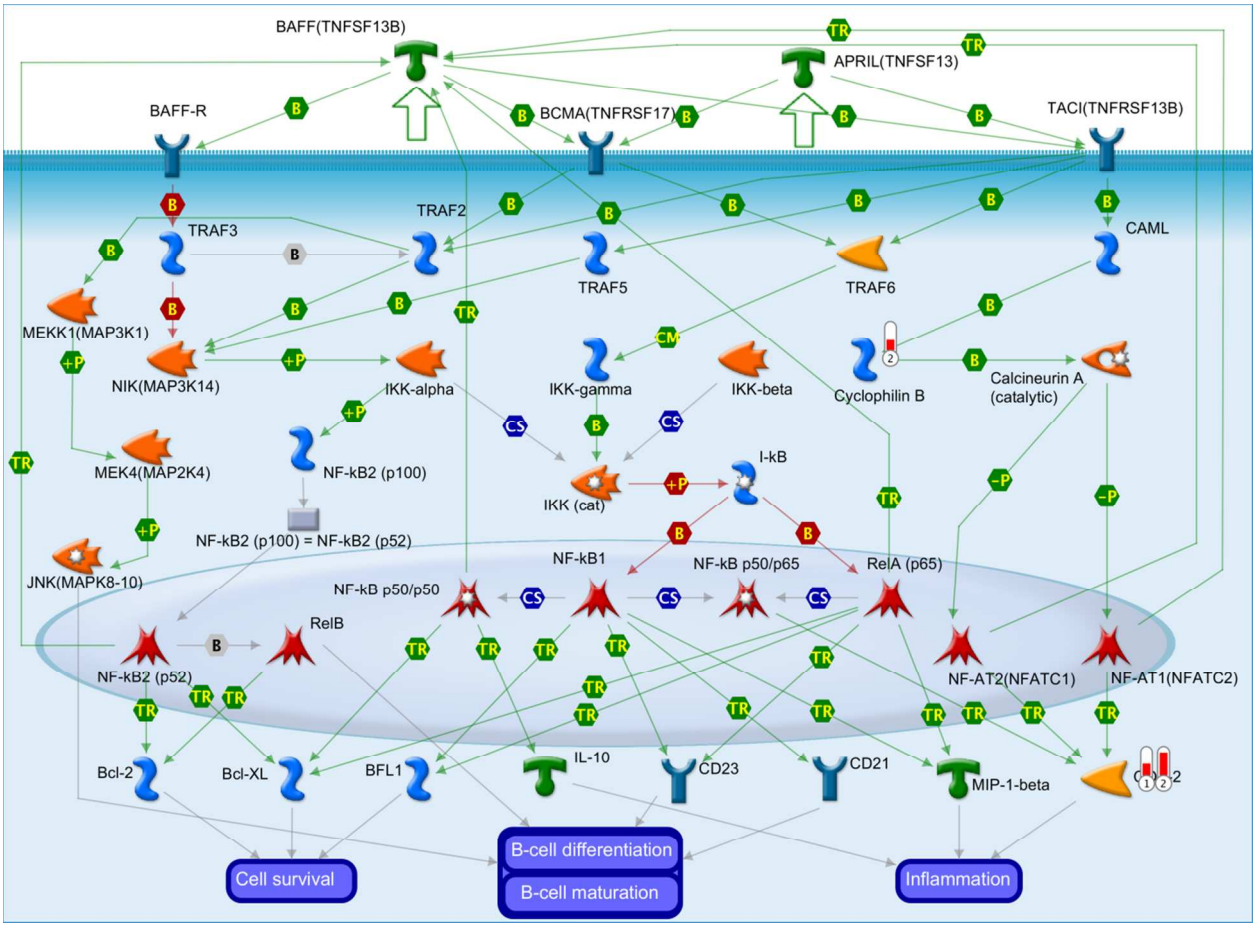


**Figure S6 Up-regulation of Cyclophilin B (peptidylprolyl cis-trans isomerase B) and COX-2 (PTGS2) in IOBA-NHC cells under osmotic stress demonstrate the involvement of APRIL and BAFF signaling pathway. Vertical bars denote fold-changes versus control with regards to the following two treatments: 1) T1 and 2) T2 analysed with iTRAQ.**

