Fam40b is required for lineage commitment of murine embryonic stem cells

V Wagh, MX Doss, D Sabour, R Niemann, K Meganathan, S Jagtap, JA Gaspar, MA Ardestani, S Papadopoulos, M Gajewski, J Winkler, J Hescheler, A Sachinidis

FAM40B (STRIP2) is a member of the striatin-interacting phosphatase and kinase (STRIPAK) complex that is involved in the regulation of various processes such as cell proliferation and differentiation. Its role for differentiation processes in embryonic stem cells (ESCs) is till now completely unknown. Short hairpin RNA (shRNA)-mediated silencing of Fam40b expression in ESCs and differentiating embryoid bodies (EBs) led to perturbed differentiation to embryonic germ layers and their derivatives including a complete abrogation of cardiomyogenesis. Pluripotency factors such as Nanog, Oct4 and Sox2 as well as epigenetic factors such as histone acetyltransferase type B (HAT1) and DNA (cytosine-5)-methyltransferase 3-β (Dnmt3b) were highly upregulated in Fam40b knockdown EBs as compared with control and scrambled EBs. To examine the relevance of Fam40b for development in vivo, Fam40b was knocked down in developing zebrafish. Morpholino-mediated knockdown of Fam40b led to severe abnormalities of the cardiovascular system, including an impaired expression of ventricular myosin heavy chain (vmhcr) and of cardiac myosin light chain 2 (cmlc2) in the heart. We identified the gene product of Fam40b in ESCs as a perinuclear and nucleolar protein with a molecular weight of 96 kDa. We conclude that the expression of Fam40b is essential for the lineage commitment of murine embryonic stem cells (mESCs) into differentiated somatic cells via mechanisms involving pluripotency and epigenetic networks.

Cell Death and Disease (2014) 5, e1320; doi:10.1038/cddis.2014.273; published online 10 July 2014

The analysis of mammalian transcriptomes, including humans and mice, led to the identification of thousands of novel transcripts of (as yet) unknown function (TUFs). Although it has recently been confirmed that TUFs, including noncoding RNAs, can participate in the regulation of biological and cellular processes, the functional role of most TUFs remains to be elucidated. The expression of many TUFs during the development is often transient and it has only recently been recognized that TUFs are indeed not just transcriptional noise but that many of them play a critical role during development. Recently, TUFs with distinct transcriptional kinetics during osteogenic and adipogenic differentiation of human mesenchymal stem cells (hMSCs) have been identified. These include a long nuclear noncoding RNA, a micro-RNA host gene and a novel small protein gene. All three were transcriptionally regulated by Wnt and protein kinase A (PKA) signaling pathways that are the key pathways for hMSC differentiation. Recently, increasing attention has been directed toward identifying and understanding the function and intracellular signaling pathways of the STRIPAK complex in regulating biological processes of multiple organisms. There is growing evidence that the TUF Fat40b (synonyms: striatin interacting protein 2 (STRIP2) or D330017J20Rik) is a member of the striatin-interacting phosphatase and kinase (STRIPAK) complex that is involved in the regulation of cell growth, proliferation, cell migration and adhesion, neural and vascular development as well as cardiac function.

Abbreviations: ESC, embryonic stem cell; EB, embryoid body; LIF, leukemia inhibitory factor; TGF-β, transforming growth factor-β; α-MHC, α-myosin heavy chain (Myh6); EGFP, enhanced green fluorescent protein; RT-PCR, reverse transcriptase-PCR; shRNA, short hairpin RNA; qPCR, quantitative PCR; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GMEM, Glasgow’s minimal essential medium; IMDM, Iscove’s modified Dulbecco’s medium; ECACC, European Collection of Cell Cultures; aRNA, amplified RNA; DET, differentially expressed transcripts; DAVID, database for annotation, visualization and integrated discovery; TBST, Tris-buffered saline Tween-20; ECL, enhanced chemiluminescence

Received 02.1.13; revised 25.4.14; accepted 21.5.14; Edited by Y Shi
Cell Death and Disease

V Wagh et al

Constitutive knockdown of Fam40b in differentiating ESCs. Fam40b was knocked down in ESCs by short hairpin RNA (shRNA) using the pGFP-V-RS shRNA vector. Expression of truncated green fluorescence protein (tGFP) and a puromycin resistance cassette allow for monitor transfection and selection of transfected transgenic cells (Figure 1a). We generated a set of ESCs clones by transfecting the shRNA construct (TR508344A, Origene Technologies, Rockville, MD, USA) by electroporation and selection of stable clones. Only clones with strong GFP expression were selected for the study. The TR508344A plasmid containing the shRNA sequence 5’-GCAAGACACTAAGGAATGGCTGGAGTTGG-3’ targeting Fam40b mRNA nucleotide positions between 365 and 393 were further used for differentiation of ESCs to embryoid bodies (EBs). Application of the hanging drop protocol resulted in spontaneous differentiation of ESCs toward different somatic cells including cardiomyocytes.14–16 Fam40b knockdown ESCs (KD ESCs) maintained their pluripotent nature and showed no differences in morphology when compared with the ESCs transfected with the control vector (without the shRNA oligonucleotide) or wild-type (WT) ESCs. EBs generated from KD ESCs will be referred to as KD EBs. Control EBs were generated from ESCs stably transfected with the empty pGFP-V-RS vector. As shown in Figures 1b and c, expression of the tGFP continued until day 12 of differentiation. The expression level of Fam40b was reduced in 4-day control EBs and increased in 8- and 12-day EBs, whereas no significant expression was observed in undifferentiated Fam40b KD ESCs and the Fam40b KD EBs. To determine whether knockdown of Fam40b results in a reduced protein level, we checked the Fam40b protein expression levels using a Fam40b antibody (sc-162799; Santa Cruz Biotechnology; 1: 500 dilution) and with the secondary donkey anti-Goat IgG antibody (1: 10,000 dilution). GAPDH has been detected using the anti-GAPDH antibody (National Institutes of Health, Bethesda, MD, USA) and the ratio of the FAM40B/GAPDH bands densities has been performed with FAM40b primary antibody (sc-162799, Santa Cruz Biotechnology; 1 : 25,000 dilution) and GAPDH has been detected using the anti-GAPDH antibody (1 : 25,000 dilution). Results of densitometric analysis of the FAM40B and the corresponding GAPDH bands densities has been performed by using the ImageJ 1.47v software.

