Identification of regulators of germ layer morphogenesis using proteomics in zebrafish

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Accepted 8 February 2006
Journal of Cell Science 119, 2073-2083 Published by The Company of Biologists 2006
doi:10.1242/jcs.02928

Summary
During vertebrate gastrulation, a well-orchestrated series of morphogenetic changes leads to the formation of the three germ layers: the ectoderm, mesoderm and endoderm. The analysis of gene expression patterns during gastrulation has been central to the identification of genes involved in germ layer formation. However, many proteins are regulated on a translational or post-translational level and are thus undetectable by gene expression analysis. Therefore, we developed a 2D-gel-based comparative proteomic approach to target proteins involved in germ layer morphogenesis during zebrafish gastrulation. Proteomes of ectodermal and mesendodermal progenitor cells were compared and 35 significantly regulated proteins were identified by mass spectrometry, including several proteins with predicted functions in cytoskeletal organization. A comparison of our proteomic results with data obtained in an accompanying microarray-based gene expression analysis revealed no significant overlap, confirming the complementary nature of proteomics and transcriptomics. The regulation of ezrin2, which was identified based on a reduction in spot intensity in mesendodermal cells, was independently validated. Furthermore, we show that ezrin2 is activated by phosphorylation in mesendodermal cells and is required for proper germ layer morphogenesis. We demonstrate the feasibility of proteomics in zebrafish, concluding that proteomics is a valuable tool for analysis of early development.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/119/10/2073/DC1

Key words: Proteomics, Ezrin, Gastrulation, Zebrafish

Introduction
The systematic analysis of gene transcription patterns during development has been instrumental in the identification of a large number of candidate genes with potential functions in various developmental processes (reviewed by Stanford et al., 2001). By contrast, proteomic screens have very rarely been used to analyze developmental processes (Gong et al., 2004) despite indications that these approaches are complementary to large scale genomics (reviewed by Hebestreit, 2001; Lopez, 1999; Patton, 1999).

Proteomics may be a powerful method to study the downstream effects of signaling pathways on protein modifications. The identification of modifications regulating tissue morphogenesis during development currently poses a major challenge in developmental biology. The first morphogenetic process in the development of most multicellular organisms is gastrulation, during which a seemingly unstructured blastula transforms into a gastrula consisting of distinct germ layers (Stern, 2004). In zebrafish, gastrulation begins with the synchronized ingression of individual mesodermal and endodermal (mesendodermal) progenitor cells at the germ-ring margin, leading to the formation of a bi-layered embryo consisting of epiblast (ectodermal progenitors) and hypoblast (mesendodermal progenitors) (Montero et al., 2005; Warga and Kimmel, 1990). Ingression is followed by progenitor cells of both germ layers converging towards the dorsal side of the gastrula and redistributing along the anterior-posterior axis in a movement commonly named convergent extension (reviewed by Wallingford et al., 2002).

Various forward and reverse genetic approaches have provided insight into the genetic pathways controlling zebrafish germ layer formation (reviewed by Schier, 2001). TGF-β-like Nodal signals play a central role in this process by both inducing mesendodermal cell fates and controlling the cell-autonomous ingestion of mesendodermal progenitors. In addition to Nodals, Wnt, PDGF and JAK/STAT signaling have all been shown to control different aspects of progenitor cell polarization and directed migration, although little is known about the precise molecular and cellular mechanisms by which these signaling pathways function during gastrulation (reviewed by Solnica-Krezel, 2005).

During germ layer formation, progenitor cells undergo pronounced changes in morphology and adhesion, suggesting that both cytoskeletal and cell adhesion proteins are crucial for this process. Wnt signals, for example, are thought to interfere with gastrulation movements by modulating the activity of adhesion molecules including cadherins (Puech et al., 2005; Torres et al., 1996; Ulrich et al., 2005) and by controlling cytoskeletal dynamics through activation of Rho kinase 2 (Rok2), a known regulator of actin-myosin contractility (Marlow et al., 2002). Interestingly, the immediate functions of
Wnts and other signals controlling gastrulation movements appear to be largely independent of changes in gene transcription (reviewed by Veeman et al., 2003), indicating that alternative mechanisms such as protein modifications are involved. However, no systematic approach has yet been undertaken to identify potential target proteins that are translationally regulated or post-translationally modified during gastrulation.

In this study, we have chosen a systematic comparative proteomic approach to identify proteins that are differentially expressed or modified between mesendodermal and ectodermal cells and might have essential roles for germ layer morphogenesis. We isolated 35 such proteins, four of which are likely to have functions in cytoskeletal organization. Comparison of our proteomic data with data obtained from an accompanying microarray-based gene expression screen revealed no significant correlation. This shows that we have identified proteins regulated on a translational or post-translational level that would not have been discovered by gene expression analysis. Finally, the functional analysis of one of the isolated proteins, ezrin2, revealed that ezrin2 is activated by phosphorylation in mesendodermal cells and is crucially required for germ layer morphogenesis during gastrulation. Our findings demonstrate that comparative proteomics in zebrafish represents an effective method to identify candidate proteins with important functions during early development.

