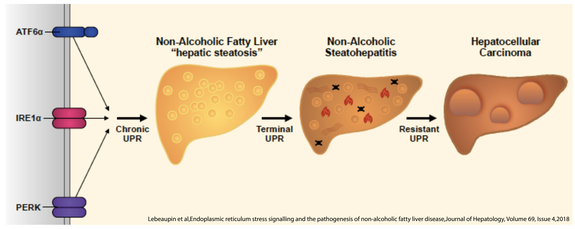
In-vivo imaging of hepatocyte endoplasmic reticulum morphology reveals correlation between the unfolded protein response and fatty liver disease in zebrafish

Anjana Ramdas Nair , Leonore Wünsche, Patrice Delaney, Jeremy Teo and Kirsten C. Sadler
Biology Program, New York University Abu Dhabi, Saadiyat Campus, 129188, Abu Dhabi, United Arab Emirates

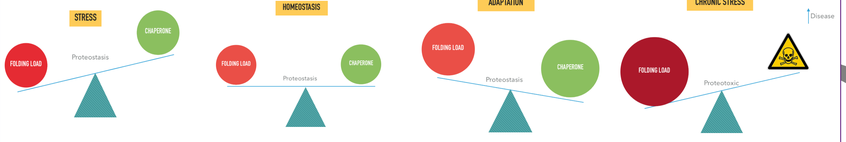
INTRODUCTION

Chronic UPR activation leads to hepatic steatosis



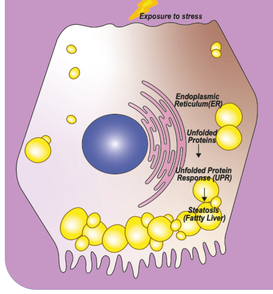
Unfolded Protein Response (UPR) is a complex pathway induced when unfolded proteins accumulate in the endoplasmic reticulum (ER). Robust or chronic UPR induction is known to cause diseases such as diabetes and fatty liver disease (FLD).

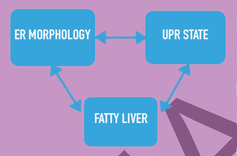
UPR acts as a stress meter



The UPR can be classified into subclasses (homeostatic, adaptive, stressed and terminal). However, the gene signature of each UPR outcome is yet to be defined.

OPEN QUESTIONS





How is the ER influenced during UPR changes?

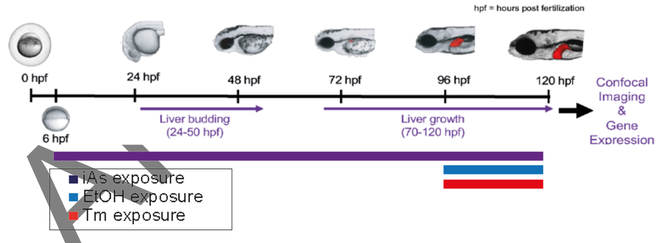
How does it correlate to Fatty Liver?

Is fat accumulation indicative of UPR dysfunction?

Is fat accumulation indicative of an adverse outcome?

EXPERIMENTAL APPROACH

(I)Treatment Scheme:



(II)UPR inducers :

Stressor	Mechanism of action
Tunicamycin (Tm)	Blocking N-linked glycosylation
Inorganic Arsenic (iAs)	Accumulation of Reactive Oxygen Species (ROS)
Ethanol (EtOH)	Accumulation of Reactive Oxygen Species (ROS) interfering with disulfide bond formation
Atf6 overexpression (nAtf6)	Transgenic fish expressing the nuclear (cleaved) form of Atf6 under the fabp10a promoter, leading to constitutive UPR activation in hepatocytes.