Analysis of the differentially expressed genes. To determine whether Fam40b levels affect the expression of genes participating in differentiation/developmental processes, the transcriptomes of both control and 12-day KD EBs were profiled and compared with the transcriptome of undifferentiated WT ESCs. Significance of variances of the

Figure 1 Generation and characterization of ESCs in which Fam40b was constitutively knocked down (KD) by transfection with pGFP-V-RS, expressing shRNA directed against the Fam40b RNA, the GFP and the puromycin resistance cassette. (a) Fluorescence microscopy of control 12-day EBs derived from ESCs transfected with the pGFP-V-RS vector without shRNA (control) and EBs derived from ESCs transfected with the pGFP-V-RS shRNA expressing vector containing 29-mers shRNA (Fam40b KD EBs) (scale bar: 50 μm). (b) Semiquantitative RT-PCR analysis of the expression of GFP and Fam40b in undifferentiated control and KD Fam40b ESCs as well as in 4-, 8- and 12-day control and KD EBs generated by the hanging drop protocol. (c) Expression of the Fam40b protein during differentiation of WT ESCs (upper panel) and during differentiation of Fam40b KD ESCs. Detection has been performed with Fam40b primary antibody (sc-162799, Santa Cruz Biotechnology; 1 : 500 dilution) and with the secondary donkey anti-Goat IgG antibody (1 : 10,000 dilution). GAPDH has been detected using the anti-GAPDH antibody (1 : 25,000 dilution). (d) Densitometric analysis of the FAM40B and the corresponding GAPDH bands densities has been performed by using the ImageJ 1.47v software.
expression levels were observed by principal component analysis (PCA). The percentage of variance at principal component (PC) 1 shows the highest variance in transcriptome variability among the biological samples and PC2 the second highest, respectively. As shown in Figure 2a, there are relatively large differences of the transcriptomes of all three cell populations. Interestingly, the transcriptome of the KD 12-day EBs was closer to undifferentiated WT ESCs in PC1 (corresponding to 60% of the variance) than the control 12-day EBs. Supplementary Table S1 shows the differentially expressed transcripts (6054 transcripts) between the control 12-day versus 12-day Fam40B KD EBs (2133 transcripts at least twofold upregulated and 3921 transcripts twofold downregulated, \( P < 0.05 \)). As indicated, several genes including cardiomyocyte-specific genes were remarkably downregulated in the KD EBs (e.g., Tnnt2 and \( \alpha \)-myosin heavy chain (Myh6) more than 300-fold downregulated).

Specific transcriptome clusters of the different cell populations were identified using \( k \)-means clustering algorithm over the transcripts significantly deregulated among different cell types at least twofold (Figure 2b).

The main representative developmental/differentiation-associated (BP) GOs and KEGG pathways identified by the annotation enrichment analysis for all the five clusters are shown in Table 1. Cluster 1 includes mainly genes that are weakly expressed in KD 12-day EBs as compared with the control 12-day EBs and ESCs and are clearly associated with developmental/differentiation processes such as heart, vascular and central nervous system development (Table 1). Moreover, we have identified several genes in clusters 2 and 3 with a high expression level in the KD 12-day EBs (red color); with a moderate expression level still in the WT ESCs but clearly with a low expression level in the control 12-day EBs (green color). GO analysis of the strongly upregulated genes in clusters 2 and 3 resulted in the identification of ncRNA metabolic processes GOs (see Table 1). Notably, cluster 3 transcripts demonstrated higher expression levels in KD 12-day EBs than in WT ESCs. Interestingly, cluster 2 includes several pluripotency-associated genes (Nanog, Rif1, Esrrb, Pou5f1, Nodal, Sox2, Piwil2, Klf4, Fgf4, Tcf1) with a high expression level, as expected in WT ESCs (red color) but also in KD 12-day EBs (Figure 2, cluster 2, Table 1, GO:0019827 ~ stem cell maintenance). As expected, the expression level of the pluripotency-associated genes should be low in 12-day EBs because of progressive differentiation after 12 days of differentiation. A similar expression pattern was observed for genes regulating gene expression via epigenetic mechanisms (Figure 2, cluster 2, Table 1, GO:0040029 ~ regulation of gene expression, epigenetic). Figure 3 shows the expression level of the epigenetic (Figures 3a and b) and pluripotency genes (Figure 3c) in all three cell populations. Moreover, we validated the gene array expression data with quantitative PCR (qPCR) methodology and examined the expression of representative genes from all three interesting GOs (Figure 3d). Cluster 4 has been identified as the largest cluster containing transcripts that show high expression levels in control 12-day EBs as compared with the WT ESCs and KD 12-day EBs (Table 1). As expected, because of the progressive differentiation, the control ESCs are capable of differentiating into various germ layer derivatives such as cardiac and neuronal cells. Besides mesodermal transcripts, ectodermal and to a lesser extent endodermal transcripts as well as transcripts participating in the KEGG signaling pathways such as the Wnt receptor and the transforming growth factor-\( \beta \) (TGF-\( \beta \)) receptor signaling pathway were expressed lower in the KD 12-day EBs as compared with the control 12-day EBs (Table 1). Cluster 5 GOs and KEGG signaling pathways include transcripts associated with developmental processes that were exclusively downregulated in the KD 12-day EBs.