### Results

#### Proteomic analysis of ectodermal versus mesendodermal progenitor cells

To identify proteins involved in germ layer formation and morphogenesis, we performed a proteomic analysis of ectodermal versus mesendodermal progenitor cells (Fig. 1). We generated highly enriched pools of ectodermal and mesendodermal progenitor cells by overexpressing the Nodal signal Cyclops in wild-type embryos to obtain embryos primarily consisting of cells with mesendodermal character (Feldman et al., 2000) and using maternal zygotic progenitor cells by overexpressing the Nodal signal Cyclops in wild-type embryos to obtain embryos primarily consisting of cells with mesendodermal character. We isolated 35 such proteins, four of which are likely to have functions in cytoskeletal organization. Comparison of our proteomic data with data obtained from an accompanying microarray-based gene expression screen revealed no significant correlation. This shows that we have identified proteins regulated on a translational or post-translational level that would not have been discovered by gene expression analysis. Finally, the functional analysis of one of the isolated proteins, ezrin2, revealed that ezrin2 is activated by phosphorylation in mesendodermal cells and is crucially required for germ layer morphogenesis during gastrulation. Our findings demonstrate that comparative proteomics in zebrafish represents an effective method to identify candidate proteins with important functions during early development.

We analyzed the samples by separating the proteins according to their molecular mass and isoelectric point (pI) on large-format two-dimensional (2D) gels, both, in the basic (pI 7-11) and acidic (pI 4-7) range (Fig. 2). We took advantage of the DIGE system for pre-separation fluorescent protein labeling with three separate dyes. To reduce gel-to-gel variability owing to lack of gel homogeneity, one dye was used as a common standard applied to all replicate gels. Spot matching, quantification and statistical analysis were carried out using ‘Proteomweaver’ software and significantly up- or downregulated spots were selected for subsequent analysis by mass spectrometry.

Comparison of ectodermal with mesendodermal cell extracts revealed 37 spots that significantly differed in their intensity on 2D gels (Table 1 and Table S1 in supplementary material). The large majority (31/37) of these were reduced in mesendodermal versus ectodermal cells. Using mass spectrometry, we were able to identify 36 out of 37 spots: four proteins have possible roles in the regulation of cytoskeletal dynamics, six proteins have less-defined but still potentially relevant functions for gastrulation (e.g. sialic acid synthase) and 25 proteins have metabolic or housekeeping functions. Two spots were identified as isoforms of the same protein.

#### Comparison of proteomic and genomic analysis

Microarray technology allows the quantitative analysis of gene expression on a genome-wide scale. To determine the overlap between comparative proteomics and gene expression profiling, we performed microarray analysis. The transcriptomes of ectodermal and mesendodermal cell populations, which were prepared with the same method as used for the proteomic screen, were analyzed on microarrays containing probes for more than 14,000 different zebrafish cDNAs (Affymetrix) (http://www.ebi.ac.uk/arrayexpress/queryentry, experiment accession number: E-MEXP-171). Based on a maximal FDR (false discovery rate) of 5% using the Benjamini-Hochberg algorithm (Benjamini and Hochberg, 1995), 220 genes showed significant regulation (Student’s t-test: $P_{\text{max}}=0.0004$). Contrary to the results from our proteomic approach, more genes were upregulated (131) than downregulated (89) in mesendodermal versus ectodermal cells (Fig. 3A and Table S2 in supplementary material). To estimate on a gene-to-gene level the correlation of the proteomics data with the gene expression data, we compared the regulation factors of the 31 genes identified by proteomics which were represented on the microarray. Plotting the regulation factor on the protein level against the regulation factor on the RNA level revealed 37 spots that significantly differed in their intensity on 2D gels (Table 1 and Table S1 in supplementary material). The large majority (31/37) of these were reduced in mesendodermal versus ectodermal cells. Using mass spectrometry, we were able to identify 36 out of 37 spots: four proteins have possible roles in the regulation of cytoskeletal dynamics, six proteins have less-defined but still potentially relevant functions for gastrulation (e.g. sialic acid synthase) and 25 proteins have metabolic or housekeeping functions. Two spots were identified as isoforms of the same protein.

### Fig. 1. Experimental layout.

Ectodermal and mesendodermal protein extracts were labeled with Cy3 or Cy5 fluorescent dyes, combined and subsequently separated by 2D gel electrophoresis (DIGE). A common standard labeled with Cy2 was used for normalization. The scanned images were analyzed with Proteowizard software to identify spots that displayed statistically significant changes. Differential spots were cut, digested by trypsin and analyzed by LC-MS/MS. Database searches were performed in Ensembl and TIGR databases using MASCOT.
Regulators of germ layer morphogenesis revealed little correlation (Fig. 3B). Thus, the majority of the proteins identified by the proteomic approach were specifically regulated on a translational or post-translational level. This emphasizes the importance of comparative proteomics to identify protein modifications undetectable by gene expression profiling.

Regulation of ezrin2
One of the significantly regulated spots turned out to be a zebrafish ERM (ezrin, moesin, radixin) protein. Analyzing the phylogenetic relationship of this ERM protein to other known ezrin, moesin and radixin gene homologues in mouse, humans and Drosophila showed that the identified protein is most closely related to *ezrin* (Fig. 4A). We therefore termed it zebrafish *ezrin2* although the same gene has previously been described as *radixin* (see ZFIN rdx). To test whether *ezrin2* is maternally supplied and/or zygotically expressed during gastrulation, we performed in situ hybridizations on 32-cell-stage (2 hpf), shield-stage (6 hpf), 70% epiboly-stage (7 hpf) and bud-stage (10 hpf) embryos. In both whole-mount and sectioned wild-type embryos, we observed strong ubiquitous expression of maternal *ezrin2* at the 32-cell stage (data not shown), weak ubiquitous zygotic/maternal expression at shield-stage and 70% epiboly-stage, and strong zygotic expression within the head mesendoderm (prechordal plate) at bud stage (Fig. 4B). Apart from the expression in the prechordal plate, no difference in expression level between ectodermal and mesendodermal tissue could be detected (Fig. 4C).

