Functions of P38 and ERK kinases in zebrafish early development

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CHAPTER IV

The P38αA and Erk2 are essential for epiboly initiation by regulating common and separate developmental genetic programs

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Abstract

The P38α and Erk2 MAPKs play an essential role in morphogenetic movements during zebrafish gastrulation, in particular during epiboly. Using time-lapse imaging we demonstrated that lethality of P38α and Erk2 morphants is correlated with defective cell migration of the blastomere margin, as a result dissociated blasoderm cells. Since knock-down of either of the two MAPKs causes similar severe morphological defects, or even arresting embryonic development before the onset of epiboly, we set out to investigate their putative functional redundancy in epiboly initiation. To identify distinct and overlapping roles for Erk2 and P38 at this early stage of developmental morhogenesis, we performed transcriptome analysis of high/oblong stage P38α and Erk2 knockdown embryos.

We found common genes affected by both P38α and Erk2 knockdown and genes specifically affected by depletion of either P38α or Erk2. The common gene set and the Erk2 knock down specific gene set include transcripts regulating cell migration and adhesion properties associated with epiboly. Epithelial cell adhesion molecule (Epcam) and bloody fingers (Blf) expression is decreased by either P38α or Erk2 morpholino treatment. The Aryl hydrocarbon receptor nuclear translocator 2 (Arnt2) is specifically up-regulated in P38α morphants. Apelin receptor b (Aplnrb), c-x-c chemokine receptor 4 (Cxcr4), snail2 (Snai2), ras homolog gene family member Ab (Rhoab) and fyn oncogene related to src (Fyna) are down-regulated by Erk2 morpholino while interferon regulatory factor 6 (Irf6) is up-regulated.

Interestingly, gene ontology analysis revealed a large pool of ribosomal proteins with low expression levels and several histone protein coding transcripts where significantly up-regulated by the P38α morpholino. Imbalanced histone and ribosomal protein expression levels are associated with loss of nuclear integrity. We found also that P38α morphants fail to induce expression of P53, a key cell cycle checkpoint regulator, upon DNA damage induced by camptothecin treatment. These results show that the function of P38α in stress signalling is already put to use during initial stages of embryogenesis. Additionally, we conclude that both P38α and ERK2 regulate epiboly initiation by controlling common and distinct genetic mechanisms.

Introduction

Mitogen Activated Protein Kinase (MAPK) signalling is involved in divers cellular processes such as cell motility, cell cycle progression, survival, protein biogenesis, and differentiation. MAPK signalling cascades consist of an upstream MAPKKK (MEKK) which activates a MAPKK(MEK) to finally activate a MAPK by phosphorylation. There are 14 MAPK proteins, which are subdivided according to their dual-phosphorylation (activation) motif (T-X-Y) into the extracellular signal-regulated kinases (Erk1,2,3,4,5,6,7), c-Jun amino (N)-terminal kinases (JNK1/2/3) and P38 isoforms (α,β,γ, and δ) [1-6].

Erk2, Erk5, P38α and Jnk1/2 MAPK family members appear to be essential for vertebrate development as knock-out mice die within the first 12 days of development [7-9]. In zebrafish all 14 MAPK are conserved and previous research accomplishments have gained insight into the functions of ERK2, P38α and JNK1/2 in the first stages of development.

P38α is asymmetrically activated on one side of the blastodisc and regulates the first cell divisions during the early cleavage period. The side of P38α activity overlaps with the expressed dorsal-specific zygotic gene dharma/bozozok however dorsal-ventral axis formation is unaffected after injection with dominant negative P38α RNA suggesting that P38α is dispensable for this process [13]. In contrast, a clear function of JNK in dorsal-ventral patterning has been demonstrated with a MO knock-down approach which leads to ventralized embryos. JNK and MEKK4 are part of a dorsalization pathway that is induced by Axin/JNK and inhibited by Aida [14]. Furthermore, ERK2 and P38α are both found to be involved in epiboly movements during gastrulation: the spreading of embryonic cells of the blastoderm over surface of the yolk until fully covered and occurs from 4.3 hpf (dome stage) to 10 hpf (tail bud). Prior to epiboly, the blastula surface cells differentiates into the enveloping layer (EVL), of which the forerunner cells delaminate (later forming the Kupples vesicles) and the yolk syncycial layer (YSL), both are the essential mediators of epiboly [15].

Activated forms of ERK2 and P38α are detected at the margin of the blastoderm throughout all stages of epiboly. When the blastoderm cells reach the equator and cover the yolk cell by half, the cells at the margin are induced into mesendoderm cell fates by a combination of Nodal and Fgf signaling. [13,16]. Fgf morphogens specificallyare shown to activate downstream Erk1/2 signalling and are involved in the specification and maintenance of mesoderm cell faith by regulation of T box transcription factors [17-19]. Erk2 levels causes defects in convergent-extension movements, and a strong knock down of Erk2 leads to a full developmental arrest in blastula stage embryos [16]. In addition, the Erk2-MAPK pathway functions downstream of Fgf [16] and is a modulator in mesendoderm formation,, which is

apparent from reduced levels of expression of the mesodermal markers No tail, Tbx6 and Tbx16 in ERK2 depleted embryos [20]. The first evidence for a role of P38α mediated signaling in epiboly came from a large genetic screen for maternal-effect mutants that display morphological defects at or after mid blastula transition (MBT) [21,22]. In this screen, the *betty boop* mutant (bbp) was identified as *bbp* mutant show strong epiboly defects characterized by a dramatic constriction of the blastoderm margin resulting in a yolk burst. The loss of function mutation in bbp was mapped in the Mitogen Activated Protein Kinase Activated Protein Kinase 2 (Mapkapk2) gene, a specific substrate of P38 MAPKs. The same phenotype was observed by injection of dominant negative P38α RNA suggesting that this MAPK isoform plays a specific non-redundant role in regulating gastrulation cell movements [23].

Since both P38α and Erk2 knockdown results in severe morphological defects the question remains what downstream target genes are regulated by the two MAPK that are essential for early embryogenesis. Erk2 and P38α were the first identified MAPKs, initially discovered as proteins which were rapidly phosphorylated after mitogen stimulation and osmotic shock respectively. Multiple cytosolic and nuclear substrates have been identified so far which was of great value for the molecular characterization of MAPK associated cellular events [24-29]. Although both MAPK are found essential for vertebrate development the molecular mechanisms are less well documented. MAPK are known to translocate to the nucleus and influence gene expression, after extracellular stimulation. In this study, we show that depletion of P38α zebrafish embryos fail to initiate epiboly, and thus phenocopies embryos that have been depleted from ERK2 by morpholino knockdown. We compare transcriptomes of these P38α and Erk knock down embryos at high/oblong stage, prior to epiboly initiation, to identify specific and common downstream target genes that are necessary for epiboly progression.

Material & Methods

Morpholino injections

ABXTL embryos were injected between 0- and 1- cell stage with either P38αA translational blocking morpholino (ATG) 5'GTGGGTCTTTCTTTCTGCGACATGC3', P38αA splice blocking morpholino (splice)5'AGCTGCATACACGAAATATGAAAGT3', ERK2 translational blocking morpholino 5'CACCCAAAAGCACCAGGAAAAGCTC3' or a standard control morpholino 5'CCTCTTACCTCAGTTACAATTTATA3'(Genetools). Morpholino's where dissolved in 1XDanieau's buffer [58mM NaCl, 0.7mM KCl, 0.4mM MgSO4, 0.6mM Ca(NO3)2, 5.0mM HEPES pH7.6] containing 1% phenol red solution.

Time Lapse Imaging

For a time course experiment of 16:40 hours embryos were dechorionated, placed on an agar coated willco dish and subsequently embedded in 1% low melting agarose and covered with eggwater. From 256 cell stage onwards, embryos where repeatedly imaged every 5 minutes with 10X magnification of the DIC microscope in a room temperature of 24°C. 200 pictures were processed in a movie of 28s with ImageJ software.

Protein isolation & western blotting

Embryos were dechorionated in 1X pronase solution (2 mg/ml Roche Applied Science) for 7 minutes and washed several times in 1X Danieau buffer. Total protein was isolated from batch embryos first by deyolking in Ca^{2+}/Mg^{2+} free solution. Cells where collected at 500 RCF and washed twice, once in Ca^{2+}/Mg^{2+} free solution and once in PBS, before final lysis (30min incubation and vortexing in lysis buffer 1M Tris-Hcl pH7,4, 0,5M EDTA, 1 tablet protease inhibitor, 0,1M Na3VO4, 0,5M NaF).

For biochemical analysis, protein extract were diluted in SDS sample buffer red +DTT (cell signaling #7723), sonificated and heated for 10 min at 95°C. 1 µg of the proteins samples were separated in a SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Schleicher & Schuell, Den Bosch, The Netherlands) by western blotting. The membranes were blocked in 5% w/v non-fat dry milk in Tris-Buffered Saline-Tween 20 (TBST). The blots were incubated with of P44/42 MAPK Kinase antibody (Cell Signaling #9102 1:1000), P38 antibody (Santa Cruz Biotechnology C-20 1:1000), pan-actin (Cell Signaling #4968 1:1000) or P53 antibody (mouse antibody, kind gift from Alyson W. MacInnes 1:1) in TBST with 3% BSA (Sigma) overnight at 4°C. Signal was detected using a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies and the enhanced chemiluminescence method (Amersham).