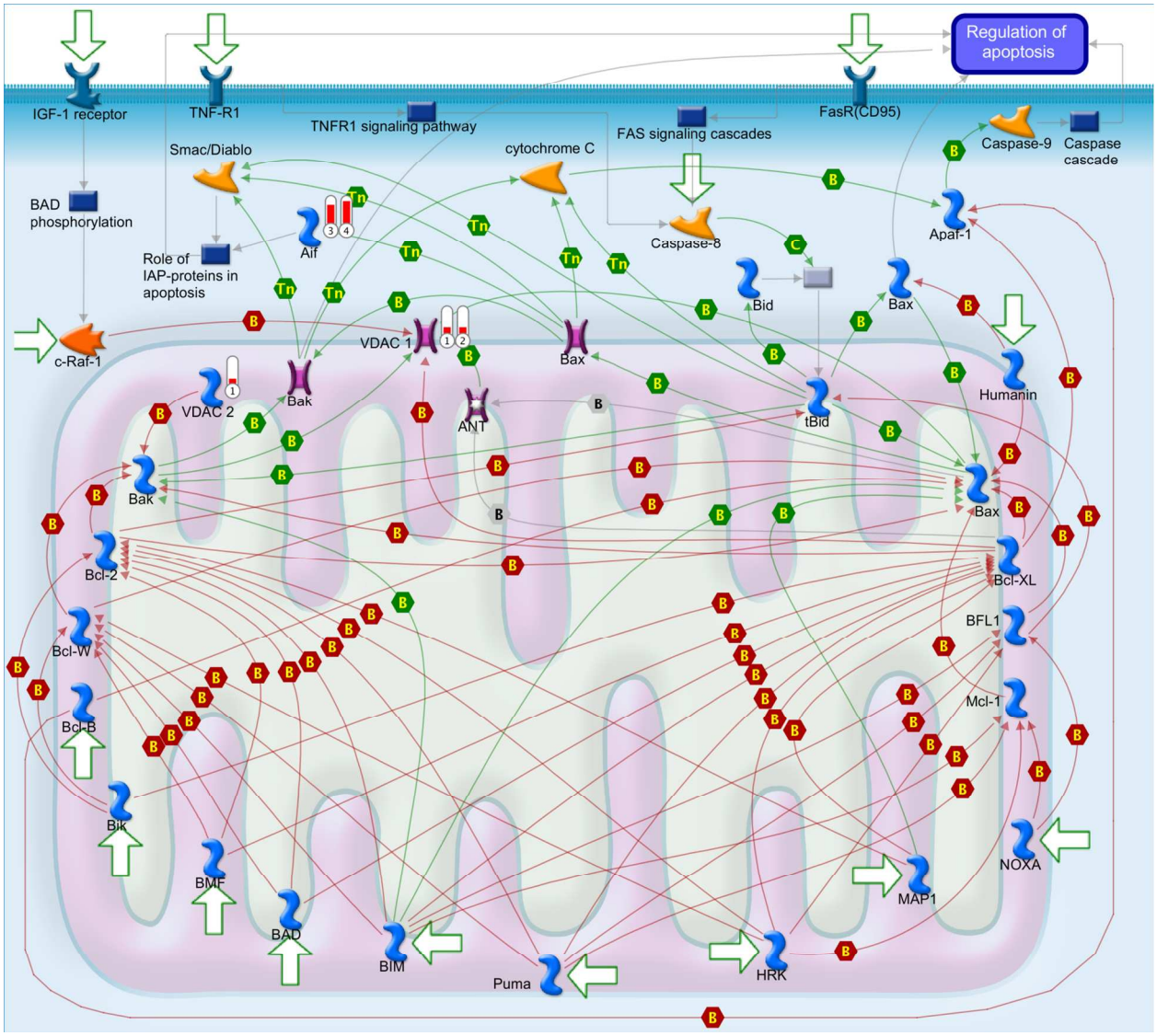


**Figure S7 Up-regulation of Aif(AIFM1) and VDAC1 in IOBA-NHC cells under osmotic stress demonstrate the involvement of regulation of apoptosis by mitochondria proteins (cytochrome C mediated). Vertical bars denote fold-changes versus control with regards to the following sets of treatment and analysis method: 1) T1 analysed with iTRAQ, 2) T2 analysed with iTRAQ, 3) T1 analysed with SWATH and 4) T2 analysed with SWATH.**