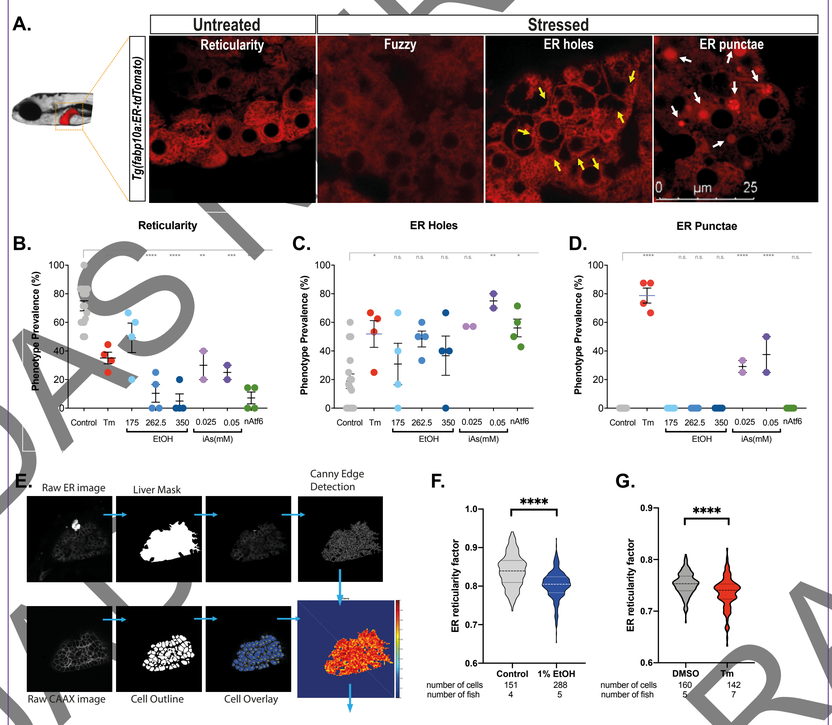
(III)Fluorescent Tools:

Cellular Structure	Tool Type	Name
Endoplasmic Reticulum	Transgenic hepatocyte expression	*Tg(*fabp10a:ER-tdTomato)
Cell Membrane	Transgenic hepatocyte expression	*Tg*(fabp10a:CAAX-EGFP)
Lipid Droplets	Vital Dye	Nile Red

This work was funded by New York University Abu Dhabi (NYUAD) and the National Institute of Health. Contact:ar5038@nyu.edu,ke27@nyu.edu