RNA isolation & transcriptome analysis

Morpholino injected embryos of high oblong stage where snap-freezed in liquid nitrogen (without dechorionation) and stored in -80°C until all samples were collected. For RNA isolation freezed embryos where homogenized with pestle or with a bullet blender after addition of 300µl Qiazol (Qiagen). 1/5 of chloroform was added to the homogenate, mixed and transferred to phase-lock gel heavy (5-Prime) containing Eppendorf tubes of 1.5ml. The RNA liquid phase (top transparent solution) was separated from the rest of the sample by 15min at 12000 g and purified with rneasy MinElute cleanup kit (Qiagen) according to manufacture protocol. RNA quality was checked with the Agilent 2100 bioanalyzer using eukaryote total RNA pico series II protocol. RNA samples were delivered to the microarray department University Amsterdam www.microarray.nl/ who performed a

two-color microarray-based gene expression analysis with treatment (P38αMO ATG or ERK2MO ATG injected embryos) labelled Cy3 hybridized against control (stCTRMO injected embryos) labelled Cy5 on an Agilent 180k chip. For analysis, data files (MAGE and JPEG files of each hybridized chip position) were imported into Rosetta Resolver 7.2 (Rosetta Inpharmatics LLC). Two biological replicate intensity profiles were combined using the default intensity experiment builder which is implemented in the Rosetta Resolver system. P38α morpholino against standard control morpholino (252823310001_3 and 252823310002_3), ERK2 morpholino against standard control morpholino (252823310001_4 and 252823310002_4)

Q-PCR

50ng of total RNA was used for a 20µl cDNA synthesis reaction with the iScript cDNA synthesis kit (Biorad). Q-PCR reactions were performed in a 25 µl volume comprised of 1 µl cDNA, 12.5 µl of 2× iQ SYBR Green Supermix (Bio-Rad) and 10 pmol of each primer.

Gene	Reference	Forward	Referse
aplnrb	ENSDART00000129643	TCTCAGGAGTTCTTGCTGCAC	CTCACCACCAGGTTGAAGTCC
cxcr4	ENSDART00000080350	TGGCTTATTACGAACACATCGTC	TGACTGACCTCCACATCACAC
dlc	ENSDART00000018514	AACCCATCTGCTTGTCTGGC	GCGTGCACTCATCACAGAGG
nav3	ENSDART00000005847	GATTGAGAATGTGGAGTGCTGTC	TCCCATTACACACCTCTTCAGC
rpsa	ENSDART00000135945	CATTGAGGCTCCTGGCAAG	GAGCAGCTGACCAATCCTCAG
map7d1	ENSDART00000090587	AAGGAGCGGTATGAATCTGCC	CAAACTGCGGGTCTCTCTGC
adrm1b	ENSDART00000081427	TGTCGCAAAGTTAACGAGTACC	AGCTGCATGAGTTGATTGTG
sall1a	ENSDART00000111842	CTCTGATGACAACATGGAAGG	TGTCTTGAGATGCATCAACTG
krt18	ENSDART00000020750	AGCTGCGCAAGAAGATCTTTG	TCTGAAGTCATCAGCTGCCAG
zgc:136359	ENSDART00000077341	AGTTGATGGATGAGATCTGGC	CAGTAGTGGCAGCTCAATCAG
rps8a	ENSDART00000078412	CAGCAAACACCAAGATTGG	ATGAGAAGTTTCCCACATCG
rpl13	ENSDART00000047391	AGTACTGAGGAGGAGCTCAAG	GCCTTCTCTTTCTTGTGCAC
rpl19	ENSDART00000018975	ACATGTACCACAGCCTGTATCTG	GATCAGCCAAGAGCTTCTTG
rpl35	ENSDART00000009241	ACAGTCATCAACCAGACACAG	AGGCTTGTACTTCTTACCCTTG

Cycling parameters were 94°C for 3 min to activate the polymerase, followed by 40 cycles of 94°C for 15 sec and 59°C for 45 sec. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers were amplified. All reactions were done in duplicate or triplicate. Data were normalized using the Genex macro provided by Bio-Rad.

Camptothecin treatment

Embryos were injected with, pronase dechorionated and washed with 1X Danieau buffer within 1hpf. At 8 cell stage (+/- 1hpf) embryos were transferred to agarose coated plates and treated with either 500nM CPT or DMSO for 8hours at 31°C. After that total protein was isolated as described above.

Results

Knock down of P38α causes blastula stage arrest and prevents epiboly initiation.

To study the function of P38αA in the first stages of zebrafish development the expression was blocked, by injection of a translation blocking morpholino (MO1) in zygote stage embryos. Development of (injected) embryos was followed with time lapse imaging starting from 256cell stage **(Fig1a)**. At 6hpf uninjected or control morpholino (stCTRMO) injected embryos progressed normally to shield stage. However, more than 80% of P38αA knockdown embryos arrested at blastula stage, and did not undergo epiboly resulting in embryonic lethality a few hours later. This phenotype was confirmed by a splice blocking morpholino (MO2) against P38αA **(Fig1b)**. Depletion of P38αA appeared to be more efficient with the translational blocking morpholino when compared to the P38αA splice blocking morpholino, an effect that is most likely due to the abundant maternally contributed spliced P38αA mRNA, that has been demonstrated previously [34]. Since knock-down of Erk2 also resulted in epiboly arrest we questioned whether the phenotypes of both morphants were identical and performed the time course experiment as well with Erk2 morphants. In uninjected wild type embryos epiboly started at 06:10 hpf (24°C) and the embryos remained viable and developed further until the time lapse measurement was terminated at 16:40 hpf. Embryos injected with P38α translational blocking morpholino (MO1) developed until 8:50 hpf when the animal pole started to show excessive movements in contrast to the not moving margin and eventually resulted in the burst of the yolk and death of the embryo. Two different phenotypes were distinguished among the Erk2 epiboly arrested morphants. The first phenotype was almost similar to the P38α morphants and embryos died by a yolk burst due to failed migration of marginal cells **(Fig1a ERK2MO upper panel).** However, the deep cells of the animal pole in the Erk2 morphant curled through excessive movements and partly dissociated from the enveloping layer (EVL). In addition, the animal pole of Erk2 morphant took on a dome shape which indicates epiboly initiation. The second Erk2 morphant phenotype was much more severe since embryos died at 7:00 hpf because the cells of the animal pole started to dissociate **(Fig1a ERK2MO lower)**. Western blotting with anti-Erk1/2 and anti-P38 antibodies confirmed that P38α morpholino1 injected embryos expressed lower levels of P38 protein compared to stCTR morpholino. Erk2 morphants contained reduced Erk1/2 levels, however they also contained reduced P38 levels, possibly explaining the observed more severe phenotype in ERK2 depleted embryos **(Fig1c)**.