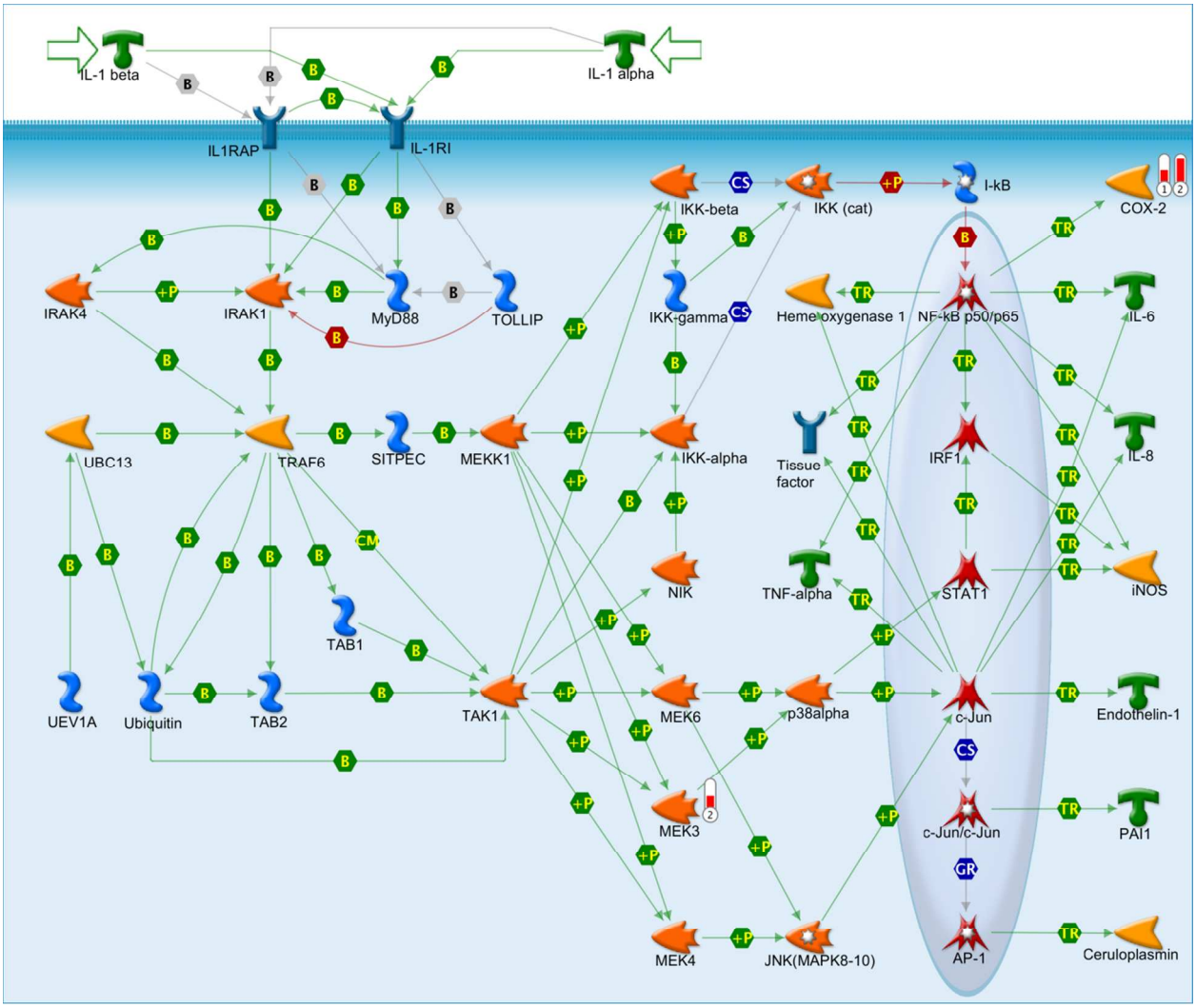


**Figure S8 Up-regulation of MEK3(MAP2K3) and COX-2 (PTGS2) in IOBA-NHC cells under osmotic stress demonstrate the involvement of Interleukin-1 signaling pathway. Vertical bars denote fold-changes versus control with regards to the following two treatments: 1) T1 and 2) T2 analysed with iTRAQ.**