On 2D gels, the *ezrin2* spot was reduced 1.6-fold in mesendodermal cells as compared with ectodermal cells. This reduction was detected in all seven gels tested and was statistically significant (*P*<0.0001) (Fig. 5A,B). To confirm the 2D gel results independently, we performed western blotting analysis on ectodermal and mesendodermal cell extracts. We applied an antibody directed against a peptide fully conserved between human ERM proteins, suggesting that it detects endogenous zebrafish ERM proteins (Fig. 5C). Comparing mesendodermal versus ectodermal cells, the signal was clearly decreased in the mesendodermal cells, confirming the regulation of *ezrin2* previously observed on 2D gels.

ERM proteins are activated by phosphorylation of a conserved threonine at their C-terminus (Matsui et al., 1998), which can be specifically detected by an antibody directed against the phosphorylated site (phospho-ERM antibody). To obtain further insight into the regulation of *ezrin2* during gastrulation, we tested whether *ezrin2* is differentially
Table 1. Differentially regulated spots identified by mass spectrometry (top scoring hits are listed)

<table>
<thead>
<tr>
<th>Cytoskeleton related</th>
<th>2D Gel</th>
<th>Gene chip</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regulation factor</td>
<td>Regulation factor</td>
</tr>
<tr>
<td></td>
<td>P value (t-test)</td>
<td>P value (t-test)</td>
</tr>
<tr>
<td>Ezrin2</td>
<td>0.64</td>
<td>0.0000</td>
</tr>
<tr>
<td>Fascin (Singled-like protein)</td>
<td>0.59</td>
<td>0.0000</td>
</tr>
<tr>
<td>Tubulin, alpha 4-like</td>
<td>0.76</td>
<td>0.0001</td>
</tr>
<tr>
<td>Actin, cytoplasmic 1 (Beta-actin)</td>
<td>0.78</td>
<td>0.0001</td>
</tr>
<tr>
<td>Others</td>
<td>0.0001</td>
<td>0.0379</td>
</tr>
<tr>
<td>Sialic acid synthase (N-acetylneuraminic synthase)</td>
<td>2.21</td>
<td>0.0000</td>
</tr>
<tr>
<td>AU-rich element RNA-binding protein 1</td>
<td>0.76</td>
<td>0.0000</td>
</tr>
<tr>
<td>Iron responsive element binding 1</td>
<td>0.57</td>
<td>0.0009</td>
</tr>
<tr>
<td>Calcylin-binding protein (Siah-interacting protein)</td>
<td>0.73</td>
<td>0.0005</td>
</tr>
<tr>
<td>NiqSnap2 protein</td>
<td>0.67</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolic and housekeeping</th>
<th>2D Gel</th>
<th>Gene chip</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP-mannose pyrophosphorylase B isoform 1</td>
<td>1.87</td>
<td>0.0000</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase 9 family, member A1</td>
<td>0.36</td>
<td>0.0000</td>
</tr>
<tr>
<td>Asparaginyl-tRNA synthetase</td>
<td>0.54</td>
<td>0.0000</td>
</tr>
<tr>
<td>ATP synthase beta chain</td>
<td>0.77</td>
<td>0.0000</td>
</tr>
<tr>
<td>Similar to proteasome 26S subunit ATPase 1 (psmc1)</td>
<td>0.32</td>
<td>0.0000</td>
</tr>
<tr>
<td>26S protease regulatory subunit 4 (P26S4)</td>
<td>1.69</td>
<td>0.0000</td>
</tr>
<tr>
<td>Delta 1-pyrroline-5-carboxylate synthase</td>
<td>0.67</td>
<td>0.0000</td>
</tr>
<tr>
<td>Delta-aminolevulinic acid dehydratase</td>
<td>0.36</td>
<td>0.0002</td>
</tr>
<tr>
<td>Carbonyl reductase 1-like</td>
<td>0.51</td>
<td>0.0011</td>
</tr>
<tr>
<td>XAA Pro Aminopeptidase 2 Precursor</td>
<td>0.22</td>
<td>0.0003</td>
</tr>
<tr>
<td>Heat shock protein 75 kDa, mitochondrial precursor (HSP 75)</td>
<td>0.41</td>
<td>0.0000</td>
</tr>
<tr>
<td>Heat shock cognate 71 kDa protein</td>
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<td>0.0001</td>
</tr>
<tr>
<td>NADH-ubiquinone oxidoreductase -42 kDa subunit, mitochondrial precursor</td>
<td>0.74</td>
<td>0.0000</td>
</tr>
<tr>
<td>Propionyl-CoA carboxylase alpha chain, mitochondrial precursor</td>
<td>2.02</td>
<td>0.0000</td>
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<tr>
<td>Protein disulfide-isomerase precursor</td>
<td>0.69</td>
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<tr>
<td>S-methyl-5-thioadenosine phosphorylase</td>
<td>1.72</td>
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<tr>
<td>Ubiquitin thiolesterase protein OTUB1</td>
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<td>0.0000</td>
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<tr>
<td>Short chain 3-hydroxacyl-CoA dehydrogenase, mitochondrial precursor</td>
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<td>0.0000</td>
</tr>
<tr>
<td>Fructose-bisphosphate aklote B</td>
<td>0.33</td>
<td>0.0000</td>
</tr>
<tr>
<td>ATP synthase alpha chain, mitochondrial precursor</td>
<td>0.63</td>
<td>0.0000</td>
</tr>
<tr>
<td>Not identified</td>
<td>0.59</td>
<td>0.0002</td>
</tr>
<tr>
<td>Voltage-dependent anion channel 2</td>
<td>0.69</td>
<td>0.0000</td>
</tr>
<tr>
<td>NADH-ubiquinone oxidoreductase 19 kDa subunit</td>
<td>0.67</td>
<td>0.0001</td>
</tr>
<tr>
<td>Aspartate aminotransferase, mitochondrial precursor</td>
<td>3.16</td>
<td>0.0000</td>
</tr>
<tr>
<td>Delta 1-pyrroline-5-carboxylate reductase</td>
<td>0.70</td>
<td>0.0001</td>
</tr>
<tr>
<td>Aspartate aminotransferase, mitochondrial precursor</td>
<td>0.37</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