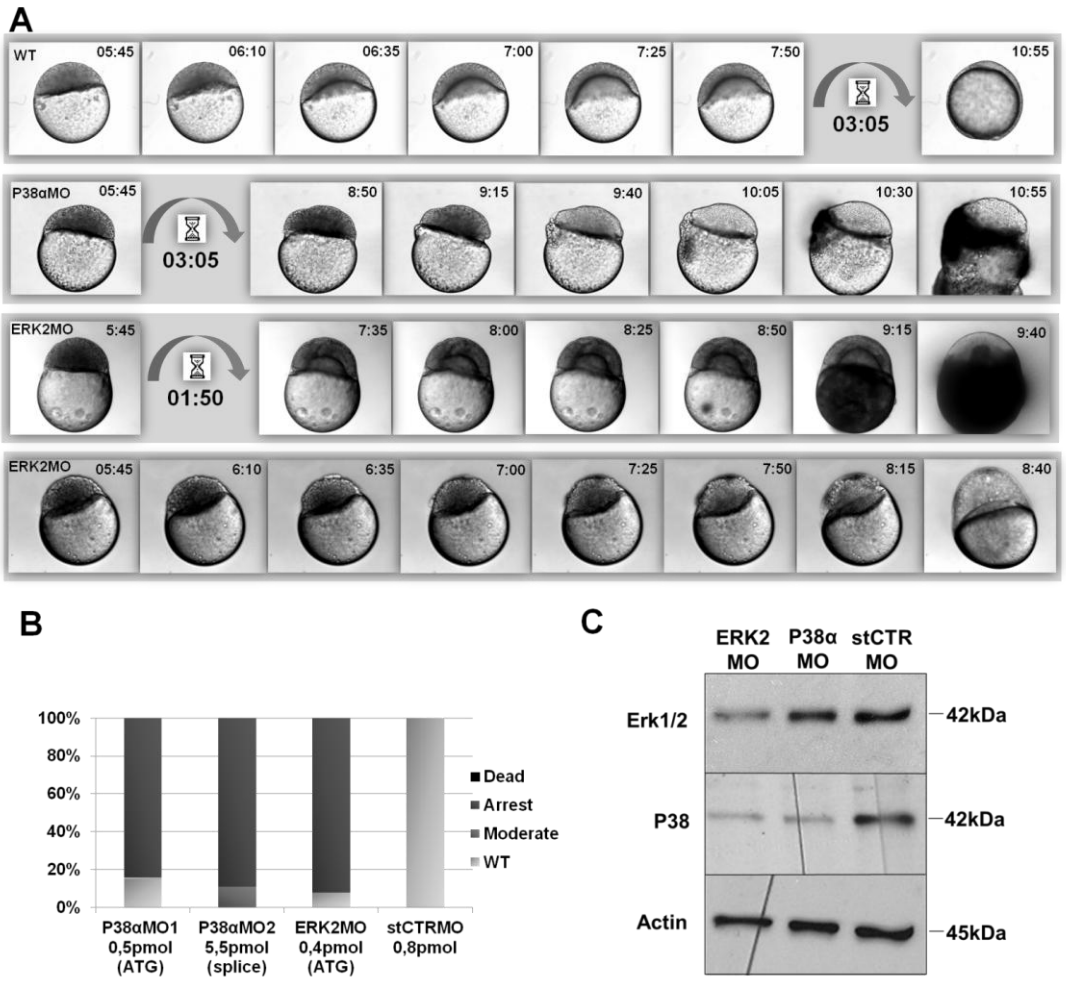


Fig1: Time lapse imaging of P38α and ERK2 morphants. P38α morphants, Erk2 morphants and WT embryos are imaged every 5 minutes starting at 256 cell stage (3hpf). Time lapse was performed with DIC microscopy using Zeiss EC Plan-Neofluar 10×/0.30 objective and extended for 16 hours and 40 minutes. **A** Fragment images of time lapse showing epiboly stage starting at 06:10hpf in WT embryos but not morpholino injected embryos. ERK2MO embryos died by a yolk burst (**upper panel**) or dissociation of the blastoderm (**lower panel**). **B** a minimum of 30 embryos for each condition was used for morpholino injections at 1-cell stage. Phenotypes were scored at 6hpf were WT embryos are developed to shield stage. Injections are performed with an ATG or a splice blocking morpholino against P38α, an Erk2 morpholino and the standard control morpholino was used as a control. **C** Western blotting with anti-Erk2 and anti-P38α antibodies on protein samples from P38αMO (ATG) and ERK2MO injected embryos prepared at high/oblong stage.

Transcriptome comparison of high/oblong stage Erk2 and P38α knock-down embryos.

To compare the effects of Erk2 and P38α knock-down in the first hours of zebrafish development, transcriptome analysis was performed of (injected) embryos from high/oblong stage using a custom designed 180k Agilent chip. With a threshold of a twofold change relative to control morpholino (stCTR) injected embryos, a total of 454 up-regulated and 455 down-regulated genes were selected as a result of P38α knock down, whereas Erk2 morphants displayed 964 up-regulated and 418 down-regulated genes (Fig2a). The Erk2 morpholino and P38α morpholino common gene pool of 229 genes shows almost equal number of genes up-regulated (114) versus down-regulated (115). Finally, we selected 1153 and 680 genes that were specifically affected by either Erk2 or P38α knock down, respectively. The P38α morpholino specific target has an equal number of up-regulated (340) and down-regulated (340) genes. However, from the Erk2 morpholino specific set of 1153 genes the largest pool of 850 genes were up-regulated and the rest of 340 genes were down-regulated. The percentage gene frequency of different fold change

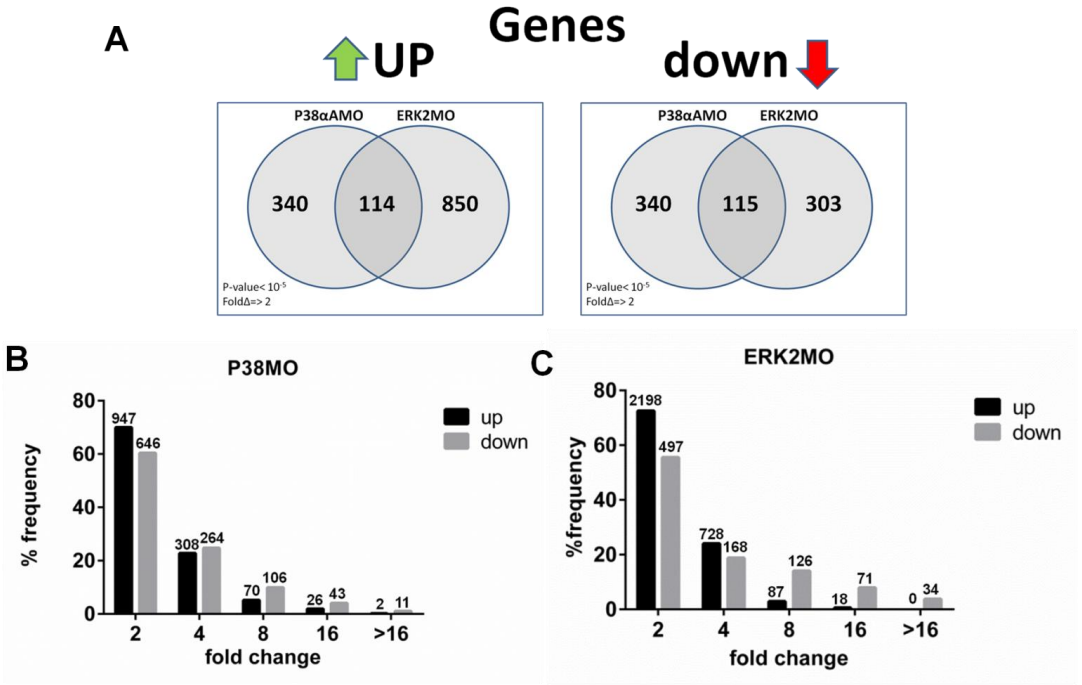
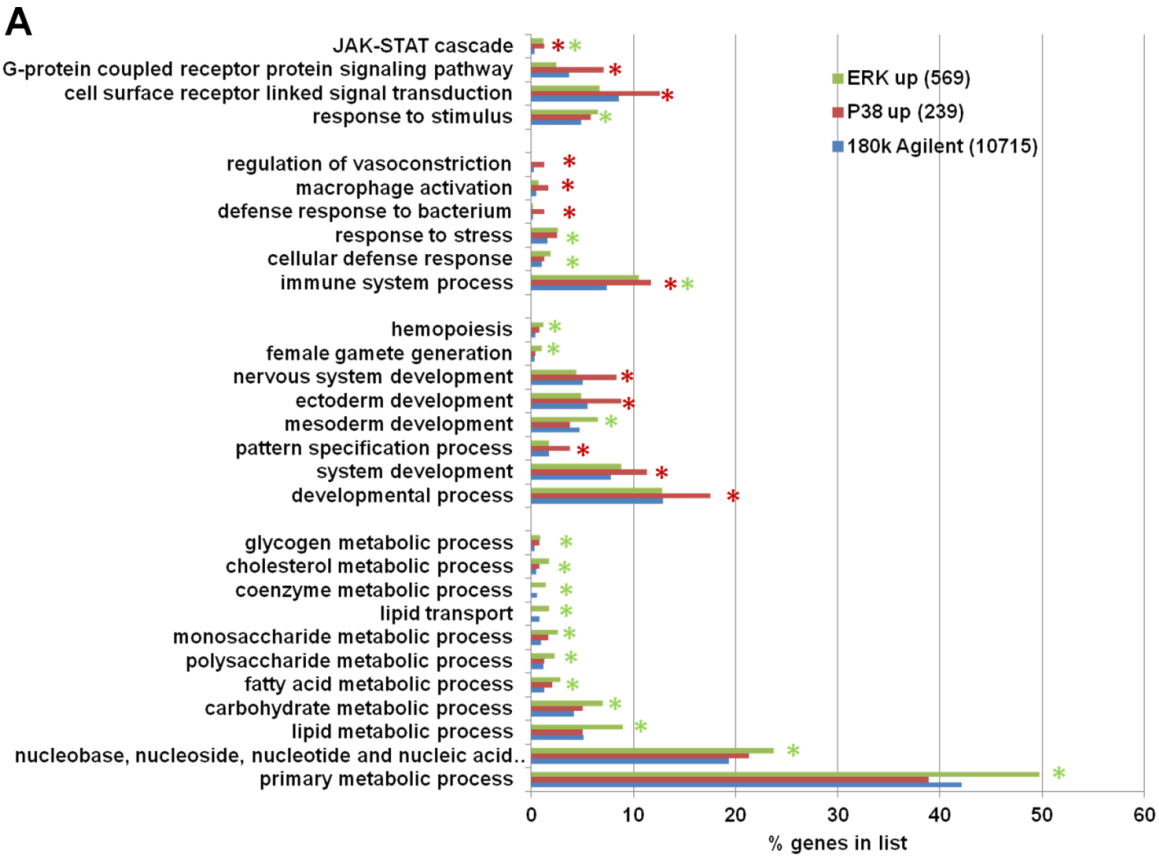


Fig2: Erk2 and P38α knock-down transcriptome analysis at high/oblong stage. A Venn diagrams of the number of genes up- or down- regulated by Erk2 and P38α translational blocking morpholinos with absolute fold change of 2 and p-value<10^{-5}. **B** Histogram of % frequency P38MO effected genes (y-axis) within a fold-change margin (x-axis) and p-value<10^{-5}. Numbers on bars represent absolute numbers of genes. Transcriptome analysis results of two independent experiments each with a pool of five embryos. **C** Histogram of ERK2MO transcriptome.

thresholds for both Erk2 and P38α knock-down were plotted. For both Erk2 and P38α knock-down significant sets of gene targets could be distinguished with fold change thresholds as high as 8 (Fig2 b and c). Interestingly, higher threshold settings resulted in more down-regulated gene expression levels than up-regulated for both Erk2 and P38α knock-down.