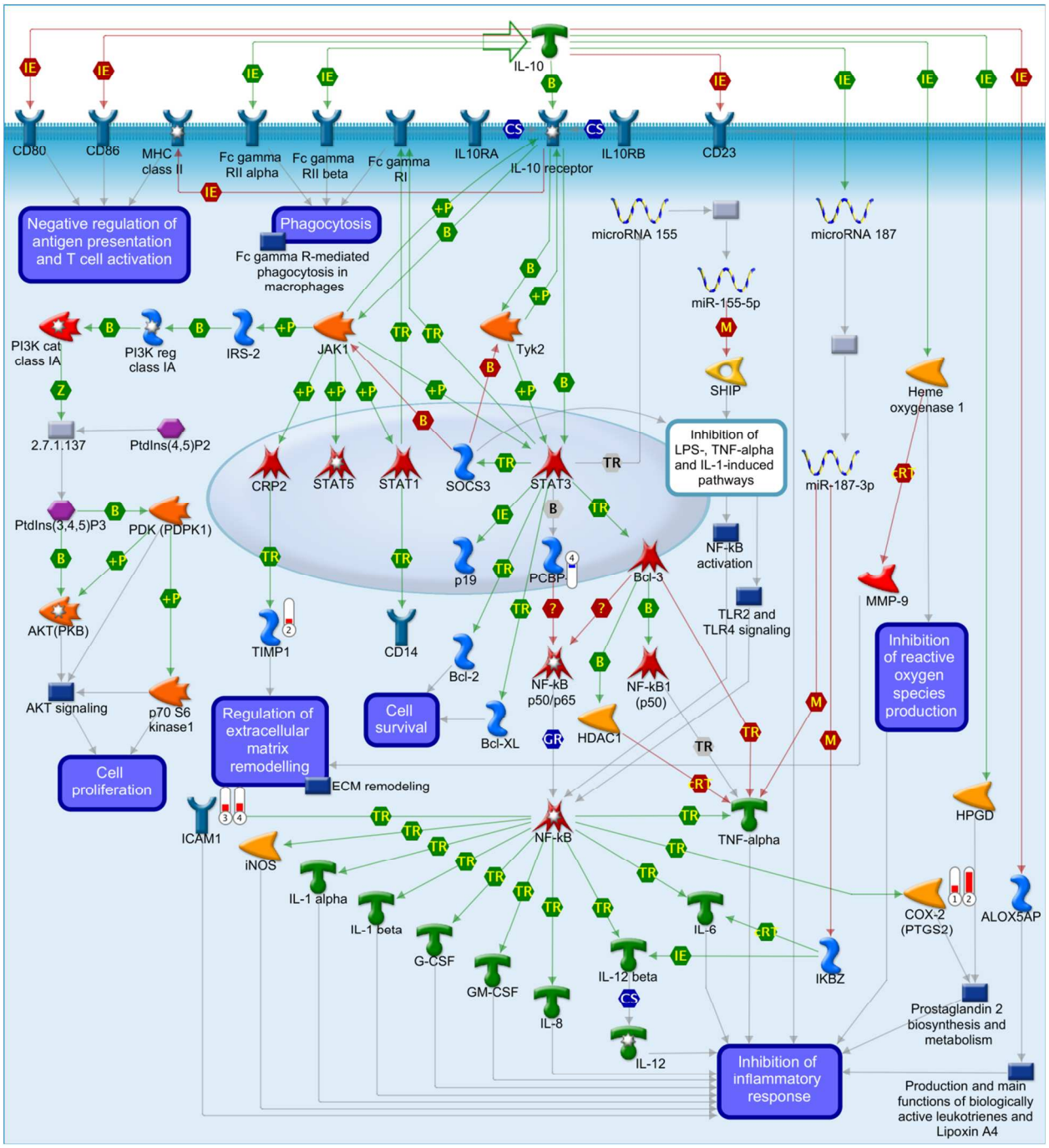


**Figure S9 Down-regulation of PCBP-1, up-regulation of COX-2(PTGS2), ICAM1 and TIMP1 in IOBA-NHC cells under osmotic stress demonstrate the involvement interleukin-10 signaling pathway. Vertical bars denote fold-changes versus control with regards to the following sets of treatment and analysis method: 1) T1 analysed with iTRAQ, 2) T2 analysed with iTRAQ, 3) T1 analysed with SWATH and 4) T2 analysed with SWATH.**