Transcriptomic analysis of Fam40b KD versus Scr and WT 12-day EBs. To give stronger evidence for the novel function of the Fam40b as a regulator of differentiation processes, we also generated scrambled ESCs (will now be referred to as Scr ESCs) applying the pGFP-V-RS shRNA approach and using the TR30013 containing the 29-mer scrambled shRNA oligonucleotide. The transcriptomes of WT ESCs, Scr ESCs as well as the transcriptomes of the Scr and KD 12-day EBs were then profiled and compared. In general, findings obtained by the comparison between

---

Figure 2  Transcriptome analysis of wild-type ESCs, control 12-day EBs and Fam40B KD 12-day EBs. (a) Principal component analysis of genome-wide gene expression. Each sphere represents an individual sample from a color-coded triplicate sample. (b) Visualization of \( k \)-means clustering of 5574 differentially expressed probe sets with Euclidean distance measurement and \( k = 5 \) group clusters. Replicates are displayed in the vertical axis and genes in the horizontal axis. Log2 transformed signal intensities are depicted in color code. The heatmap indicates high expression levels in red, intermediate expression level in dark gray and low expression levels in green.
Table 1: Developmental GO BPs as well as KEGG pathways differentially regulated in KD 12-day EBs in comparison with control 12-day EBs and WT ESCs

| Cluster | GOs and KEGG pathways including highly upregulated genes in control 12-day and moderate expressed in WT ESCs but very low expressed in KD 12-day EBs | Transcripts | P-value |
|---------|---------------------------------------------------------------------------------------------------------------------------------|-------------|---------|
| Cluster 1 | GO:0007507 ~ heart development | 20 | 9.47E−05 |
| | GO:0045597 ~ positive regulation of cell differentiation | 16 | 4.77E−04 |
| | GO:0030705 ~ cytoskeleton-dependent intracellular transport | 7 | 5.37E−04 |
| | GO:0001568 ~ blood vessel development | 19 | 8.02E−04 |
| | GO:0001944 ~ vasculature development | 19 | 0.001092 |
| | GO:0001649 ~ osteoblast differentiation | 7 | 0.003581 |
| | GO:007417 ~ central nervous system development | 23 | 0.00363 |
| | GO:0060348 ~ bone development | 11 | 0.004406 |
| | GO:007420 ~ brain development | 19 | 0.005137 |
| | GO:0043009 ~ chordate embryonic development | 24 | 0.008354 |
| | GO:0030324 ~ lung development | 10 | 0.008945 |
| | mmu4310:Wnt signaling pathway | 17 | 1.00E−05 |
| | mmu4340:Regulation of actin cytoskeleton | 19 | 9.47E−05 |
| | mmu4340:Hedgehog signaling pathway | 8 | 0.001149 |
| | mmu5414:Dilated cardiomyopathy | 10 | 0.001831 |
| | mmu4360:Axon guidance | 12 | 0.002053 |

Cluster 2: GOs including upregulated genes in WT ESCs and KD 12-day EBs as compared with control 12-day EBs

| GO:0034660 ~ ncRNA metabolic process | 42 | 1.16E−13 |
| GO:0034470 ~ ncRNA processing | 34 | 1.34E−11 |
| GO:0060348 ~ RNA processing | 53 | 6.00E−08 |
| GO:019827 ~ stem cell maintenance | 10 | 9.91E−07 |
| GO:0040029 ~ regulation of gene expression, epigenetic | 14 | 8.25E−05 |

Cluster 3: GOs including upregulated genes KD 12-day EBs as compared with control 12-day EBs and WT ESCs

| GO:0006396 ~ RNA processing | 27 | 8.80E−06 |
| GO:0016070 ~ RNA metabolic process | 33 | 5.44E−05 |
| GO:0000154 ~ rRNA modification | 4 | 6.17E−04 |
| GO:0034470 ~ ncRNA processing | 12 | 0.001053 |
| GO:0043414 ~ biopolymer methylation | 8 | 0.001189 |
| GO:009451 ~ RNA modification | 6 | 0.002623 |
| GO:0034660 ~ ncRNA metabolic process | 12 | 0.007034 |

Cluster 4: GOs and KEGG pathways including genes that are upregulated in control 12-day EBs as compared with WT ESCs and KD 12-day EBs

| GO:0007507 ~ heart development | 54 | 1.98E−17 |
| GO:001568 ~ blood vessel development | 56 | 6.00E−17 |
| GO:0057472 ~ gland development | 38 | 2.42E−09 |
| GO:0060348 ~ bone development | 28 | 4.45E−09 |
| GO:0022008 ~ neurogenesis | 71 | 6.88E−09 |
| GO:0030323 ~ respiratory tube development | 27 | 7.60E−09 |
| GO:001822 ~ kidney development | 26 | 1.06E−08 |
| GO:0051216 ~ cartilage development | 20 | 3.07E−07 |
| GO:0016055 ~ Wnt receptor signaling pathway | 25 | 2.15E−06 |
| GO:0048565 ~ gut development | 13 | 3.38E−06 |
| GO:0035108 ~ limb morphogenesis | 22 | 1.10E−05 |
| GO:0048666 ~ neuron development | 39 | 2.16E−05 |
| GO:008016 ~ regulation of heart contraction | 13 | 5.47E−05 |
| GO:007417 ~ central nervous system development | 44 | 9.31E−05 |
| GO:007219 ~ Notch signaling pathway | 13 | 1.22E−04 |
| GO:0030111 ~ regulation of Wnt receptor signaling pathway | 11 | 1.26E−04 |
| GO:0045666 ~ regulation of neuron differentiation | 18 | 2.37E−04 |
| GO:0031016 ~ pancreas development | 9 | 0.001984 |
| GO:004886 ~ lung alveolus development | 7 | 0.0020672 |
| GO:0030900 ~ forebrain development | 22 | 0.0022447 |
| GO:007498 ~ mesoderm development | 11 | 0.0038993 |
| GO:001889 ~ liver development | 9 | 0.00531 |
| GO:0048567 ~ ectodermal gut morphogenesis | 5 | 0.0099191 |
| mmu5414:Dilated cardiomyopathy | 22 | 2.44E−07 |
| mmu4350:TNF-α signaling pathway | 17 | 1.15E−04 |
| mmu4310:Wnt signaling pathway | 21 | 0.0013417 |

Cluster 5: GOs and KEGG pathways including genes with low expression level only in KD 12-day EBs as compared with the other cell population

| GO:0007507 ~ heart development | 24 | 0.001835 |
| GO:0002076 ~ osteoblast development | 5 | 0.001952 |
| GO:0043414 ~ biopolymer methylation | 11 | 0.004197 |
| GO:0060348 ~ RNA processing | 37 | 0.006022 |
| mmu4910:Insulin signaling pathway | 18 | 3.68E−04 |
| mmu4810:Regulation of actin cytoskeleton | 23 | 8.59E−04 |
| mmu4310:Wnt signaling pathway | 18 | 8.99E−04 |