To address the biological processes represented by the Erk2 and P38α knockdown transcriptomes, gene ontology enrichment analysis was performed with total up-regulated and down-regulated gene sets (Fig3). 258 out of 455 P38α down-regulated genes were assigned to the gene ontology classification (GO). For Erk2 knock-down 218 out of 418 could be analyzed with GO enrichment analysis. Compared to the 180k Agilent chip both Erk2 and P38α down-regulated transcriptomes have overrepresentation of genes involved in processes of early development such as mesoderm/ectoderm development and pattern/system specification. Embryonic patterning is regulated by morphogens such as Fgf, Wnt and the Tgf-β family members Nodal and Bmp. We found various morphogen pathway components to be affected by Erk2 or P38α knock-down (**Supplemental tables1-4**). Germ line specification is dependent on positional cues, provided by



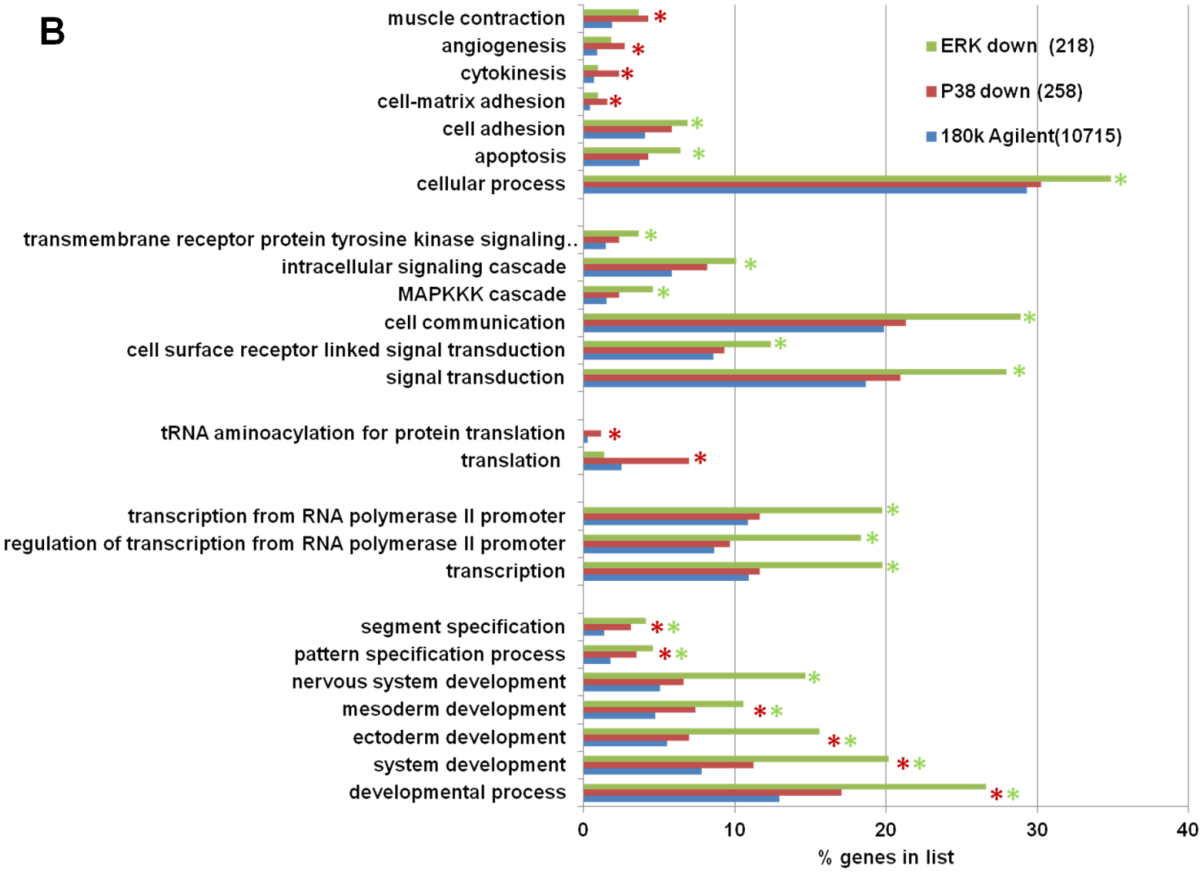


Fig3: Biological processes enriched in Erk2 and P38α knock-down transcriptome data. **A** Bar graph of significantly enriched PANTHER™ GO slim biological processes compared to 180k Agilent array using upregulated gene sets of P38α morphants in red (239/454) and Erk2 morphants in green (569/964). **B** Bar graph of significantly enriched biological processes using downregulated gene sets of P38α morphants in red (258 of 455) and Erk2 morphants in green (218 of 418). * are significant overrepresented in Erk2 morphants and red * significantly overrepresented in P38α morphants. Comparison is made of %genes of the GO category in gene list of experimental condition compared to the %genes of the category in the 180k Agilent chip. Gene ontology enrichment analysis was performed with Panther software www.pantherdb.org using statistical overrepresentation function without Bonferroni correction. Only significant overrepresented biological processes in Erk2 or P38α morphant are shown with p-value ≤ 0.05 calculated with binominal statistics.

morphogens, and the same pathways are involved in regulating cell migration and adhesion properties of blastoderm cells during epiboly. We found also genes directly involved in cell adhesion to be overrepresented in both Erk2 and P38α down-regulated gene pools. Different from Erk2 morpholino, the P38αMO1 down-regulated gene pool contained overrepresentation of genes involved in protein

translation, cytokinesis, angiogenesis and muscle contraction. Erk2 knockdown resulted in the down-regulation of a large set of genes involved in transcription, predominantly transcription factors, and cell signaling cascades including the MAPK pathway.

239 (of 340) P38α morpholino1 up-regulated and 569 (of 850) Erk2 morpholino up-regulated genes were found by GO classification and enrichment analysis showed again overrepresentation of developmental genes (Fig3). Many P38α morpholino up-regulated genes seem to be involved in pattern specification and system development while the genes assigned to the mesoderm differentiation ontology were up-regulated (as well as down-regulated) by Erk2 morpholino.

Identification of common and P38α, Erk2 specific gene targets involved in cell migration and adhesion

Combined Transcriptome profiling and gene ontology analysis of epiboly arrested P38α and Erk2 knock-down embryos revealed drastic deregulation of genes regulating cell migration and adhesion underlying **(supplemental Table 5)**. Interestingly, also a number tissue specific genes expressed in the EVL, YSL or margin were down-

regulated either in both Erk2 and P38α morphants or specifically in Erk2 morphants. Erk2 and P38α morpholinos both down-regulated keratin 4 (Krt4), bloody fingers (Blf), epithelial cell adhesion molecule (Epcam), neuron navigator 3 (Nav3), and up-regulated echinoderm microtubule associated protein like 3 (Eml3) and junction plakoglobin (Jup). Epcam, and Blf are known regulators of epiboly cell movements in zebrafish [35,36]. Transcriptome data suggested that keratin 18 is specifically down-regulated by Erk2 morpholino, however keratin 4 as well as keratin 8 appeared to be dramatically down-regulated by Erk2 as well as P38α morpholino when validated with Q-pcr **(Fig4a)**. Down-regulation of Nav3, zgc136359 and Ctsh by P38α and Erk2 knock-down was also confirmed with quantitative rtPCR. In addition transcriptome data suggest that the aryl hydrocarbon receptor nuclear translocator 2 (Arnt2) is specifically up-regulated in P38α morphants which is known to regulate cell-cell adhesion during gastrulation [37]. Six previously identified epiboly critical regulators were present in the Erk2 morpholino transcriptome including apelin receptor b (Aplnrb), c-x-c chemokine receptor 4 (Cxcr4), snail2 (Snai2), ras homolog gene family member ab (Rhoab) and fyn oncogene related to src (Fyna) (down-regulated) and interferon regulatory factor 6 (Irf6) (up-regulated) [38-43]. Dlc, Cxcr4a, Aplnrb and Rpsa were indeed specifically down-regulated by Erk2 morpholino as validated with Q-pcr **(Fig4b).** Down-regulation of two P38α morphoplino specific cell adhesion regulating genes Map7d1/Zgc;172238 and Adrm1b were validated which have a yet unknown function during development **(Fig4c)**.