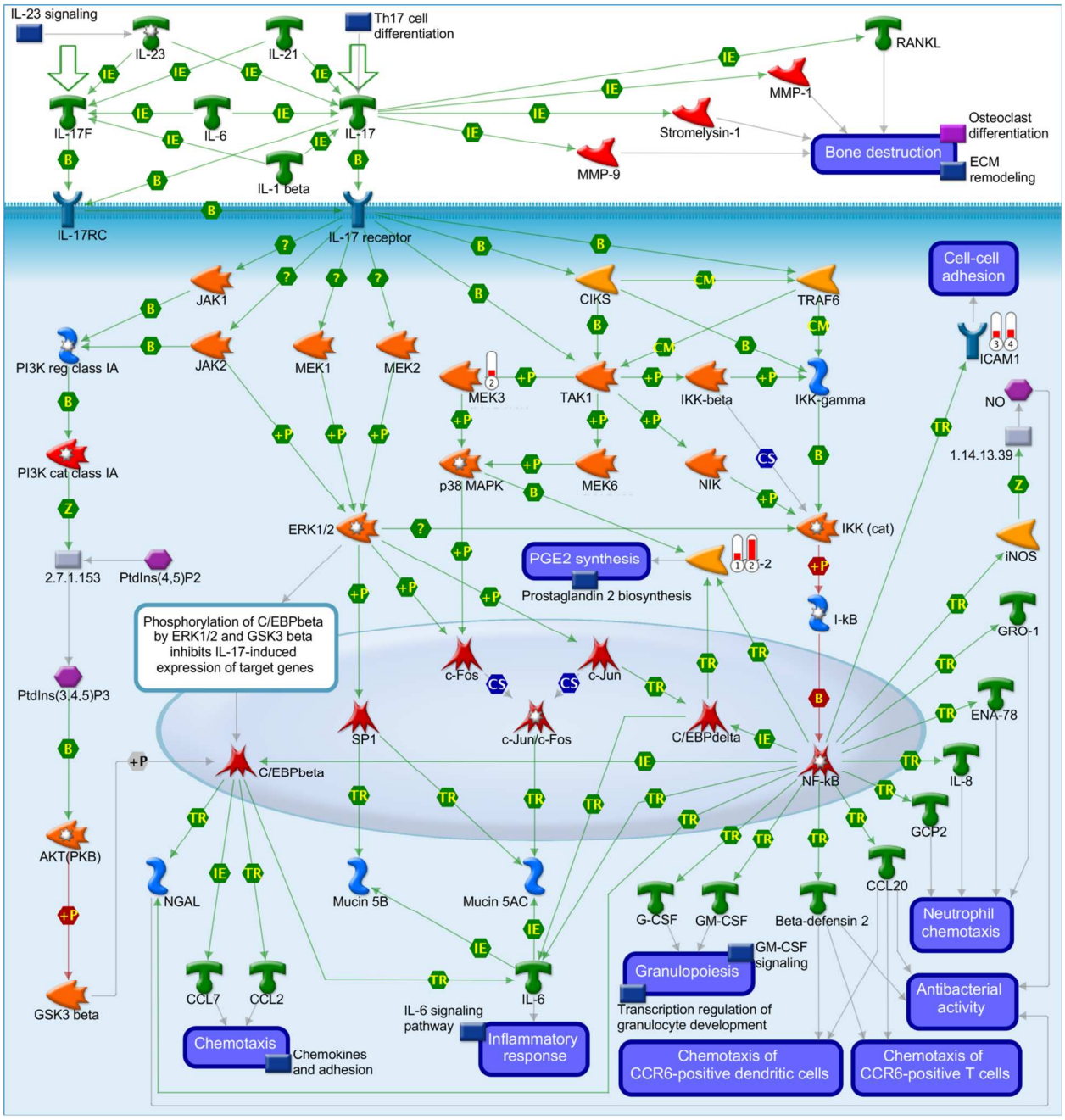


**Figure S10 Up-regulation of COX-2 (PTGS2,) ICAM1 and MEK3(MAP2K3) in IOBA-NHC cells under osmotic stress demonstrate the involvement interleukin-17 signaling pathway. Vertical bars denote fold-changes versus control with regards to the following sets of treatment and analysis method: 1) T1 analysed with iTRAQ, 2) T2 analysed with iTRAQ, 3) T1 analysed with SWATH and 4) T2 analysed with SWATH.**