GOs include genes that are at least twofold up- or downregulated
control and KD 12-day EBs were confirmed by the comparison between Scr and WT versus Fam40b KD at 12-day post differentiation EBs. As shown in Figure 4a, there are relatively large differences between Scr 12-day and the KD 12-day EBs. Again, the transcriptome of the KD EBs was closer to undifferentiated WT, Scr and KD ESCs (all grouped together) than to the WT and Scr 12-day EBs (both grouped together). Compared with the transcriptome of the undifferentiated ESCs (WT, Scr and KD ESCs), the transcriptome of the KD 12-day EBs differed only in PC2 direction with a variance of <10%, whereas differences in PC2 were marginal. The k-means clustering shows the five clusters containing genes with a similar expression pattern (Figure 4b). Transcripts of the different five clusters and their representative annotation enrichment analysis are shown in Table 2. Cluster 1 transcripts indicated a high expression level in the WT, Scr and KD ESCs and in KD 12-day EBs but with a low expression level in the WT and Scr 12-day EBs. Again, genes associated with ncRNA processing, stem cell maintenance and epigenetic genes were identified to be highly upregulated not only in the WT, Scr and KD ESCs, but also in KD 12-day EBs as compared
Table 2 Selected significantly regulated GO-BPs as well as KEGG pathways in KD 12-day EBs versus Scr 12-day EBs

| Term | Transcripts | P-value |
|------|-------------|---------|
| **Cluster 1: GOs including genes indicated a high expression level in the WT, Scr and KD ESCs and in KD 12-day EBs but with a low expression level in the WT and Scr 12-day EBs** | | |
| GO:0034460 ~ ncRNA metabolic process | 74 | 1.95E–20 |
| GO:0034470 ~ ncRNA processing | 62 | 6.29E–19 |
| GO:0006396 ~ RNA processing | 109 | 2.77E–15 |
| GO:0043414 ~ biopolymer methylation | 25 | 5.71E–07 |
| GO:0040029 ~ regulation of gene expression, epigenetic | 22 | 2.35E–05 |
| GO:019827 ~ stem cell maintenance | 11 | 6.81E–05 |

| **Cluster 2: GOs including genes upregulated in the KD 12-day EBs as compared with the WT, Scr and 12-day EBs** | | |
| GO:0016125 ~ sterol metabolic process | 17 | 8.87E–08 |
| GO:006749 ~ glutathione metabolic process | 7 | 3.66E–04 |
| GO:001568 ~ blood vessel development | 23 | 5.78E–04 |
| GO:0006631 ~ fatty acid metabolic process | 19 | 7.00E–04 |
| mmu00100: Steroid biosynthesis | 6 | 6.18E–04 |
| mmu00052: Galactose metabolism | 7 | 8.98E–04 |
| mmu03320: PPAR signaling pathway | 11 | 0.002151043 |
| mmu00051: Fructose and mannose metabolism | 7 | 0.00489396 |

| **Cluster 3: GOs including genes with low expression levels in WT, Scr and KD ESCs and also in KD 12-day EBs as compared with WT and Scr 12-day EBs** | | |
| GO:0007507 ~ heart development | 79 | 1.70E–18 |
| GO:001568 ~ blood vessel development | 74 | 3.02E–13 |
| GO:0060537 ~ muscle tissue development | 48 | 2.20E–11 |
| GO:0048666 ~ gland development | 59 | 1.71E–10 |
| GO:001822 ~ kidney development | 40 | 1.82E–10 |
| GO:0060348 ~ bone development | 42 | 3.45E–10 |
| GO:0051216 ~ cartilage development | 32 | 1.14E–09 |
| GO:0048732 ~ regulation of signal transduction | 33 | 1.52E–09 |
| mmu007420: brain development | 71 | 2.16E–08 |
| GO:007417 ~ central nervous system development | 83 | 9.98E–08 |
| GO:0022008 ~ neurogenesis | 107 | 8.16E–07 |
| GO:0030324 ~ lung development | 34 | 1.14E–06 |
| GO:0016055 ~ Wnt receptor signaling pathway | 37 | 2.45E–06 |
| GO:0017015 ~ regulation of transforming growth factor-β receptor signaling pathway | 14 | 2.69E–05 |

| **Cluster 4: GOs including upregulated in all 12-day EBs populations in comparison with the undifferentiated ESCs populations** | | |
| GO:0001568 ~ blood vessel development | 30 | 2.17E–05 |
| GO:0001944 ~ vasculature development | 30 | 3.42E–05 |
| GO:0009966 ~ regulation of signal transduction | 57 | 1.91E–04 |
| GO:0019220 ~ regulation of phosphate metabolic process | 32 | 1.76E–04 |
| GO:0045597 ~ positive regulation of cell differentiation | 22 | 2.54E–04 |
| GO:0030324 ~ lung development | 14 | 0.0045939 |
| GO:001822 ~ kidney development | 13 | 0.0087518 |
| GO:0048666 ~ neuron development | 25 | 0.01569382 |
| GO:0022008 ~ neurogenesis | 40 | 0.0181755 |

| **Cluster 5: GOs including slightly downregulated genes in the KD 12-day EBs as compared with WT and Scr 12-day EBs** | | |
| GO:0007507 ~ heart development | 18 | 2.06E–04 |
| GO:0022008 ~ neurogenesis | 30 | 6.40E–04 |
| mmu04010: MAPK signaling pathway | 18 | 8.45E–04 |

with the WT and Scr 12-day EBs (Table 2). Figure 5 shows the high expression level of pluripotent genes (Figure 5a) and epigenetic genes regulating gene expression (Figure 5b) in the different cell populations. In cluster 2, primary metabolic processes associated with lipid metabolisms and some GOs involved in developmental processes (e.g., vessel development) were identified to be upregulated in the KD 12-day EBs as compared with the WT, Scr and 12-day EBs. Cluster 3 has been identified as the largest cluster containing transcripts that show low expression levels in WT, Scr and KD ESCs and also in KD 12-day EBs as compared with WT and Scr 12-day EBs. As expected, progressive differentiation of the WT and Scr ESCs was accompanied by highly enriched developmental GOs and KEGG pathways in the WTs and Scr 12-day EBs as compared with the KD 12-day EBs (Table 2). Cluster 4 genes show upregulation of genes in all 12-day EBs populations in comparison with the undifferentiated ESCs populations. These genes belong to developmental processes and signaling pathways and are apparently not affected by knockdown of Fam40b. The expression of genes in cluster 5 are slightly decreased in the KD 12-day EBs as compared with WT and Scr 12-day EBs. These genes mainly belong to developmental processes such as heart development and blood vessel development.