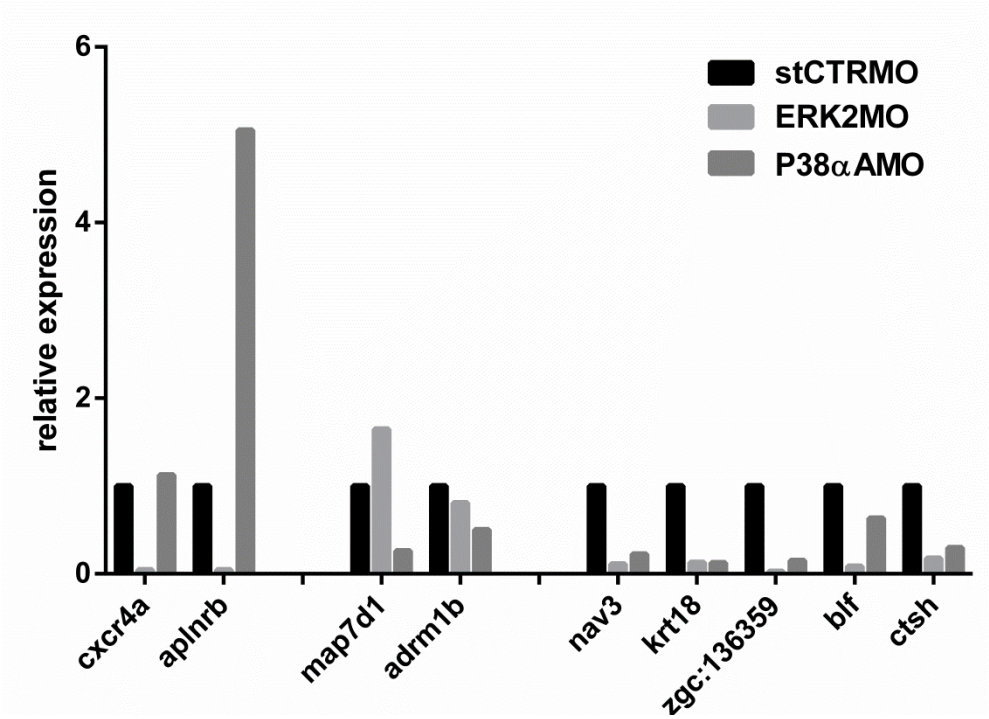


Fig4: Quantitative PCR of Erk2MO downregulated genes, P38αMO downregulated genes and common regulated genes. Graph depicts fold changes relative to stCTRMO. Results are confirmed with a second independent experiment**.**

P38α induces expression of ribosomal proteins, inhibits expression of histone proteins and maintains P53 protein levels after DNA damage in the gastrulating embryo.

The transcriptome data of P38α knock down embryos from high/oblong stage includes a significant number of down-regulated genes with a function in biological protein synthesis. The majority of the genes listed in supplemental **Table 6** are ribosomal proteins of which Rps8, Rpl13, Rpl19 and Rpl35 are known to have a function in nuclear stress signaling when not assembled into the ribosome [44,45]. Ribosomal proteins maintain cellular homeostasis most likely by inducing levels of P53 through binding to the P53-inhibitor MDM2 during stress. In zebrafish, Rps8, Rpl13, Rpl19 or Rpl35 recessive mutations are associated with growth impairment and tumor predisposition in zebrafish [46,47]. We identified several cancer associated ribosomal proteins downstream of P38α with transcriptome analysis followed by Q-pcr of morpholino knock-down embryos (**Fig5a)**. In addition, we found a significant pool of histone protein genes being highly up-regulated. For example histone 3.2 was 16 fold higher expressed in P38α knock-down embryos (**supplemental Table 7**). Histone protein levels are tightly controlled during cell

cycle progression since elevated histone protein levels is essential for proliferation however an excess can cause

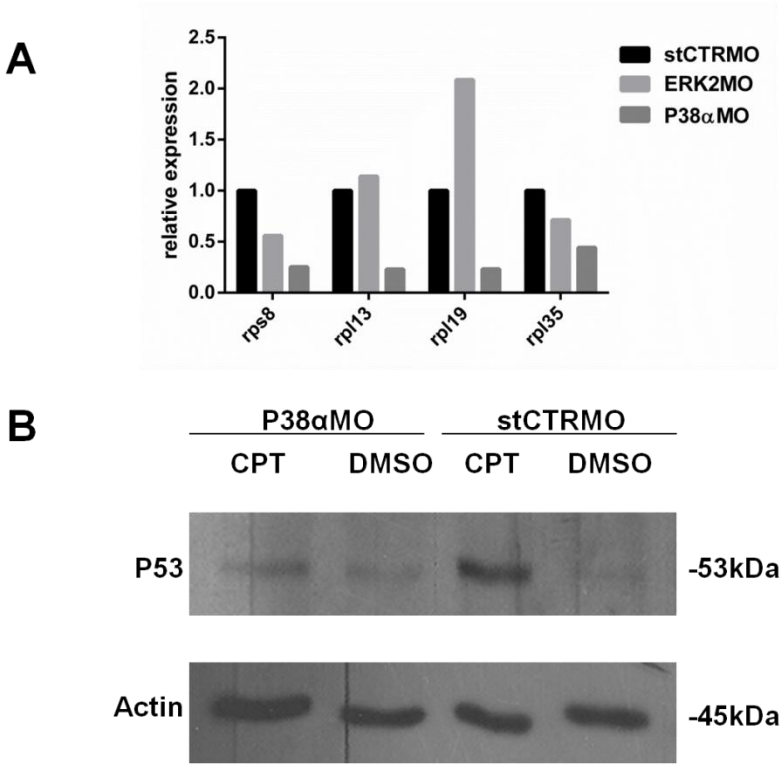


Fig5: P38α regulates expression of ribosomal proteins and maintains P53 protein levels after DNA damage during early zebrafish development. **A** Graph of quantitative real time PCR results with rps8, rpl13, rpl19 and rpl35 fold changes in Erk2 and P38 morphants compared to stCTR morpholino injected. Results are confirmed twice with independant biological experiments **B** Western blotting with ant-P53 and anti-actin antibody on protein samples from P38αMO1 or stCTRMO injected embryos treated for 7hours with either 500nM DMSO or Camptothecin.

genomic instability in the form of excessive chromosome loss, enhanced sensitivity to DNA damaging agents and cytotoxicity [48,49]. Furthermore, P38α and P53 are both stress induced proteins and functional in the same DNA-damage induced stress signaling pathway since P38α directly phosphorylates and activates P53 at Ser33 and Ser46 residues[50]. Interestingly in this report we were able to detect ribosomal protein down-regulation and histone protein up-regulation, stress associated cellular events, by knock-down of basal levels of P38α. Since we obtained these results from an early embryonic stage, were the presence of the P53 protein is questionable, we investigated the presence of P53 protein with western

blotting. We used an anti-P53 antibody in the gastrulating embryo with and without the DNA damage inducing agent camptothecin (CPT) (**Fig5b)**. P53 was indeed undetectable at 8 hpf after 7 hour treatment with DMSO, however, the level was induced by CPT in stCTRMO injected embryos. Importantly, P53 induction by CPT was reduced in P38α morphants suggesting the function of P38 in regulation of P53 protein stability during stress.

Discussion

The P38α and ERK2 MAPKs are crucial factors that regulate the first stages of vertebrate development. Knock down of either of the two MAPKs arrests embryonic development in zebrafish before the onset of epiboly. We performed transcriptome analysis of high/oblong stage P38α and ERK2 knockdown embryos to identify distinct and overlapping roles for Erk2 and P38α at this early stage of development.

Our results are in line of previous studies published by *Holloway et al.,* who discovered a role for P38α in regulation of the marginal actin ring constriction in embryos undergoing epiboly by injecting dominant negative P38α RNA [23]. Additionally, morpholino knockdown of P38α totally abolished initiation of epiboly in this study. Time-lapse imaging revealed that P38α morpholino inhibits migration of marginal cells of the blastula at a stage before 50% epiboly when the F-actin ring is normally established [51]. Previously we reported the indispensability of ERK2 for zebrafish epiboly. In this study, we elaborate on these findings by directly comparing the knock-down phenotypes of P38α and ERK2, using time-lapse imaging and transcriptome profiling. Live imaging showed that either Erk2 or P38α knock-down induced epiboly arrest due to failed migration of marginal cells. In contrast, the deep cells remained to increase in motility and protruding forces against EVL and margin resulting in lethality of embryo (see movie4 ERK2MO). The Erk2 morpholino is more potent to block epiboly, than the P38α morpholino, and injection can result in failure of EVL to maintain embryonic cell integrity and finally lead to total dissociation of the blastoderm. Therefore knock-down of either Erk2 or P38α results in failed epiboly initiation, however the phenotypes are not completely identical. In addition, western blot analysis showed lower level of Erk2 and P38α protein in Erk2 morpholino treated embryos what can account for more severe but not completely Erk2 specific phenotype of these morphants. However, it is also possible that ERK knock-down indirectly effects P38 levels.