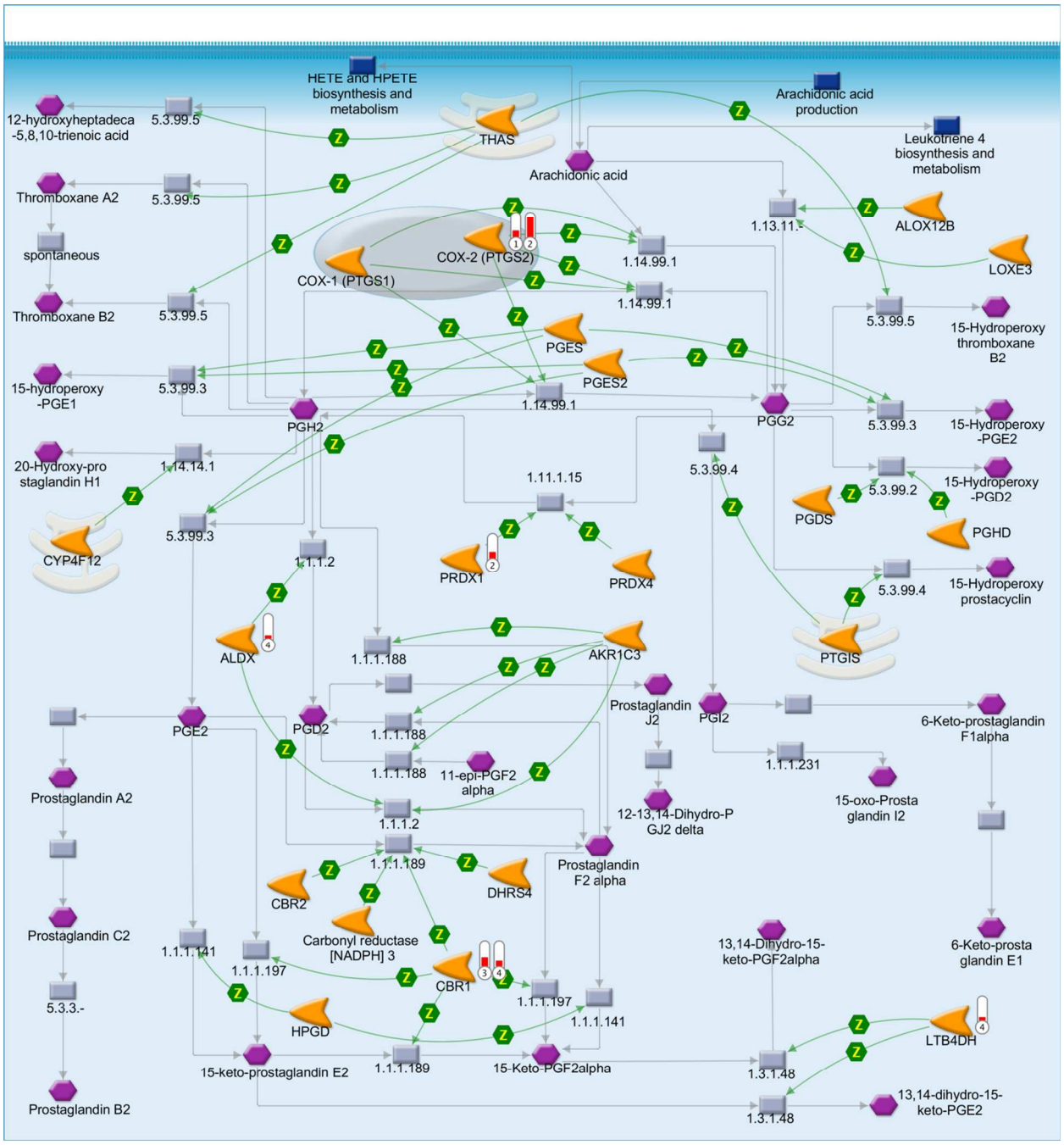


**Figure S11 Up-regulation of CBR1, COX-2 (PTGS2) and PRDX1 in IOBA-NHC cells under osmotic stress demonstrate the involvement of prostaglandin 2 synthesis and metabolism. Vertical bars denote fold-changes versus control with regards to the following sets of treatment and analysis method: 1) T1 analysed with iTRAQ, 2) T2 analysed with iTRAQ, 3) T1 analysed with SWATH and 4) T2 analysed with SWATH. The levels of ALDX (AK1A1) and LTB4DH (PTGR1) under T2 were 1.34 and 1.36 times that of the controls, and did not meet the threshold for significance.**