Cellular detection of Fam40b in undifferentiated ESCs. According to the Cell-PLoc 2.0 bioinformatics tool for
in Figure 6f (lower panel), the presence of Fam40b is not restricted only to the nucleus but also extends to the perinuclear and cytoplasmic domains of the ESCs.

**Fam40b KD ESCs fail to differentiate into beating cardiomyocytes.** Perturbation of cardiomyocyte differentiation processes of ESCs can be monitored by examining the increasing beating activity of EBs during differentiation of ESCs to cardiomyocytes. Therefore, to determine whether Fam40b is essential for differentiation of ESCs toward different cell types, we differentiated cells in ‘hanging drops’ using KD ESCs in comparison with the Scr ESCs and WT ESCs. On days 8, 10, 12 and 16 of differentiation, the number of beating EBs was counted from 50 EBs performed (= 100%). No cardiomyocyte beating activity was observed after differentiation of the KD ESCs (Supplementary Figure S1). Representatively, the beating activity of the control and KD 12-day EBs is shown in the video recordings in the Supplementary Movies M1 and M2, respectively. The control and Scr ESCs were able to differentiate into functional beating cardiomyocytes, in contrast to KD ESCs. These data show that Fam40b knockdown can cause a complete loss of differentiation potential toward functional cardiomyocytes.

**Fam40b knockdown in zebrafish causes heart defects.** To validate the *in vitro* studies suggesting an important role of Fam40 for differentiation/developmental processes including cardiomyogenesis under *in vivo* conditions, we knocked down Fam40b in zebrafish. Two morpholino oligonucleotides (MOs) directed against Fam40b and one control MO were injected into one- to two-cell-stage embryos of the *TG(fli:EGFP)* zebrafish line. To monitor a potential perturbation in the expression of cardiomyocyte-specific markers, we looked at the expression levels of cardiac *vmhc* and of cardiac *cmlc2* in 2-day MO1 Fam40b knockdown embryos. As shown, the Fam40b morphants exhibit impaired expression of *vmhc* and *cmlc2* as compared with control animals (Figure 7). Knockdown of Fam40b resulted in heart defects observable 48 h post injection. The most prominent effects were ventricle stagnancy, enlarged atrium and vascular defects. Ventricular stagnancy was observed in 54% (42 out of 78 embryos) of morpholino I (MO1)-treated embryos and in 51% (57 out of 111) of morpholino II (MO2)-treated embryos (Figure 7b). In addition, vascular defects were observed in 23% and 35% of the MO1 and MO2 morphants, respectively. In contrast, none of the control MO-injected embryos displayed any of these defects.

**Discussion**

Until now, the function of the novel transcript Fam40b was unknown. However, more recently there is increasing evidence that Fam40b is a part of the STRIPAK complex and therefore may be involved in the regulation of cell differentiation and cardiac function. To elucidate the role of Fam40b for cell differentiation, we silenced Fam40b in ESCs and investigated its role for the differentiation of ESCs. We were able to demonstrate that Fam40b encodes for a protein with a molecular weight of 96 kDa that is also located perinuclearly in cytosol and in the nucleoli of the ESCs. Knockdown of the Fam40b compromises the differentiation of...
mouse ESCs including cardiomyocytes. Key transcription factors required for maintaining pluripotency of stem cells include Pou5f1, Nanog and Sox2 (for review see Niwa19). These molecules are vital for maintaining the identity of the ICM during mouse preimplantation development.20–23 In addition to transcriptional regulation, epigenetic modifications, miRNAs and the cell-to-cell communications also participate in the maintenance of pluripotency and differentiation. We identified several additional genes such as Pou5f1 (also known as Oct4) and Nanog participating into the pluripotency
of ESCs as well as genes participating in the epigenetic regulation of the gene expression such as Hat1, Lin28a and Dnmt3b that are upregulated in the undifferentiated ESCs and in Fam40b KD 12-day EBs but downregulated in control 12-day EBs. In general, epigenetic regulation of gene expression occurs mainly via DNA methylation and/or histone deacetylation, both of which induced suppression of the gene expression. Gene silencing via hypermethylation of mainly CpG dinucleotides involves enzymatic methylation by DNA methyltransferases of cytosine residues. Approximately 60 to 70% of gene promoter regions overlap with CpG islands. Gene silencing by hypermethylation of the CpG islands plays an important role in the switch between the pluripotent and differentiation state of ESCs.24 There are two types of DNA methyltransferases: DNMT1 is a ‘maintenance’ DNA methyltransferase and is responsible for methylating cytosines at hemimethylated CpG sites. DNMT3A and DNMT3B are ‘de novo’ DNA methyltransferases and methylate completely unmethylated loci. During early embryonic development, Dnmt3a and Dnmt3b are expressed at high levels and apparently define the normal embryonic methylation pattern.25,26 In ESCs, DNMT3A and DNMT3B methylate Pou5f1 and Nanog promoters during differentiation. It has been established that the DNA methylation in vivo and in ESCs is indeed critical for early developmental processes, but is required for differentiation rather than for maintenance of the pluripotent state.24-26 Hat1 is significantly upregulated in Fam40b KD 12-day EBs, suggesting a possible role of Fam40b in the Hat1-associated epigenetic modulations. Recently, there is increasing evidence that Hat1 plays an important epigenetic role in the chromatin assembly of both nonmammalian and mammalian cells (for review see Parthun30). Epigenetic mechanisms including chromatin rearrangements by DNA methylation and histone modifications are implicated in mammalian development and are characteristic of differentiation of ESCs toward somatic cells.31 Hat1 is overexpressed in adult stem cells such as hematopoietic stem cells (HSCs)32 and neural stem cells.33 Interestingly, more recently, LIN28 has been identified as a pluripotency factor in mature nucleoli in mouse preimplantation embryos and also in ESCs where it colocalizes with nucleophosmin 1,34