Specific gene pools for Erk2 and P38α depleted embryos are found, which are at least three times larger than the common pool which is similarly affected by Erk2 and P38α knock-down. A number of genes that can be associated with cell migration are found in the common pool (**supplemental Table 5**) of which Blf and

Epcam are previously found to be essential for a proper progression of epiboly movements[35,36]. Keratin 18 is down-regulated specifically by Erk2 according to the transcriptome analysis, however with Q-pcr we found that the expression of this gene is also down-regulated by P38α morpholino, but less drastically. Keratin 4 and keratin 18 are intermediate filaments that are part of the cytoskeleton of the EVL [52]. Down-regulation of these EVL markers suggest that the EVL does not differentiate properly. As a consequence the blastoderm of Erk2 morphants dissociates, most likely through impaired cytoskeleton of the EVL and loss of cell adhesion. Within the Erk2 morpholino specific gene pool we found additional epiboly related genes such as Aplnrb, Cxcr4, Snail2, Rhoab, Fyna and Irf6. The p38α specific down-regulated gene set contains a pool of adhesion and cell migration functional genes, however only one that was previously found to be essential for epiboly. Arnt2 knock-down caused mild defects of the blastoderm during epiboly and we found it to be up-regulated in P38α morphants. Some P38α targets are conserved in mammals, also humans, of which the function is still largely unresolved. These targets such as si:dkey-30c15.12, a cadherin domain containing protein, and Map7d1, a microtubule association domain containing protein, are interesting candidates for further expression and functional studies in zebrafish. Functional studies of the adhesion regulating molecule 1 (Adrm1) have also not yet been performed in zebrafish. However *in vitro* studies have shown that adrm1 is a functional ubiquitin receptor of the proteasome complex, is inducible by interferon-γ and increases adhesion to endothelial cells when overexpressed in macrophages [53,54]. Therefore genes such as Adrm1 and Map7d1 can be studied further, with morpholino knock-down, to investigate involvement in epiboly cell movements. Theoretically it is possible that P38α exerts its function in zebrafish epiboly primarily by regulating cytosolic proteins rather than controlling gene expression. In smooth muscle and endothelial cells the P38 substrate Mapkapk2 regulates activity of heat shock protein 27 (Hsp27) an F-actin polymerization modulator [55,56].

Gene ontology analysis of P38α morpholino down-regulated gene set revealed specific enrichment of genes characterized with the GO-term ‘protein translation’. The protein translation gene ontology category consists of genes predominantly coding for ribosomal proteins (RP). A possible link of P38α and RP gene expression can be found in P53 regulation during stress signaling. Both RP and P38α have a positive effect on P53 stability and are perhaps involved in a similar mechanism where stress induced P38α activity maintains RP excess [57]. RP are in excess when they exceed rRNA levels and cannot be assembled into the 40s or 60s ribosome subunits. Unbound RP have the capacity to bind Mdm2 which, in absence of stress, binds and targets P53 to the proteasome complex for degradation [44].

In line with this hypothesis, a second mechanism appeared from the transcriptome data involving suppression of histone proteins by P38α. This suggests that P38α

exerts its function in the cell cycle not only by controlling checkpoints, through P53 and RP, but also cell cycle progression by regulating expression of histone proteins. Interestingly, our transcriptome analysis reveals that the neuroepithelial cell transforming gene 1(net1) is 25 fold down-regulated in Erk2 morphants. Net1 binds RNA polymerase 1 directly and stimulates the synthesis of rRNA in yeast [58]. Therefore, Erk2 and P38α MAPK activities may form a mechanism to maintain cellular homeostasis by having opposing roles in controlling the ribosome assembly and this mechanism is established already in the first stages of development.

We have not detected P53 protein in DMSO treated stCTR morpholino injected embryos of 8hpf. Furthermore, P53 was drastically induced during initial stages of epiboly with the DNA damage, causing agent CPT in stCTR morpholino injected embryos but not in P38α morphants. These results suggest that P38α regulates P53 stability and stress associated ribosomal protein expression in the zebrafish embryo. Further study investigating the association between P38α activity, P53 stability, ribosomal protein expression and histone protein expression would be valuable to gain more understanding about the role of P38α in tumor formation.

Conclusion

Depletion of P38α prevents epiboly initiation and blocks further development of the embryo. With the help of transcriptome profiling, we found that the expression of multiple cell migration and adhesion functional genes to be disregulated, along with components of Nodal, Fgf, Wnt and Bmp morphogen pathways, which have a critical role ininterplay to regulate migratory properties of embryonic cells for developmental patterning. Many of these P38α target genes are also affected by Erk2 morpholino despite the fact that much larger gene sets do not overlap between the two conditions. Interestingly, P38α knock-down results in down-regulation of a large pool of ribosomal proteins while Erk2 Morpholino specifically down-regulates net1 a protein also involved in ribosome biogenesis. These results suggest that, in zebrafish, both ERK2 and P38α are crucial for epiboly initiation, but despite similar phenotypic outcomes have separate roles in transcriptional regulation. This is illustrated by the involvement of both ERK2 and p38 in maintenance of cellular homeostasis during early zebrafish development, however by playing seemingly opposing roles in ribosome biogenesis.

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Supplemental tables 1-7

Nodal								
Gene name	Ensemble	nr probes	P38αMO Δ fold	P38αMO p-value		ERK2MO Δ fold	ERK2MO p-value	
smad1	ENSDART00000033566	2	5,3	2,1E-04	↑	4,2	9,9E-13	↑
ndr2	ENSDART00000058969	1	-2,7	4E-17	↓	1,3	0,2721	X
Jun	ENSDART00000063912	1	2,3	3,0E-07	↑	-1,1	8,0E-01	X
JunB	ENSDART00000109640	6	1,1	8,4E-01	X	3,3	7,3E-43	↑
bambi	ENSDART00000077732	1	-1,2	1,0E-04	X	-2,1	3,8E-12	↓
oep	ENSDART00000042441	1	1,6	1,0E-05	X	1,9	2,0E-12	↑
eng2a/Dharma	ENSDART00000031700	4	1,5	6,2E-01	X	9,2	2,8E-06	↑
stat3	ENSDART00000080854	1	1,4	1,3E-01	X	2,7	2,5E-17	↑

sTable 1

WNT								
Gene name	Ensemble	nr probes	P38αMO Δ fold	P38αMO p-value		ERK2MO Δ fold	ERK2MO p-value	
dia1	ENSDART00000015374	4	3,6	0,0E+00	↑	3,1	1,1E-40	↑
drl	ENSDART00000097364	7	-3,7	0,0E+00	↓	-11,6	1,5E-20	↓
wnt2	ENSDART00000060259	2	2,6	3,2E-11	↑	1,9	4,3E-02	X
fzd8a	ENSDART00000066835	1	2,3	1,9E-07	↑	-1,1	8,3E-01	X
fzd1	ENSDART00000060789	1	2,6	1,3E-07	↑	1,2	4,0E-01	X
fzd9	ENSDART00000024087	2	2,6	4,5E-11	↑	1,4	2,7E-04	X
limk2	ENSDART00000020532	1	-2,8	8,1E-14	↓	-1,1	6,6E-01	X
tcf4	ENSDART00000009237	1	-3,9	1,6E-06	↓	-2,7	2,5E-04	X
sfrp2	ENSDART00000102498	1	4,6	4,0E-08	↑	1,6	2,1E-01	X
ctbp2	ENSDART00000114215	1	4,4	1,3E-10	↑	-2,0	1,3E-01	X
wnt11r	ENSDART00000099880	1	-1,2	2,9E-01	X	-2,1	3,2E-09	↓
wnt3l	ENSDART00000098394	2	-1,2	7,6E-01	X	3,7	2,8E-07	↑
fzd4	ENSDART00000112320	1	1,2	4,3E-01	X	-3,8	1,8E-07	↓
has2	ENSDART00000053754	2	1,1	5,9E-01	X	-8,0	4,6E-09	↓
stat3	ENSDART00000080854	1	1,4	1,3E-01	X	2,7	2,5E-17	↑
Stat5.2	ENSDART00000077952	1	2,1	5,0E-05	X	2,3	6,4E-09	↑
dact1	ENSDART00000058001	3	1,0	9,2E-01	X	-13,7	0,0E+00	↓
gsk3b	ENSDART00000018228	2	1,2	2,3E-01	X	2,6	3,3E-07	↑