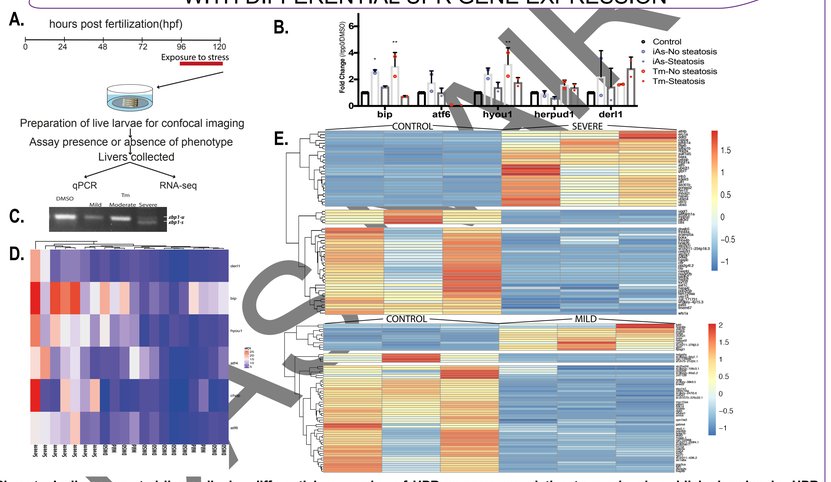
RESULTS

1.ER STRESS CAUSES STRESS DEPENDENT ALTERATIONS TO ER STRUCTURE



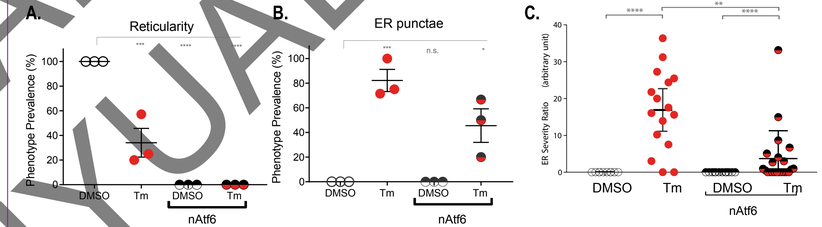
ER morphology reflects different stress conditions, making it a useful tool for assessing ER stress and UPR activation. Loss of reticularity is the most common phenotype when ER is subjected to stress and it can be quantified using an edge detection code.
(A) Representative in vivo confocal images of hepatocyte-specific driven ER localization signal tagged with tdTomato displaying the dynamic changes in the ER during different stress conditions. Untreated 5 dpf larval hepatocyte display fine, reticular ER distribution pattern. Three key phenotypes were identified when hepatocytes were subjected to stress by UPR inducers, namely: Fuzzy (loss of reticularity), ER holes (appearance of gaps or holes in the ER) indicated by yellow arrows) and ER punctae (appearance of spherical ER punctae within hepatocytes, indicated by white arrows). (B) Quantification of the different ER stress phenotype prevalence in larval livers treated with Tm, EtOH, iAs or hepatocyte specific chronic Atf6 activation (nAtf6). Values are plotted as mean±SEM, with significance levels relative to the control group (one-way ANOVA). (E)Analysis workflow for edge detection in MATLAB.(F&G)Plot of the ER reticularity factor generated by the code per cell after filtering based on hepatocyte size for different treatments. Values plotted are the median , with significance values relative to the control/DMSO (Unpaired two-tailed t test; ****p<0.0001)

3.HETEROGENOUS PHENOTYPIC RESPONSE IS ASSOCIATED WITH DIFFERENTIAL UPR GENE EXPRESSION



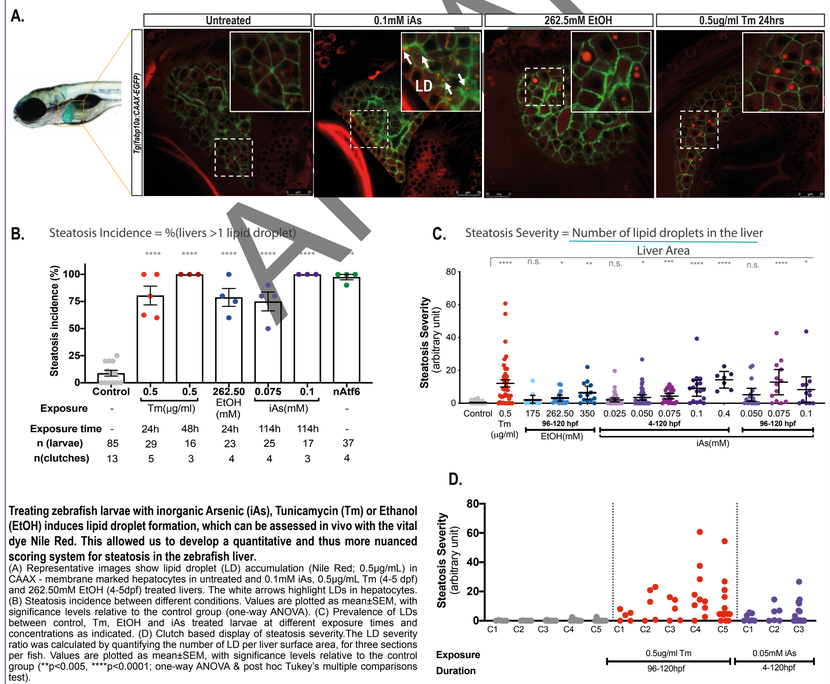
Phenotypically segregated livers display differential expression of UPR sensors, correlating to previously published molecular UPR patterns.
(A) Experimental design. (B) Fold Change of UPR gene expression based on qPCR livers segregated by steatosis, normalized to rplp0 and DMSO. (C) PCR analysis of unspliced (xbp1-u) and spliced (xbp1-s) xbp1 mRNA in single liver of 5dpf larvae treated with DMSO/Tm for 24 hour and segregated by severity of ER phenotype. Heatmap of UPR target gene expression based on qPCR ,with genes in columns and each single-liver sample in rows. White and red indicates below and above average, respectively, for each column. Target gene expression was normalized to rplp and DCt values were plotted; n= 10 for DMSO, n= 12 for Tm (4 mild, 8 severe). (E) Heatmap of signficant differentially upregulated and downregulated UPR genes (>2log2FC>2) based on RNA-seq between control and tm-treated livers segregated by phenotype.