See Table S3 in supplementary material for additional hits. Protein names are based on annotation by ZFIN or Vega or on Ensembl orthologue prediction. In the latter case, the result type of reciprocal BLAST analysis is provided alongside with the orthologue species (HS human, MM mouse, GG chicken). The average regulation factor together with the Student’s t-test (two-sided, unpaired) is provided for the results of 2D gel and gene-chip analysis. ID 9, 24, 26 and 29 lack the gene-chip values because the corresponding gene was not represented on the chip. MOWSE-score was determined by MASCOT (1.8). Molecular mass and isoelectric point (pl) as predicted by Ensembl or TIGR.
Regulators of germ layer morphogenesis

phosphorylated between mesendodermal and ectodermal cells. Applying the phospho-ERM antibody, we detected increased levels of phosphorylated ERMs in mesendodermal versus ectodermal cells (Fig. 5C), indicating that ERMs, including ezrin2, are specifically phosphorylated in the mesendoderm. This regulation conflicts with the results obtained on the 2D gels and with the ERM antibody. It is likely that this inverse regulation observed with the ERM and phospho-ERM antibodies is due to specific detection of only unphosphorylated ERMs by the ERM antibody. This assumption is supported by the fact that the monoclonal ERM antibody we used in this study was raised against an unphosphorylated 11 amino acid peptide that is positioned at the threonine phosphorylation site. Consequently, phosphorylation of ezrin2 in mesendodermal cells would result in increased staining with the phospho-ERM antibody and decreased staining with the ERM antibody, as we have found. With respect to the observed regulation of ezrin2 on 2D gels, we speculate that the identified spot corresponds to the unphosphorylated form of ezrin2, which has been separated from its phosphorylated form as is often observed with protein phosphorylation on 2D gels.

To test for in vivo differences in ezrin expression or phosphorylation between the ectodermal and mesendodermal germ layers, we stained sections of gastrulating wild-type embryos with the ERM and phospho-ERM antibodies. Similar to our western blotting results, we found that mesendodermal progenitor cells, once ingressed at the germ ring margin, displayed elevated levels of phosphorylated ERM proteins on the cell membrane compared with adjacent non-ingressing ectodermal progenitor cells (Fig. 5D).

Fig. 3. Gene expression analysis. Gene expression changes between ectodermal and mesendodermal cells. Wild-type embryos consisting of both ectodermal and mesendodermal cells were compared with samples of ectodermal or mesendodermal cells using Affymetrix zebrafish gene arrays in triplicate. (A) The 220 most significantly regulated genes were selected based on an expected false discovery rate (FDR) of 5%. After centering and normalizing, the genes were clustered according to their regulation pattern. Color coding: up-regulated, red; unchanged, black; downregulated, green. (B) The regulation factor comparing mesendodermal with ectodermal cells on a transcriptional level was plotted against the regulation factor of 2D gel spot intensities. Areas of regulation in the same direction are shaded gray. The overall correlation between gene expression and spot intensity is weak.
ERM phosphorylation/dephosphorylation is dynamically regulated in these cells. In contrast to the phospho-ERM stainings, we only obtained weak, possibly nonspecific staining results using the ERM antibody, suggesting that this antibody, although working well on western blots, is not suitable for detecting ezrin2 expression in zebrafish tissue sections (data not shown).

Function of ezrin2
To analyze the function of ezrin2 during germ layer formation and morphogenesis, we knocked down ezrin2 translation by injecting morpholino antisense oligonucleotides (MO) targeted against the translation initiation site of the ezrin2 gene (ezrin2 MO1; Fig. 6A). Injection of wild-type embryos with 4 and 8 ng of ezrin2 MO1 led to a 1.4- and 2.2-fold reduction, respectively, of endogenous ezrin2 protein levels as determined on western blots (Fig. 6B), suggesting that the ezrin2 MO1 effectively knocked down ezrin2 expression and that the ERM antibody detects ezrin2. This reduction in ezrin2 expression could be reversed by co-injecting 100-200 pg of a 5′-modified version of ezrin2 mRNA, which does not bind the ezrin2 MO1 (Fig. 6B).