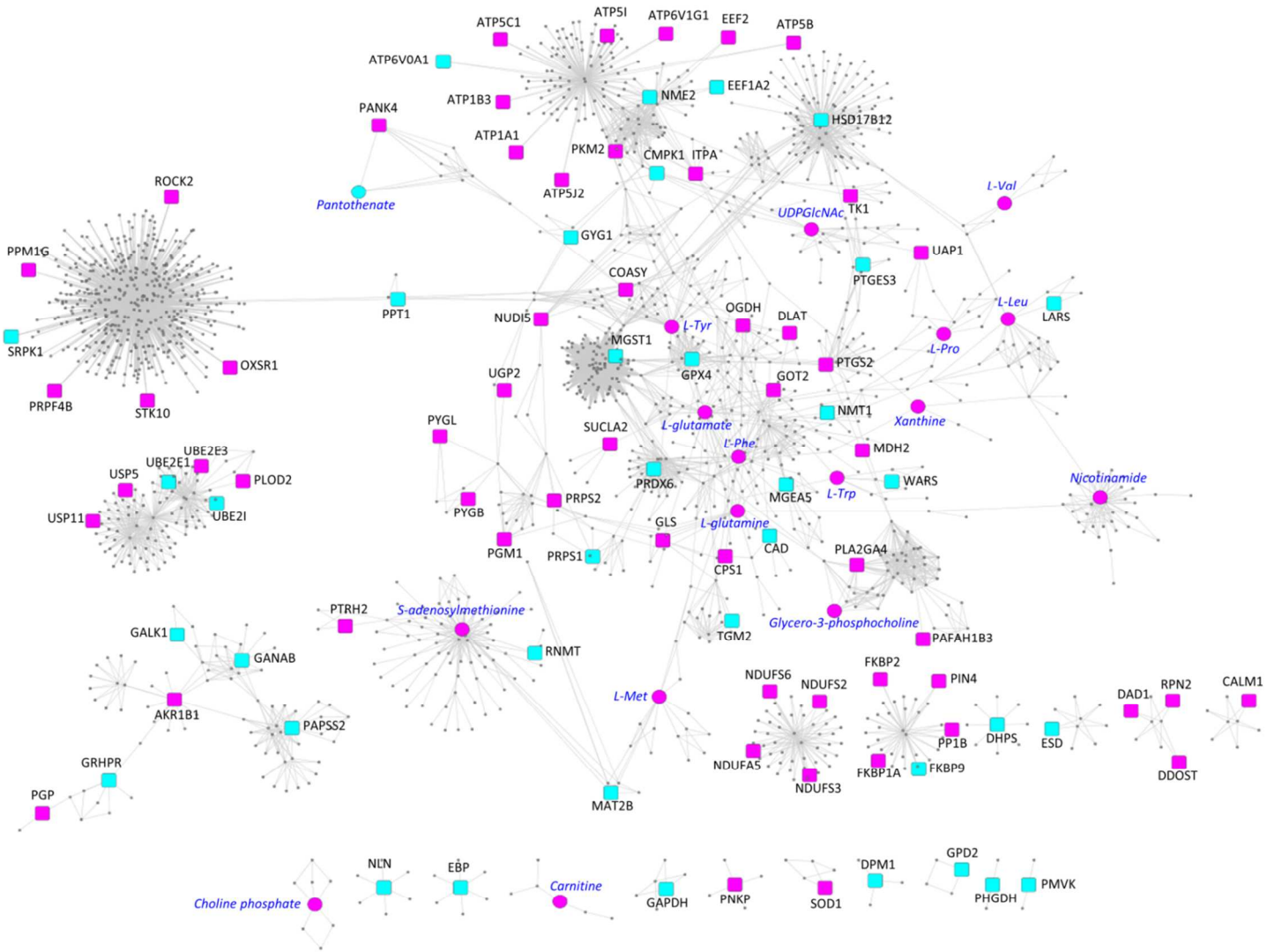


**Figure S12 Network map of metabolites (circles) and proteins (squares) with increased (pink) and decreased (blue) levels in IOBA-NHC cells under hyperosmotic condition T2 (480 mOsm). Networks were constructed in Metscape. For visual clarity, only proteins and metabolites identified in the study are labeled whereas linker molecules (required for network construction but not identified in the study) are represented as unlabeled dots.**