Our findings suggest that Fam40b during development suppresses the expression of genes associated with RNA metabolic processes, as is indicated by our observation of an upregulation of RNA metabolic processes, including ncRNA processing, upon knockdown of Fam40. Although the role of ncRNA for the regulation of various biological processes remains unclear, more recently, some ncRNAs have attracted a great deal of attention because they seem to regulate gene expression via epigenetic regulation thereby, regulating embryogenesis (for review see Pauli et al.35). The ncRNAs include the so-called ‘housekeeping’ ncRNAs (ribosomal RNA, transfer RNA, small nuclear RNA and small nucleolar RNA), regulatory ncRNAs such as microRNA (miRNAs), endogenous siRNAs (endo-siRNAs) PIWI-interacting RNAs (piRNAs), long noncoding RNA (lncRNA) and several poorly characterized ncRNAs originating from regulatory elements.35

These results suggest that Fam40b, which has also been identified in the nucleoli of ESCs, might be involved in the regulation of ribosomal RNA (rRNA) expression and in the expression of other ncRNAs, thereby regulating the differentiation processes of ESCs. In the nucleolus, different processes for ribosomal biogenesis occur such as transcription of rDNA to rRNA and maturation of rRNAs that further assemble with ribosomal proteins. Thereafter, intact ribosomes can be released from the nucleoli to the cytosol to initiate the translation process (for review see Hernandez-Verdun et al.36). Therefore, the size and the organization of the nucleoli, which defines the number, and the maturation of the ribosomes are associated with cellular processes such as proliferation and differentiation.

For instance, the size of the nucleolus represents a diagnostic marker for the proliferative potential of cancer cells.36,37 We therefore suggest that Fam40b may interact with the ribosomal machinery, thereby participating in the control of the ESC differentiation status. Apparently, because of the absence of Fam40b, a perturbation of the ESC differentiation processes occurred including differentiation to cardiomycocytes.

More recently, it has been demonstrated that Fam40b is located in the cytosol and is involved in the regulation of cell morphology of HeLa and PC3 prostate cancer cells.11 Fam40B-depleted HeLa or Fam40B-depleted PC3 cells appeared to detach from each other or to reduce migration, respectively.10-12 Fam40B depletion in PC3 cancer cells increased elongation of the cells, suggesting a potential role in the regulation of cytoskeletal organization and cell morphology.10-12 It has lately been recognized that the cytoskeletal organization is different in undifferentiated ESCs. It has been demonstrated that the pluripotency of ESCs is associated with disorganized perinuclear actin cytoskeleton, whereas progressive differentiation of ESCs is associated with an organized actin cytoskeleton.38,39 Another regulator of the cytoskeleton involved in differentiation processes is Sirt2. Recently, it has been shown that knockdown of Sirt2 by the shRNA approach resulted in promotion of mesodermal and endodermal cells while inhibiting ectodermal cells.39 In summary, we may conclude that the novel gene Fam40b is essential for early and late lineage commitment of mESCs via mechanisms involving pluripotency and epigenetic networks.

Materials and Methods
Culturing and differentiation assays of ESCs. The loss-of-function experiments were performed with murine CGR8 ESC (European Collection of Cell Cultures (ECACC) No. 95011018). The cells were maintained on (0.2%) gelatinized tissue-culture dishes in feeder-free conditions in a standard ES culture medium consisting of Glasgow’s minimum essential medium (GMEM, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, GibCO, Grand Island, NY, USA), 2 mmol/l -glutamine, 100 units/ml leukemia inhibitory factor (LIF-1, Calbiochem, San Diego, CA, USA) and 50 µmol/l β-mercaptoethanol (Invitrogen GmbH) as described previously.40 The cells were passaged on alternate days and maintained confluence between 60 and 70%. ESC differentiation was induced by the conventional ‘hanging drop’ protocol, as described previously.42 Briefly, 20 µl hanging drops were made in a 10 cm low adhesion dishes from trypsin-dissociated ESC suspension (2.5 × 10⁴ cells/ml) prepared in differentiation medium (Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies, Darmstadt, Germany) supplemented with 20% fetal calf serum, 1% nonessential amino acids, 2 mM l-glutamine and 100 µM β-ME. Plates were incubated at 37°C, 5% CO₂ in a humidified incubator for 2 days. EBs thus formed were harvested by washing and resuspended in differentiation medium. The EBs were incubated at 37°C in 5% in an CO₂ incubator under shaking conditions, with a medium change every alternate days. EBs were monitored for beating areas
called cardiomyocyte foci starting from day 7 after differentiation on an inverted phase contrast microscope (Zeiss Axiosvert25, Oberkochen, Germany). The numbers of beating EBs were counted and representative videos were captured (Sony DFW-X700, Sony Corporation, Tokyo, Japan).

**Transfection of vectors into undifferentiated ESCs and generation of a constitutive Fam40b KD ESC line.** The shRNA expression vector pGFP-V-RS targeting mouse Fam40b (TR508344A plasmid) and the scrambled plasmid (TR30013) were purchased from OriGene (Rockville, MD, USA). The shFam40b target sequences on its mRNA were 5'-GCAAGACACT-GCACTACCAGAGCTAACTCAGAT-3', pGFP-V-RS control, the shRNA vector (TR589344A plasmid) and the scr shRNA vector (TR30013) were linearized. Using Bio-Rad Gene Pulser (Hercules, CA, USA) electroporator, 25 μg of linearized vector was transfected into 10^6 ESCs suspended in phosphate-buffered saline (PBS) free of Ca^2+ and Mg^2+ salts. The electroporation conditions were 500 μF and 240 V as described previously. The electroporated cells were cultured on gelatinized tissue culture flasks for 2 days and eventually selected by treatment with 2 μg/ml puromycin. On day 10 of selection, green fluorescence was monitored under blue excitation light through a fluorescence microscope, after which the resistant clones were picked for further experiments and amplified following standard ESC culture conditions. The clones were passaged at least four times before use in the experiments to obtain a stable gene expression profile. The shFam40b cell line generated with the TR508344A plasmid was used to study gene expression changes during differentiation by forming EBs.