sTable 2

BMP								
			P38αMO			ERK2MO		
Gene name	Ensemble	nr probes	Δ fold	p-value		Δ fold	p-value	
smad1	ENSDART00000033566	2	5,3	2,1E-04	↑	4,2	9,9E-13	↑
gata3	ENSDART00000025153	5	-2,6	1,4E-06	↓	-21,9	1,6E-40	↓
vent	ENSDART00000017456	1	4,2	1,2E-09	↑	-2,9	1,5E-29	↓
noggin 1	ENSDART00000081766	4	1,3	4,5E-04	X	-3,1	9,5E-11	↓
nog2	ENSDART00000063231	1	-2,9	1,2E-01	X	3,3	8,5E-06	↑
chd	ENSDART00000045110	1	-1,9	8,0E-05	X	-2,6	1,0E-05	↓
fst	ENSDART00000051065	3	3,1	6,0E-05	X	5,0	1,3E-06	↑
fstl1	ENSDART00000081824	1	-1,2	7,0E-01	X	6,7	4,4E-06	↑
bmp2b	ENSDART00000100482	3	1,6	6,2E-03	X	-6,5	1,4E-09	↓
bmp7b	ENSDART00000092214	1	2,2	1,7E-02	X	3,8	1,0E-10	↑
bmper	ENSDART00000097348	3	-1,1	7,9E-01	X	-9,1	0,0E+00	↓
admp	ENSDART00000037557	3	-1,2	1,2E-01	X	-10,5	3,0E-15	↓
bambi	ENSDART00000077732	2	-1,2	1,4E-01	X	-2,2	1,7E-18	↓
snai2	ENSDART00000058571	3	-1,7	2,0E-04	X	-3,0	4,8E-08	↓
dlc	ENSDART00000018514	3	1,3	5,2E-01	X	-15,0	4,0E-43	↓
id3	ENSDART00000077087	2	1,1	5,7E-01	X	-2,6	1,1E-11	↓
gata2a	ENSDART00000082420	1	-1,2	3,5E-01	X	-6,7	5,4E-11	↓
otul	ENSDART00000064320	2	1,5	3,0E-05	X	3,6	9,7E-16	↑

sTable 3

FGF								
Gene name	Ensemble	nr probes	P38αMO			ERK2MO		
			Δ fold	p-value		Δ fold	p-value	
mych	ENSDART00000115049	1	2,4	2,2E-20	↑	-1,2	1,7E-01	x
mych	ENSDART00000115049	1	1,1	6,2E-01	x	-4,6	6,2E-21	↓
RALA	ENSDART00000027587	4	-3,1	3,1E-09	↓	-6,5	8,6E-13	↓
DUSP14	ENSDART00000080338	2	3,2	1,2E-06	↑	4,1	5,5E-10	↑
RASSF4	ENSDART00000100803	1	2,0	8,5E-06	↑	2,2	9,1E-40	↑
hrasl	ENSDART00000015040	1	-2,4	7,1E-20	↓	-1,9	4,2E-17	↓
fgf4	ENSDART00000108971	2	-2,8	3,7E-06	↓	-34,1	0,0E+00	↓
MAPK organizer 1	ENSDART00000109873	2	2,0	1,9E-25	↑	1,3	4,9E-04	x
mapk12	ENSDART00000018502	1	3,9	8,0E-10	↑	1,6	1,7E-01	x
mapk14a	ENSDART00000040362	1	-2,2	3,2E-37	↓	-1,5	4,6E-08	x
mapksp1	ENSDART00000079669	4	2,5	4,3E-11	↑	1,3	9,8E-02	x
rasgef1bb	ENSDART00000064968	1	-2,1	1,6E-12	↓	1,4	2,7E-04	x
a-raf	ENSDART00000076687	1	5,9	2,3E-08	↑	2,9	1,5E-03	x
b-raf	ENSDART00000023894	1	-4,7	1,1E-27	↓	-2,2	7,0E-05	x
SPRY2	ENSDART00000052423	2	4,7	2,9E-06	↑	1,2	7,1E-01	x
Spry4	ENSDART00000099528	4	-3,0	1,1E-08	↓	-2,6	1,1E-02	x
eef2k	ENSDART00000052001	1	-3,6	1,2E-06	↓	-2,7	1,3E-02	x
cFOS-like	ENSDART00000043298	1	3,4	6,8E-11	↑	1,5	1,8E-01	x
c-jun	ENSDART00000063912	1	2,3	3,0E-07	↑	-1,1	8,0E-01	x
Jun dimerization protein	ENSDART00000014166	3	-2,1	4,1E-09	↓	1,1	6,6E-01	x
CRTC1	ENSDART00000113356	1	-2,2	1,0E-16	↓	1,1	5,3E-01	x
pea3	ENSDART00000013033	1	3,9	3,2E-07	↑	2,1	2,5E-02	x
fgf17	ENSDART00000029630	4	-1,4	1,3E-01	x	-44,3	1,1E-25	↓
fgf3	ENSDART00000111524	2	1,2	5,3E-01	x	-8,5	1,3E-08	↓
fgfr1b	ENSDART00000004803	1	-1,2	6,6E-01	x	-2,7	6,0E-31	↓
fgfr4	ENSDART00000100286	3	-1,7	6,3E-02	x	-43,6	1,1E-25	↓
12b6 ras-like	ENSDART00000015755	2	-1,2	7,8E-02	x	-2,3	1,0E-05	↓
rasl11b	ENSDART00000015755	1	-1,2	4,5E-02	x	-2,4	5,3E-06	↓
rasa1	ENSDART00000051518	1	1,5	1,8E-08	x	2,2	6,5E-15	↑
spry2	ENSDART00000114948	1	1,3	3,9E-01	x	2,0	4,0E-26	↑
dusp1	ENSDART00000014515	1	1,5	6,8E-07	x	2,1	4,4E-07	↑
DUSP6	ENSDART00000104496	16	-1,4	1,4E-02	x	-89,5	0,0E+00	↓
mycn	ENSDART00000024104	6	1,4	2,4E-01	x	-8,6	1,2E-20	↓
bad	ENSDART00000077219	1	1,6	5,4E-12	x	2,0	8,6E-07	↑
srf	ENSDART00000076035	1	1,0	9,9E-01	x	2,2	3,0E-06	↑
JunB	ENSDART00000109640	6	1,1	8,4E-01	x	3,3	7,3E-43	↑
ATF4	ENSDART00000055609	3	-1,5	4,5E-04	x	-3,3	2,2E-06	↓

sTable 4

Cell migration								
			P38αMO			ERK2MO		
	Ensemble	nr probes	Δ fold	p-value		Δ fold	p-value	
nav3	ENSDART00000036886	1	-5,8	0,0E+00	↓	-5,9	0,0E+00	↓
blf	ENSDART00000063318	2	-2,1	1,4E-30	↓	-12,8	0,0E+00	↓
EpCAM/ zgc:110304	ENSDART00000110637	2	-100,8	0,0E+00	↓	-58,8	3,5E-33	↓
krt4	ENSDART00000012644	2	-2,6	5,7E-10	↓	-8,7	4,2E-45	↓
ncam1	ENSDART00000017229	1	-2,4	2,6E-09	↓	-2,7	3,4E-08	↓
ncam2	ENSDART00000100681	1	-2,4	4,2E-27	↓	-3,5	3,4E-13	↓
net1	ENSDART00000049066	6	-2,4	3,9E-06	↓	-13,3	3,3E-09	↓
zic3	ENSDART00000105761	3	-6,7	6,1E-06	↓	-64,1	0,0E+00	↓
EML3	ENSDART00000080834	1	2,1	2,5E-22	↑	3,0	2,1E-21	↑
arpc5b	ENSDART00000010329	5	2,2	1,2E-15	↑	2,7	1,5E-10	↑
tgfb2	ENSDART00000030271	1	2,2	3,1E-07	↑	3,7	6,9E-21	↑
jup	ENSDART00000003891	1	2,3	1,8E-15	↑	2,6	2,8E-24	↑
stka	ENSDART00000054823	3	-2,4	2,9E-36	↓	-1,3	2,3E-03	X
Spry4	ENSDART00000099528	3	-3,0	1,1E-08	↓	-2,6	1,1E-02	X
zgc:172238	ENSDART00000090587	9	-7,7	0,0E+00	↓	-2,5	4,7E-02	X
map1a	ENSDART00000067321	1	-3,9	7,8E-07	↓	-2,8	7,5E-03	X
mibp	ENSDART00000114380	2	-2,3	1,9E-11	↓	-1,4	8,5E-03	X
adrm1a	ENSDART00000081433	4	-4,0	7,3E-39	↓	-1,4	3,9E-04	X
adrm1b	ENSDART00000081427	2	-2,2	1,6E-11	↓	1,3	1,1E-02	X
flot2a	ENSDART00000109770	1	-7,5	3,7E-06	↓	-8,9	1,2E-04	X
cldn10	ENSDART00000081900	1	-2,4	5,9E-07	↓	1,4	1,7E-04	X
actn3a	ENSDART00000005682	2	-4,7	0,0E+00	↓	1,7	1,9E-01	X
pcdh2ab1	ENSDART00000081898	1	-2,8	6,3E-08	↓	1,1	2,3E-01	X
rhoj	ENSDART00000002507	1	2,0	7,9E-07	↑	1,8	5,1E-02	X
rnd1l	ENSDART00000109712	1	3,7	8,8E-11	↑	1,3	5,9E-01	X
twf1l	ENSDART00000065006	2	2,8	1,5E-32	↑	1,2	3,2E-01	X
si:dkey-30c15.12	ENSDART00000081386	1	8,4	3,8E-18	↑	3,6	1,7E-02	X
cldna	ENSDART00000102086	2	6,0	5,2E-16	↑	-1,1	7,4E-01	X
arnt2	ENSDART00000031712	1	2,5	2,4E-12	↑	1,3	1,0E-01	X
rhoh	ENSDART00000102646	1	3,1	4,9E-10	↑	1,7	1,4E-01	X
aplnrb	ENSDART00000053264	3	1,2	5,5E-01	X	-17,3	2,2E-20	↓
cxcr4a	ENSDART00000080350	1	1,2	7,8E-02	X	-9,7	3,6E-39	↓
epd	ENSDART00000049895	5	-1,4	3,4E-03	X	-16,8	4,6E-35	↓
delta C	ENSDART00000018514	3	1,3	5,2E-01	X	-15,0	4,0E-43	↓
snail2/slug	ENSDART00000058571	2	-1,7	2,0E-04	X	-3,0	4,8E-08	↓
rhoab	ENSDART00000024009	15	-1,9	6,5E-03	X	-4,0	6,6E-09	↓
krt18	ENSDART00000020750	1	-1,2	6,1E-01	X	-5,3	3,7E-08	↓
rpsa	ENSDART00000114282	4	-1,4	1,2E-02	X	-3,1	5,9E-31	↓
fyna	ENSDART00000046414	1	-1,8	5,8E-06	X	-2,2	1,2E-36	↓
irf6	ENSDART00000063562	1	2,1	1,7E-04	X	2,0	2,7E-14	↑
adam9	ENSDART00000109805	1	1,5	6,2E-06	X	2,2	5,1E-06	↑
limk2	ENSDART00000020532	2	1,8	8,2E-18	X	2,1	3,9E-07	↑
hmmr	ENSDART00000031785	2	1,7	4,0E-06	X	2,3	1,8E-37	↑
clauding	ENSDART00000015547	2	1,5	2,9E-03	X	2,1	9,3E-12	↑