ERM proteins are known to connect transmembrane proteins to the cytoskeleton (reviewed by Bretscher et al., 2002). Loss of function may therefore influence cell shape, adhesion and motility. Embryos injected with ezrin2 MO1 showed reduced epiboly movements and in the most severe cases, the blastoderm completely detached from the yolk cell at early gastrulation (Fig. 7A). This phenotype appeared to be specific for ezrin2 because we were able to both produce similar phenotypes using a second MO (MO2) targeted against the 5′UTR of ezrin2 (Fig. 6A, Fig. 7C) and to rescue the ezrin2 morphant phenotype by co-injection of ezrin2 mRNA not targeted by the MOs (Fig. 6C). In addition, ezrin2 morphant embryos displayed a shortened and broadened body axis at the end of gastrulation, indicating that convergent extension movements are affected (Fig. 7C,D). We found this phenotype in morphant embryos both before and after completion of epiboly, suggesting that reduced convergent extension is not just a secondary consequence of delayed development. By contrast, dorso-ventral patterning of early gastrula stage embryos was unaffected in ezrin2 morphant embryos (data not shown).

To analyze the epiboly phenotype in more detail, we performed DIC microscopy of cells within the animal pole of late blastula/early gastrula stage embryos (4-5 hpf). Wild-type cells at the animal pole were typically organized into a compact assembly of round cells as seen by both DIC microscopy and phalloidin staining of cortical actin. By contrast, morphant blastodermal cells appeared more loosely associated and exhibited more spread-out and amorphic shapes (Fig. 7B). The degree of disorganization of cell shape and assembly correlated

Fig. 4. Zebrafish ezrin2. (A) Un-rooted tree of human (Hs), mouse (Mm), zebrafish (Dr) and Drosophila (Dm) ERM-family proteins. Corresponding NCBI accession numbers: Dr Ezrin2 NP_001025456, Hs Ezrin NP_003370, Mm Ezrin P26040, Dr Ezrin1 XP_699992, Hs Radixin AAH74109, Mm Radixin NP_033067, Mm Moesin NP_034963, Hs Moesin NP_034963, Dr Moesin-like NP_001004296, Dr Moesin XP_700327, Dm Moesin P46150, Dm Merlin AAF49005, Dr Merlin-like NP_998116, Mm Merlin P46662, Hs Merlin P35240, Dr Merlin XP_689682. Scale bar indicates point mutations per site. (B) In situ hybridization of shield-stage (6 hpf), 70% epiboly-stage (7 hpf) and bud-stage (10 hpf) sectioned and whole-mount wild-type embryos using an ezrin2-antisense probe. Lateral views with dorsal side to the right. (C) Close-up view of the dorsal side of a sectioned 70% epiboly-stage (7 hpf) embryo after in situ hybridization using ezrin2 antisense probe.
Fig. 5. Ezrin2 regulation analyzed by 2D gels, western blotting and immunostaining of sections. (A) Close-up view of three out of seven gels (Cy3 and Cy5 labeled) in the region of the ezrin2 spot (circled red). (B) Quantification of ezrin2 spot intensities of the DIGE 2D gel experiment. (C) Western blot analysis of wild-type, nz-oep (ectoderm) and cyclops mRNA injected (mesendoderm) embryos at 8 hpf (corresponding to 80% epiboly stage in wild-type embryos). After analysis with phospho-ERM (p-ERM) antibody, blots were stripped and re-probed with ERM antibody. The lower part of the blot was probed for tubulin as a loading control. (D) Wild-type embryos were sectioned at the shield stage (6 hpf; sagittal section through the shield region) and at 80% epiboly (8 hpf; transverse section through the emerging body axis, paraxial region) and stained with phospho-ERM (p-ERM) antibody. In mesendodermal cells the level of phosphorylated ERM proteins is increased. Insets show section planes.

Fig. 6. Ezrin2 morpholino antisense oligonucleotides efficiently reduce ezrin2 protein level and impair gastrulation. (A) Placement of the ezrin2 MOs used in this study (MO1 and MO2) on the ezrin2 mRNA. MO1 targets the translation initiation site and MO2 is placed in the upstream 5’UTR of ezrin2 mRNA. (B) Injection of ezrin2 MO1 efficiently reduces ezrin2 protein as detected by western blotting with ERM antibody (tubulin antibody was used as loading control). Co-injection of 100 pg ezrin2 mRNA with 8 ng of ezrin2 MO1 restores ezrin2 protein expression. (C) Quantification of the early loss-of-function phenotypes and partial rescue by ezrin2 mRNA injection. One-cell-stage embryos were injected with morpholino MO1 or MO2 and scored for blastoderm detachment or epiboly defects (wt 0/122, 2 ng MO1 37/91, 4 ng MO1 53/81, 4 ng MO2 38/109, 8 ng MO2 42/89). To rescue ezrin2 morpholino-induced phenotypes 4 ng ezrin2 MO2 followed by 100 pg ezrin2 mRNA were injected. Embryos were scored for blastoderm detachment or epiboly defects (MO2 20/107; MO2 + ezrin2 mRNA 9/90).
with the strength of the epiboly phenotype. This suggests that, at the onset of zebrafish gastrulation, ezrin2 is required for the arrangement of blastodermal cells in compact cell assemblies needed for proper blastoderm epiboly.