**Microarray analysis.** Total RNA was isolated from the Fam40b KD 12-day EBs, control 12-day EBs and WT undifferentiated ESCs using RNeasy mini kit (Qiagen, Hilden, Germany). Then, 100 ng total RNA was used for aRNA amplification with GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA). After 16 h of biotinylated in vitro transcription, amplified RNA (aRNA) was purified and 15 μg of purified aRNA was fragmented with fragmentation buffer. Next, 12.5 μg of fragmented aRNA was hybridized with Mouse Genome 430 2.0 arrays (Affymetrix) for 16 h at 45°C. Arrays were washed and stained with fluorescence. Microarray analysis. The raw data were background corrected, summarized and normalized using RMA algorithm executed by R microarray packages. A PCA was performed to observe the samples transcriptional variability. Significantly regulated transcripts were determined by empirical Bayesian linear model applied using the LIMMA package in R. The significance of the change was calculated correcting the P-value of the t-score with false discovery rate using Benjamini–Hochberg method at significance of 0.01. The mRNA levels were normalized against endogenous control (β-actin) levels and calculated using the cycle threshold (Ct) method. The primers used in real-time PCR analysis are summarized in Supplementary Table S2.

**Western blotting.** Equal quantities (40 μg) of protein lysate prepared in sodium dodecyl sulfate (SDS) cell lysis buffer (Thermo Fisher Scientific) were resolved in denaturing (10%) polyacrylamide gels. Separated proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to Immobilon-P or nitrocellulose membranes at 4 °C for 2 h at 250 mA, membranes were blocked for 1 h in 5% non-fat milk/Tris-buffered saline and Tween-20 (TBST) buffer, incubated with primary antibody overnight, washed three times for 10 min, incubated in secondary antibody for 1 h, washed five times for 10 min and developed using enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Inc., Rockford, IL, USA) on a photographic film (Kodak Biomax, Rochester, NY, USA).

Polyclonal antibodies against Fam40b were generated by Thermo Fisher Scientific by immunization of rabbits with the peptide Fam40b433-450 (KVRQKDIEHFLEMSRNKF). The Fam40b polyclonal antibody (sc-162799) was purchased from Santa Cruz Biotechnology. Anti-β-actin (A2282) and anti-GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology. Anti-FAM40B IgG (SA5-10052) antibodies and anti-Mouse IgG alkaline phosphatase (A3562) were purchased from Thermo Fisher Scientific and Sigma. Donkey anti-Goat IgG (secondary antibody 926-32214) was obtained from LI-COR Biosciences (Lincoln, NE, USA).

**Immunohistochemistry.** To visualize the localization of Fam40b, the ESCs were seeded at ~2 × 10^6 cells/cm^2 on 0.1% gelatin-coated cover slips and cultured at 37°C and 5% CO_2 at 24 h before experiments. At a confluence of 70%, the cells were fixed with 4% paraformaldehyde (PFA) in PBS (10 mM sodium phosphate, 2.7 mM KCl, 140 mM NaCl, pH 7.4) for 15 min and then permeabilized with 0.4% Triton X-100 diluted in blocking buffer (5% BSA diluted in PBS) for 15 min at room temperature (RT). After washing the cells three times with PBS, 5 min each, and blocking for 45 min at RT, the cells were incubated with Fam40b antibody (sc-162799; Santa Cruz Biotechnology) diluted 1:200 in blocking buffer for 1 h at RT. Excessive antibodies were washed off with PBS and cells were incubated with donkey anti-goat IgG-FITC secondary antibody (sc-2024 Santa Cruz Biotechnology) diluted 1:200 in blocking buffer for further 1 h, followed by 3 washes with PBS, 5 min each. To visualize the nucleus the cells were incubated with Hoechst 33342 (Life Technologies, Carlsbad, CA, USA) diluted (1:1000) in blocking buffer for 1 h. After the final wash, the cells were fixed on microscopic slides with PolyGlass cover sealing medium (Polysciences, Warrington, PA, USA). Images were captured with an inverted fluorescence microscope (Zeiss Axiovert 200) or, for higher subcellular resolution of nuclei and nucleoli, with the Olympus (Hamburg, Germany) Fluoview1000 confocal system as described earlier.

**Detection of Fam40b in ESCs via HaloTag.** To detect the localization of the Fam40b protein we applied the HaloTag technology (Promega Corporation, Madison, WI, USA) after transient transfection of undifferentiated ESCs with pFN21K HaloTag CMV Flexi vector containing the Fam40b cDNA. The HaloTag reporter protein (~33 kDa) is an engineered, catinally inactive derivative of a hydrolase that forms a covalent bond with HaloTag ligands. ESCs (WT, 10^3 cells) were plated and cultured in gelatinized six-well plates at a confluence of ~50 to 70%. The cells were then transfected with a mixture of 1 μg vector and 2 μl TurboFect (Thermo Fisher Scientific) in 200 μl DMEM. After 6 h of culturing, culture medium (4 ml) was replaced with fresh culture medium. After 48 h, Fam40b protein was detected using the HaloTag Oregon Green ligand by confocal fluorescence microscopy as described in the manual. In addition, the protein was also detected by immunocytochemistry after fixing the cells with 4% paraformaldehyde/0.2 M sucrose/1 × PBS using primary Anti HaloTag pAb and Alexa Fluor 594 anti-mouse IgG as a secondary antibody (1:500 dilution; Life Technologies).