sTable 5

Protein Translation								
Gene symbol	Ensemble	Nr. probes	P38αMO Δ fold	P38αMO p-value		ERK2MO Δ fold	ERK2MO p-value	
rpl3	ENSDART00000011251	2	-2,2	6,4E-25	↓	-1,5	9,5E-03	x
rpL9	ENSDART00000054340	6	-2,3	1,2E-35	↓	-1,7	4,3E-04	x
rpl10	ENSDART00000032744	3	-2,9	1,2E-13	↓	-1,6	1,3E-04	x
rpl13	ENSDART00000047391	1	-2,5	3,1E-06	↓	-1,1	1,9E-01	x
rpl19	ENSDART00000018975	2	-4,4	7,5E-13	↓	-1,3	4,5E-04	x
rpl23a	ENSDART00000003001	3	-3,2	7,5E-11	↓	-1,5	9,5E-02	x
rpl24	ENSDART00000019227	1	-3,2	1,2E-08	↓	-1,4	3,0E-02	x
rpl35	ENSDART00000009241	3	-2,6	1,9E-26	↓	-1,9	1,6E-12	x
rps2	ENSDART00000113070	1	-3,2	1,1E-09	↓	-1,6	6,6E-02	x
rps8	ENSDART00000078412	7	-7,0	2,3E-17	↓	1,4	4,7E-03	x
mrps15	ENSDART00000048727	1	-3,1	1,4E-12	↓	1,0	8,4E-01	x
mrpl24	ENSDART00000010862	2	-2,9	9,1E-18	↓	1,1	7,4E-01	x
mrpl37	ENSDART00000091479	2	-2,1	5,7E-07	↓	-1,1	3,9E-01	x
eif2b2	ENSDART00000060689	3	-2,3	1,7E-07	↓	-1,2	1,1E-01	x
cwc22	ENSDART00000023684	1	-2,0	2,1E-10	↓	-1,0	5,9E-01	x
tyw3	ENSDART00000055737	3	-6,2	4,2E-23	↓	-1,1	3,4E-01	x
yars	ENSDART00000052126	4	-3,6	1,1E-18	↓	1,1	1,3E-01	x
farsa	ENSDART00000033738	4	-2,8	0,0E+00	↓	-1,3	3,8E-02	x
iars	ENSDART00000004423	1	-2,0	1,7E-13	↓	-1,8	5,0E-05	x
hars2	ENSDART00000065566	2	-2,1	6,7E-25	↓	-1,2	7,8E-02	x
pus3	ENSDART00000062722	3	-2,7	6,1E-20	↓	-1,3	9,1E-04	x
NSA2	ENSDART00000021976	3	-5,3	2,9E-08	↓	-1,0	8,8E-01	x
eif3s7	ENSDART00000011052	1	-2,9	1,0E-05	↓	-1,1	3,5E-01	x
eef2k	ENSDART00000052001	1	-3,6	1,2E-06	↓	-2,7	1,3E-02	x
fhla	ENSDART00000105767	1	2,2	1,1E-10	↑	1,1	8,0E-01	x
eef1a1	ENSDART00000075340	1	3,1	6,8E-14	↑	1,7	1,2E-01	x
eif2ak3	ENSDART00000113051	2	1,7	3,6E-04	x	2,7	8,2E-06	↑
eif3h	ENSDART00000110583	1	-1,1	6,7E-01	x	2,5	1,8E-14	↑
eef1b2	ENSDART00000065373	1	-1,5	1,0E-01	x	2,3	6,3E-22	↑
eif4a3	ENSDART00000014887	1	2,2	2,5E-11	↑	2,5	5,8E-07	↑
IGFN1	ENSDART00000114121	1	3,2	1,4E-07	↑	5,8	2,8E-06	↑

sTable 6

Epigenetic modification								
	Ensemble	nr probes	P38αMO			ERK2MO		
			Δ fold	p-value		Δ fold	p-value	
h2afx	ENSDART00000040328	6	7,2	2,3E-25	↑	-3,5	5,7E-36	↓
zgc:163047	ENSDART00000103024	1	11,2	0,0E+00	↑	-2,7	1,2E-07	↓
histone H4-like	ENSDART00000114073	6	5,5	0,0E+00	↑	-2,9	0,0E+00	↓
prmt1	ENSDART00000012630	1	2,0	2,0E-14	↑	2,1	2,7E-22	↑
ppme1	ENSDART00000016219	1	2,4	2,3E-12	↑	2,7	5,7E-12	↑
Histone H3.2	ENSDART00000111408	8	15,9	9,4E-42	↑	-3,6	8,5E-02	x
Histone H2B 1/2	ENSDART00000097813	6	7,0	0,0E+00	↑	-2,5	8,7E-04	x
Histone H2B 1/2	ENSDART00000109316	3	4,2	1,0E-14	↑	-1,3	7,0E-02	x
histone H2a	ENSDART00000109856	1	4,4	3,5E-08	↑	-2,4	5,1E-02	x
histone H3	ENSDART00000115371	1	15,2	0,0E+00	↑	-1,4	5,0E-01	x
sirt5	ENSDART00000112030	3	-2,2	4,0E-06	↓	-1,6	6,9E-03	x
sirt3	ENSDART00000051973	1	-2,1	3,8E-14	↓	-1,1	7,4E-01	x
amt2	ENSDART00000051813	1	2,5	2,4E-12	↑	1,3	1,0E-01	x
gamt	ENSDART00000104360	3	-2,2	9,6E-08	↓	1,0	5,8E-01	x
mri1	ENSDART00000111671	1	3,7	1,0E-05	↑	1,5	1,9E-01	x
mybbp1a	ENSDART00000038767	5	-3,1	1,1E-39	↓	1,3	3,9E-04	x
mych	ENSDART00000115049	2	2,4	2,2E-20	↑	-1,2	1,8E-01	x
rbb4	ENSDART00000048144	4	1,5	3,0E-02	x	4,3	3,0E-38	↑
rbb4l	ENSDART00000008144	1	1,5	3,3E-07	x	2,0	3,5E-28	↑
histh1l	ENSDART00000124705	1	1,1	9,3E-01	x	-2,8	2,8E-11	↓
asf1bb	ENSDART00000064194	6	1,6	1,4E-04	x	3,8	2,6E-17	↑
TGS1	ENSDART00000097868	2	1,7	2,7E-03	x	2,7	3,3E-26	↑
METTL9	ENSDART00000084076	1	1,3	2,3E-01	x	2,2	4,7E-15	↑
kdm2aa	ENSDART00000083127	1	1,5	1,3E-03	x	2,4	7,6E-06	↑
cMyca	ENSDART00000067178	6	1,1	9,2E-01	x	6,3	3,3E-19	↑

sTable 7