4.ATF6 IS SUFFICIENT TO RESCUE ER PUNCTAE FORMATION IN STRESSED LIVERS



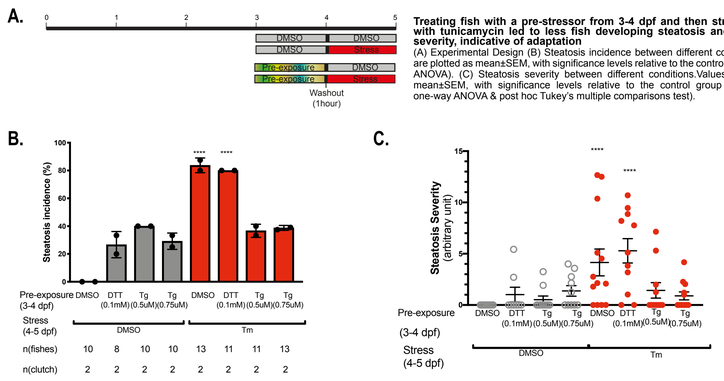
The ER punctae phenotype in Tm-treated livers is dramatically reduced in zebrafish expressing hepatocyte-specific chronic activation of Atf6
(A,B)Quantification of the different ER stress phenotype prevalence in larval livers treated with tunicamycin (Tm) in wild type background and transgenic fish overexpressing nAtf6. Values are plotted as mean±SEM, with significance levels relative to the control group (one-way ANOVA). (B) Overview of data from confocal experiments showing the ER severity ratio, which was derived from the number of punctae and liver surface area. Each data point corresponds to one zebrafish, based on the average value of 2 sections per fish (ntotal=163). *p<0.05, **p<0.005, ****p<0.0001 (Kuskal-Wallis and Dunn's post hoc). Values are plotted as mean±SEM (N=15-20 fish, 3 clutches)

2.*IN-VIVO* LIPID ASSESSMENT REVEALS HETEROGENEITY IN STEATOSIS OUTCOME



Treating zebrafish larvae with inorganic Arsenic (iAs), Tunicamycin (Tm) or Ethanol (EtOH) induces lipid droplet formation, which can be assessed in vivo with the vital dye Nile Red. This allowed us to develop a quantitative and thus more nuanced scoring system for steatosis in the zebrafish liver.
(A) Representative images show lipid droplet (LD) accumulation (Nile Red; 0.5µg/mL) in CAAX - membrane marked hepatocytes in untreated and 0.1mM iAs, 0.5µg/mL Tm (4-5 dpf) and 262.50mM EtOH (4-5dpf) treated livers. The white arrows highlight LDs in hepatocytes. (B) Steatosis incidence between different conditions. Values are plotted as mean±SEM, with significance levels relative to the control group (one-way ANOVA). (C) Prevalence of LDs between control, Tm, EtOH and iAs treated larvae at different exposure times and concentrations as indicated. (D) Clutch based display of steatosis severity.The LD severity ratio was calculated by quantifying the number of LD per liver surface area, for three sections per fish. Values are plotted as mean±SEM, with significance levels relative to the control group (**p<0.005, ****p<0.0001; one-way ANOVA & post hoc Tukey's multiple comparisons test).

5.PRECONDITIONED LIVERS DISPLAY LOWER STEATOSIS INCIDENCE



Treating fish with a pre-stressor from 3-4 dpf and then stresssing them with tunicamycin led to less fish developing steatosis and much lower severity, indicative of adaptation
(A) Experimental Design (B) Steatosis incidence between different conditions. Values are plotted as mean±SEM, with significance levels relative to the control group (one-way ANOVA). (C) Steatosis severity between different conditions.Values are plotted as mean±SEM, with significance levels relative to the control group (****p<0.0001; one-way ANOVA & post hoc Tukey's multiple comparisons test).

MODEL AND CONCLUSION

The ER shape changes and lipid acumulates in response to chemically-induced stress and UPR activation. However, there is high variation between fish with regard to phenotypic response which is reflected in their UPR gene expression profiles. ATF6 over-expression protects against Tm-induced punctae formation indicating that ATF6 may have a role in ER morphology changes during stress. Most imortantly, both the ER and lipid can be reliably used as a marker to predict UPR stages.