The observation that in ezrin2 morphant embryos, convergent extension movements are reduced at the end of gastrulation (Fig. 7C,D), together with the finding that ERM phosphorylation is increased in mesendodermal progenitor cells (Fig. 5C,D), suggests that ezrin2 phosphorylation and hence activation is required for proper mesendodermal progenitor cell migration. To obtain insight into the potential role of ezrin2 in this process, we recorded high-resolution two-photon confocal time-lapse movies of the cellular rearrangements within the axial germ ring margin (shield) of wild-type and ezrin2 morphant embryos (MO2) starting at shield stage (6 hpf) by confocal time-lapse microscopy. Images correspond to timepoints 0 minutes and 60 minutes of Movies 1 and 2 in supplementary material. One exemplary mesendodermal progenitor cell was labeled in red to mark its position at 0 and 60 minutes of the time-lapse movie. Note that during this time interval the labeled cell has moved further away from the germ ring margin in the wild-type embryo compared with the morphant embryo, suggesting that mesendodermal cell migration is reduced within the shield of ezrin2 morphant embryos. Lateral view, dorsal to the right. Bar, 40 μm.

Discussion
We have used comparative proteomics in zebrafish to identify proteins that are differentially expressed or modified in the embryonic mesendodermal and ectodermal germ layers and are therefore likely to be involved in morphogenesis of these tissues. Several proteins with presumed cytoskeletal functions were isolated as being regulated on a translational or post-translational level. One of these proteins, ezrin2, is activated cells away from the germ ring margin towards the animal pole (Montero et al., 2005). By contrast, mesendodermal progenitors in morphant embryos were less motile and exhibited reduced animal-pole-directed migration (Fig. 7E and Movies 1 and 2 in supplementary material). This suggests that ezrin2, in addition to its earlier function in blastoderm epiboly, is required for proper mesendodermal progenitor cell migration.
by phosphorylation in the mesendodermal cells and is required for proper germ layer morphogenesis during gastrulation. We conclude (1) that proteomics is feasible in zebrafish and (2) that comparative proteomics can be used to identify proteins that are specifically regulated on a translational or post-translational level and have essential functions during early development.

Gene expression profiles are usually compared by hybridizing microarrays that contain probes for a large number of cDNAs. In addition, large-scale enhancer, gene trap and in situ hybridization screens are performed to analyze the temporal and spatial pattern of gene expression. However, by comparing gene transcription only, potential differences due to translational or post-translational regulation of proteins cannot be detected. Comparative proteomics, by contrast, allows screening for differences on both translational and post-translational levels and therefore complements the transcriptional approaches. Although comparative proteomics is a powerful method, as demonstrated here, several technical limitations must be taken into account. First of all, despite remarkable advances of proteomic techniques in the last years, the dynamic range of this technology remains limited. Despite newly developed fluorescent dye techniques that allow quantitative analysis at nanogram levels, proteins of low abundance, which are estimated to compromise more than half the proteome, are commonly missed. Moreover, certain physicochemical properties such as hydrophobicity, molecular mass or isoelectric point can strongly decrease the recovery of specific proteins on 2D gels. Together, this ultimately narrows the range of proteins that can be identified using 2D-gel-based proteomics.

Although our proteomic approach did not cover the whole proteome owing to the intrinsic limitations of 2D gel technology, we were still able to quantitatively analyze more than 1000 different spots on our 2D gels. In addition to many proteins with metabolic or housekeeping functions, whose specific roles in germ layer morphogenesis are difficult to predict, we identified several regulatory and cytoskeleton-related proteins. Importantly, most of the genes identified by proteomics were not significantly regulated on a transcriptional level. Therefore, gene expression profiling alone would not have identified these genes.

It is noteworthy that, comparing mesendoderm and ectoderm, more of the significantly regulated spots were decreased than increased. This could be due to mesendoderm-specifying signals, such as Nodals, that modify proteins in epiblast (ectodermal) cells during their transition from epiblast into hypoblast (mesendodermal) cells. Considering that modifications increase protein heterogeneity, the resulting different protein isoforms in mesendodermal cells might fall below the detection limit as a result of their relatively low abundance. Consequently, the increase of these modified isoforms in mesendodermal cells might be undetectable whereas the decrease of the unmodified form is detectable.

Although most of the proteins identified here have not been investigated with regard to a specific function in development, previous studies suggest potential morphogenetic functions. For example, the small actin-bundling protein fascin is involved in the formation and regulation of cellular protrusions mediating motility, cell adhesion and cell interactions (reviewed by Adams, 2004), processes also involved in the regulation of gastrulation movements. Fascin activity is regulated by phosphorylation in response to signals from the extracellular matrix (ECM) or other external cues (reviewed by Kureishy et al., 2002). Furthermore, fascin expression in epithelial cell lines increased their 2D and 3D motility (Jawhari et al., 2003), again supporting the possibility that fascin has an important function in regulating cell movements during gastrulation. Another protein we identified here, sialic acid synthase, triggers the formation of sialic acids, which are terminal sugars on cell surface glycoproteins. Attachment of polysialic acid to the neural cell adhesion molecule (NCAM) serves to modulate cell interactions in the nervous system (Rutishauser and Landmesser, 1996). In addition, sialic acids serve as markers for cell-cell recognition events (reviewed by Varki, 1997). Sialic acids therefore may have the potential to influence gastrulation movements by modulating cell-cell adhesion and/or migration. Finally, 14-3-3 proteins have an extraordinarily widespread influence on cellular processes in all eukaryotes. They bind to phosphorylated-serine-containing peptides to regulate the activity and interactions of more than 200 proteins (reviewed by Mhawech, 2005). Interestingly, 14-3-3 proteins have also been reported to regulate cell migration and spreading by interactions with β1 integrin, Raf and Cas (Rodriguez and Guan, 2005) and therefore are also likely to control cell migration during gastrulation.