**Morpholino design and microinjection in zebrafish embryo.** WT and transgenic (fli1;EGFP, a kind gift from Dr. Gerd-Jörg Rauch, Heidelberg, Germany) zebrafish stocks were maintained at 28.5 °C at 14-h light/10-h dark cycle.
Embryos were obtained from natural spawning and staged as previously described. Embryos were treated with 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma) after 24 hpf to inhibit pigmentation. Morphological changes were described under a stereomicroscope (Leica, Heerbrugge, Switzerland). Morpholino antisense oligonucleotides (MOs) were obtained from Gene Tools (Corvallis, OR, USA). To identify putative zebrafish orthologs, BLAST searches were performed using predicted peptide sequences (NCBI Unigene, http://www.ncbi.nlm.nih.gov/unigene) as a query for the Ensembl Zebrafish peptide database. The CDNA sequences of the transcripts of interest were obtained from NetAffx (Affymetrix). Respectively, two MOs were designed against zFam40b and targeted to inhibit translation. Control injections to assess off-target effects were performed with a mismatch MO. Morpholino antisense oligonucleotide sequences are as follows: zFam40b I-5'-TAGACATACAAACCCGAGCCGCTCCAT-3'; zFam40b II-5' mismatch MO 5'-CTCTTACCTGCTCGTTAAATTTAA-3'. The MOs were diluted to 0.6 to 1.0 mM in 2× H2O, 0.1 M KCl and 0.2% phenol red. Embryos were injected at the one-to-two cell stage using a Femtojet (Eppendorf, Hamburg, Germany) and fixed at appropriate time points. MO-injected embryos were compared with uninjected and mismatch MO (MMO)-injected controls from the same clutch.

**In situ hybridization.** Whole mount in situ hybridization was performed as previously described using an automated InsituPro system (Abimed, Langenfeld, Germany). Digoxigenin-labeled RNA probes were prepared using an RNA labeling kit (Roche, Indianapolis, IN, USA) and stained using BM purple (Roche). Whole-mount embryos were imaged on a Leica stereo microscop fitted with a Zeiss AxioCam color camera. For generating in situ probes, following PCOR primers were used (forward and reverse primers are flanked with T3 and T7 promoter sequences respectively): zFam40b-Forw-T3-5'-AATTAACCTCCTAATAGGGAGAAGGAGGATGAGACT-3'; zFam40b-Rev-T7-5'-TAATACGACTCACTATAGGGTTCGCTCGTGGTCTT-3'; rhm-Forw-T3-5'-AATTAACCTCCTAATAGGGAGGAAAGAGCAACCCCG-3'; rhm-Rev-T7-5'-TAATACGACTCACTATAGGGTTCGCTCGTGGTCTT-3'; amhc-Forw-T3-5'-AATTAACCTCCTAATAGGGAGGATGAGACT-3'; amhc-Rev-T7-5'-TAATACGACTCACTATAGGGTTCGCTCGTGGTCTT-3'. Statistical analysis. If not indicated in the text, analysis was performed by one-way pairwise ANOVA test. The P-values of <0.05 are considered as statistically significant.

**Conflict of Interest**

The authors declare no conflict of interest.

**Acknowledgements**

This work was supported by a DFG grant to Agpios Sachindis (SA 568/17-1). 1. Ikuchi K, Fukuda M, Ito T, Inoue M, Yokoi T, Chiku S et al. Transcripts of unknown function in multiple signaling pathways involved in human stem cell differentiation. Nucleic Acids Res 2009; 37: 4987–5000. 2. Campero P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N et al. The transcriptional landscape of the mammalian genome. Science 2005; 309: 1559–1563. 3. Cheng J, Kapranov P, Drenkow J, Dike S, Brubaker S, Patel S et al. Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. Science 2005; 308: 1149–1154. 4. Bertone P, Stocic V, Royce TE, Rozowsky JS, Urban AE, Lander ES et al. Global identification of human transcribed sequences with genome tiling arrays. Science 2004; 306: 2242–2246. 5. Kapranov P, Willingham AT, Gingeras TR. Genome-wide transcription and the implications for genomic organization. Nat Rev Genet 2007; 8: 413–423. 6. Willingham AT, Gingeras TR. TUF love for 'junk' DNA. Cell 2008; 125: 1215–1220. 7. Mattick JS. The genetic signatures of noncoding RNAs. PLoS Genet 2008; 4: e1000459. 8. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155–159. 9. Gingeras TR. Origin of phenotypes: genes and transcripts. Genome Res 2007; 17: 682–690. 10. Hessing J, Pallas DC. STRIPAK complex: structure, biological function, and involvement in human diseases. Int J Biochem Cell Biol 2013; 47C: 118–148. 11. Bai SW, Herrera-Abreu MT, Rohn JL, Racine V, Tajudra V, Ryu YS et al. Identification and characterization of a set of conserved and new regulators of cytoskeletal organization, cell morphology and migration. BMC Biol 2011; 9: 54.
41. Andressen C, Stocker E, Klinz FJ, Lenka N, Hescheler J, Fleischmann B et al. Nestin-specific green fluorescent protein expression in embryonic stem cell-derived neural precursor cells used for transplantation. Stem Cells 2001; 19: 419–424.
42. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003; 19: 185–193.
43. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004; 3: Article 3.
44. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998; 95: 14863–14868.
45. Dennis G, Sherman BT, Lane HC, Lemicki RA. Identifying biological themes within lists of genes with EASE. Genome Biol 2003; 4: R70.
46. Potta SP, Sheng X, Gaspar JA, Meganathan K, Jagtap S, Ptasnikuste K et al. Functional characterization and gene expression profiling of alpha-smooth muscle actin expressing cardiomyocytes derived from murine induced pluripotent stem cells. Stem Cell Rev Rep 2012; 8: 229–242.
47. Westerfield M. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio). 2nd edn. University of Oregon Press: Eugene, 1993.
48. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203: 253–310.
50. Nasevicius A, Eisser SC. Effective targeted gene knockdown in zebrafish. Nat Genet 2000; 26: 216–220.
51. Schulte-Merker S, Ho RK, Herrmann B, Nusslein-Volhard C. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development 1992; 116: 1021–1032.
52. Pickett G, Gajewski M, Gehlke G, Gausepohl H, Schloscher J, Ibrahim H. Automated in situ detection (AISD) of biomolecules. Dev Genes Evol 1997; 207: 362–367.

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)