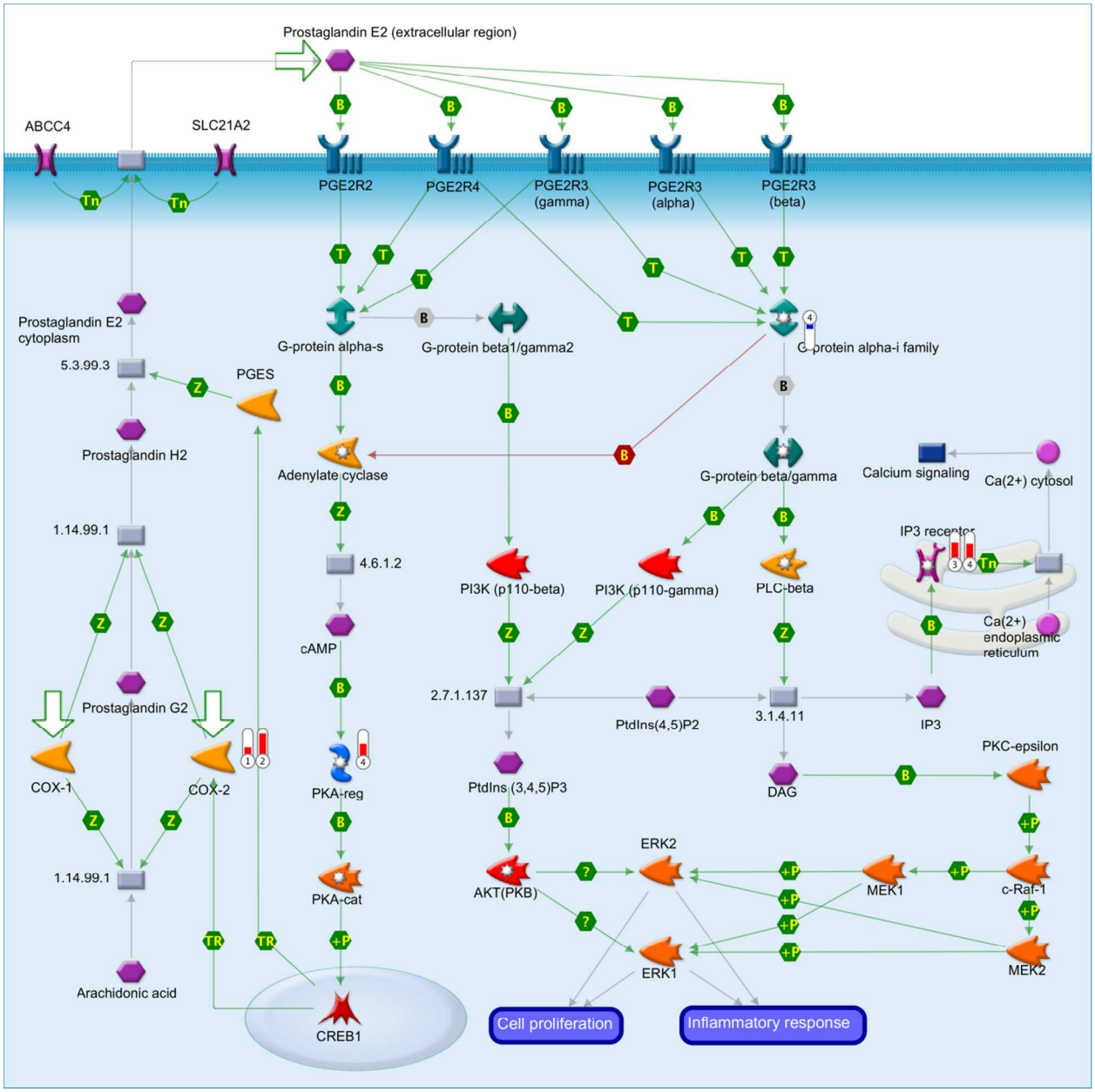


**Figure S13. Up-regulation of COX-2 (PTGS2) and IP3 receptor and the down-regulation of G-protein alpha-i family in IOBA-NHC cells under osmotic stress demonstrate the involvement of prostaglandin 2 common pathways. Vertical bars denote fold-changes versus control with regards to the following sets of treatment and analysis method: 1) T1 analysed with iTRAQ, 2) T2 analysed with iTRAQ, 3) T1 analysed with SWATH and 4) T2 analysed with SWATH. The level of PKA-reg under T2 was 1.35 times that of the controls and did not meet the threshold for significance.**