Although we identified several regulatory and cytoskeleton-related proteins, we did not find any secreted/extracellular proteins or transcription factors, including those that previously have been identified as Nodal signaling targets (reviewed by Schier, 2003). Presumably, secreted proteins were lost during sample preparation because we dissociated the embryos during the de-yolkling process, and transcription factors, which are generally known to be expressed at low levels, fell below the 2D detection limit.

Our experimental approach took advantage of the observations that Nodal signaling is both required and sufficient to induce mesendodermal cell fates in shield-stage embryos (reviewed by Schier, 2003). Although our assay was designed to reflect differential expression or modification between ectoderm and mesendoderm, we cannot exclude the detection of proteins targeted by Nodal signaling that is involved in other early developmental events such as left-right axis determination. Therefore, distribution and modification of every target protein must be independently analyzed in wild-type embryos to distinguish between direct Nodal targets and proteins regulated specifically in the mesoderm or ectoderm.

In this study, we have analyzed ezrin2 function during gastrulation. Ezrin2 belongs to the family of closely related ERM proteins (Sato et al., 1992) that link filamentous actin to integral plasma membrane proteins upon phosphorylation mediated activation (Matsui et al., 1998), thereby regulating cell shape, surface structures, adhesion and motility. Furthermore, ERMs have been implicated in signal transduction regulation by altering membrane protein localization and internalization (reviewed by Bretscher et al., 2002). Studies in cell culture have suggested that ezrin is concentrated at the leading edge of migrating cells and that blocking ezrin function leads to defective extension of pseudopods and collapse of the leading edge (Crepaldi et al., 1997; Lamb et al., 1997). Our finding that in ingressing mesendodermal progenitors, ezrin2 gets phosphorylated and...
hence activated, suggests that ezrin2 activity is involved in mesendodermal cell ingestion and subsequent migration. Evidence for a crucial role of ezrin2 in the forming mesendoderm came also from our morpholino-based loss-of-function analysis, which showed that proper mesendodermal progenitor migration depends on normal ezrin2 expression.

In addition to the mesendodermal phenotype in ezrin2 morphant embryos, we observed distortions of cell shape and assembly within the blastoderm of pre-gastrula-stage embryos, followed by reduced epiboly movements and, in the most severe cases, complete delamination of the blastoderm from the yolk cell. Ezrin has previously been shown to interfere with compaction and cavitation of the blastocyst in pre-implantation mouse embryos (Dard et al., 2001), suggesting a similar function in regulating blastoderm cell compaction in zebrafish and mouse. Future studies will have to elucidate the molecular and cellular mechanisms by which ezrin functions in cell compaction and how this function is related to its effects on epiboly and convergent extension movements during gastrulation.

In conclusion, we have demonstrated that proteomics can be used in zebrafish to identify proteins that are regulated on a translational or post-translational level. Further characterization of one of the identified proteins, ezrin2, revealed that ezrin2 is phosphorylated in mesendodermal cells and required for proper germ layer morphogenesis during gastrulation. Proteomics can therefore be used to identify proteins with essential functions during zebrafish gastrulation.

**Materials and Methods**

**Embryo maintenance**

All wild-type embryos were obtained from zebrafish TL, AB and Golden lines. Fish maintenance and embryo collection was carried out as described (Mullins et al., 1994). Embryos were staged as previously described, grown at 31°C and manipulated in E3 zebrafish embryo media (Kimmel et al., 1995).

**Sample preparation**

To obtain mesendodermal progenitor cells, wild-type embryos were injected with 100 pg cyclops mRNA. Ectodermal progenitor cells were obtained from maternal-zygotic one-eyed-pinhead (oep) mutant embryos (Gritsman et al., 1999). The embryos were dechorionated and deyolked with two extra washing steps as previously described (Link et al., 2006).

**DIGE labeling and 2D gel electrophoresis**

20 μl lysis buffer (7 M urea, 2 M thiourea, 4% Chaps, 30 mM Tris-HCl pH 9.2) was added to the cells of 100-de-yolked embryos. The sample was incubated shaking for 15 minutes at 8°C after the addition of 0.5 μl benzamidine (25 U/μl. >99% purity, Novagen) to degrade DNA/RNA. Protein concentration was determined by DC DC Protein assay (Bio-Rad). Insoluble particles were removed by centrifugation for 1 hour at 60,000 g. 50 μg protein was then added with 200 pmol Cy dye as described in the user manual (Amersham Biosciences). The samples prepared with different dyes were combined. For 2D gels of proteins in the acidic range (pI 4–7), labeled samples were mixed with 450 μl rehydration solution (pI 4-7: 7 M urea, 2 M thiourea, 4% Chaps, 5% isopropanol, 2.5% glycerol, 1% DTT, 500 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) followed by two washes and detection was performed as previously described (Link et al., 2006).

**Microarray analysis**

RNA was extracted from de-yolked cells using NucleoSpin RNA II kit (protocol 5.1, Macherey-Nagel, Dueren, Germany). RNA was then precipitated with sodium acetate and 80% ethanol (7×10^6 washed twice with 80% ethanol and re-suspended to a concentration of 1 μg/μl. ServiceXS (Leiden, The Netherlands) performed the labeling and hybridization experiments using MessageAMPII kit (Ambion, Austin TX, USA) and Affymetrix Zebrafish Genome arrays (Affymetrix, Santa Clara CA, USA). Statistical analysis was performed with ArrayAssist software (Stratagene, La Jolla CA, USA). The raw data (CEL-format) was processed by PLIER (www.affymetrix.com) probe level analysis and logarithmic transformation. Three independent replicates of each condition were analyzed. Student’s t-test was applied with a maximal FDR (false discovery rate) of 5% (Benjamini-Hochberg algorithm). Significantly regulated genes were centered, normalized and then hierarchically clustered with ‘Cluster 3.0’ software (Eisen et al., 1998). Fig. 3A was generated with ‘Gene Treeview’.

**Phylogenetic tree**

Sequences fetched from NCBI were aligned using Clustal X (Thompson et al., 1997). The tree was generated with PHYLIP (Felsenstein, 1989), distance-matrix protist, 100 iterations calculated using the fitch algorithm.

**Western blotting**

De-yolked samples were dissolved in 2 μl 2× SDS sample buffer per embryo and incubated for 5 minutes at 95°C and loaded on SDS gels. Electrophoresis, blotting and detection was performed as previously described (Link et al., 2006). Before re-probing, membranes were incubated for 30 minutes at 50°C in stripping solution (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) followed by two washes for 5 minutes each with PBST. Antibodies used were anti-MEK1 (Cell Signaling #9112, #1:1000), anti-α-tubulin (Sigma T6199, #1:2000), anti-ERM (BD Biosciences 610401, #1:1000) and anti-phosphorylated-ERM (Cell Signaling #3141, #1:1000).

**Morpholino oligonucleotide and mRNA injection**

For MO injections the following MOs directed against zebrafish ezrin2 were injected into one-cell-stage embryos: (MO1 directed against the 5’ coding sequence 5’-GCCGAACATTACCTGTTAGGCT-3’ and MO2 directed against the 5’UTR) 5’-GATGATAGCAGCCATCCTCCGTGC-3’ (Gene Tools, Philomath, OR). MOs were designed according to Gene Tools targeting guidelines. MO sequences were then compared with the Ensembl database by using BLAST, and no significant similarities were found to any sequences other than zebrafish ezrin2. For mRNA injection, ezrin2 full-length cDNA was cloned by PCR into pcDNA3 vector. Seven mutations changing two valines to isoleucines were introduced at the 3’UTR of the gene index (ftp://ftp.tigr.org/pub/data/tgi/Danio_rerio/ZGI.release_16.zip) and the Ensembl zebrafish peptide database (http://wwwensembl.org/info/data/download.html#zebrafish-peptide-＞-fa.gz). MAGSCOT search parameters were peptide precursor mass tolerance, ±0.5 Da; mass tolerance fragment ions, ±0.5 Da; enzyme specificity, trypsin; missed cleavages tolerated, 1; fixed modifications, carbamidomethyl; no variable modifications.
pCS2-erz2n2 by using the Sp6 mMessage mMachine (Ambion, Austin, TX) and injected into one-cell-stage embryos.

In situ hybridization and sectioning
Whole-mount in situ hybridization was performed as previously described (Barth and Wilson, 1995). For erz2n, sense and antisense RNA probes were synthesized from the erz2n full-length cDNA. For hgg, nl, and dl5x in situ hybridization, antisense RNA probes were synthesized from partial sequences of the respective cDNAs. For sectioning, in situ-stained embryos were equilibrated in gelatin/albumin solution (0.49% gelatin, 30% egg albumin, 20% sucrose in PBS), transferred into fresh polymerization solution (25% glutaraldehyde in gelatine/albumin solution, 1:10) and polymerized for 15 minutes at room temperature. 4 μm serial sections were taken using a Leica Vibratome VT1000S.

Immunostaining of sectioned embryos
Embryos were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and embedded in paraffin wax. 10 μm serial sections were taken on a microtome, dried overnight at 37°C and then rehydrated. Immunostaining was done according to manufacturer’s instructions for the anti-phosphorylated-ERM antibody (phospho-ERM) (Cell Signaling #3141). Briefly, for antigen retrieval, the slides were boiled in 10 mM sodium citrate buffer pH 6.0 and kept at sub-boiling temperature for 10 minutes. Samples were washed with TBST (Tris-buffered saline, 1% Tween-20) and blocked with 5% normal goat serum in TBST for 2 hours at room temperature. Then, they were incubated overnight at 4°C with phospho-ERM antibody 1:25 in blocking solution, followed by several washes with TBST and incubation with secondary antibody (Alexa Fluor 488 anti-rabbit 1:1000; Invitrogen), for 2 hours at room temperature. After several washing steps, sections were mounted in DABCO medium and images were acquired with a Leica TCS SP2 confocal microscope.

Image acquisition and quantification
DIC images were taken as previously described (Montero et al., 2005) using a BioRad Radiance 2000 multiphoton confocal microscope with a 60x water-immersion lens.

We thank Jennifer Geiger, Masa Tada and Laurel Rohde for reading earlier versions of this manuscript and Bianca Habermann for generating the phylogenetic tree of erz2n. We are grateful to G. Junghans, E. Lehmann, M. Fischer and J. Hueckmann for help with the fish care and to S. Witzel and S. Schneider for technical help. This work was supported by the DFG priority program SP 1049 (Molecular Control Mechanisms of Cell Migration) and the EC FP6 Zf-Models project.

References
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