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Early embryonic lethality in gene trap mice with disruption of the *Arfgef2* gene

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ABSTRACT The switching of ADP-ribosylation factors from the inactive form to the active form is catalyzed by ARF-GEF (ADP ribosylation factor - guanine nucleotide exchange protein) proteins containing a Sec7 domain. The murine *Arfgef2* gene encoding the BIG2 protein belongs to the class of high molecular mass (>100 kDa) ARF-GEF proteins. BIG2 is believed to be associated with the trans-Golgi network and the recycling endosomes. In humans, mutations in the *ARFGEF2* gene cause autosomal recessive periventricular heterotopia with microcephaly. To elucidate the function of BIG2 in mouse we studied a gene-trap mouse line with a functional disruption of the *Arfgef2* gene. Heterozygous mutants did not reveal phenotypic abnormalities and were fertile. However, no homozygous embryos were obtained from breeding heterozygous females and males. To explore the reason for embryonic lethality, we analysed the pattern of expression of *Arfgef2*. *Arfgef2* transcripts were detected in several adult tissues. Interestingly, *Arfgef2* undergoes alternative splicing and the splicing pattern differs among tissues from adult animals. Moreover, the *LacZ* reporter gene of the gene-trap construct was used to reveal the expression of *Arfgef2* during embryonic development. Here, we show that *Arfgef2* mRNA is stored in the oocyte and is likely translated during the first embryonic divisions. SNP (Single Nucleotide Polymorphism) markers were used to demonstrate that the embryonic *Arfgef2* gene is activated first at the 4-cell stage, suggesting an important role for embryonic development. This assumption is supported by the failure of *Arfgef2*-deficient oocytes fertilized with *Arfgef2*-deficient sperm to develop into 4-cell stage embryos. Our results indicate that murine BIG2 is essential for early embryonic development.

KEY WORDS: *gene-trap, embryonic development, zygotic gene activation, BFA, ADP-ribozylation*

Introduction

The recruitment of coat proteins to membrane for the generation of transporting vesicles at intracellular sites between Golgi, ER and plasma membrane is controlled by the ADP ribosylation factors, small GTPases that cycle between GTP-bound active form and GDP-bound inactive form. The activation of ARFs requires the activity of guanine nucleotide exchange proteins (GEF, Moss and Vaughan, 1998; Kirchhausen, 2000). All ARF–GEFs identified so far possess a Sec7 domain composed of 200 amino acids as a minimum unit for the catalysis of replacement of GDP to GTP (reviewed in Donaldson and Jackson, 2000; Jackson and Casanova, 2000). The ARF–GEF protein family can be divided into high and low molecular weight proteins, on the basis of sequence similarity and functional differences (Donaldson and Jackson, 2000; Jackson and Casanova, 2000). High-molecular GEFs are further subdivided into Gea/GBF and Sec7/BIG groups

Abbreviations used in this paper: ARF, ADP ribosylation factor; BFA, brefeldin A; GEF, guanine nucleotide exchange proteins; LacZ, β-galactosidase; TGN, trans-Golgi network.

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(Ramaen *et al*. 2007; Ishizaki *et al*. 2008). So far in mammals only one member of the Gea/GBF group, the GBF1 has been described, that primarily functions in the trafficking between the cis-Golgi and ER-Golgi intermediate compartment (Claude *et al*. 1999; Kawamoto *et al*. 2002; Zhao *et al*. 2002; 2006; Garcia-Mata *et al*. 2003). From the Sec7/BIG group BIG1 and BIG2 proteins were purified from bovine brain together with macromolecular complexes (>670 kDa) (Morinaga *et al*. 1996). Several studies reported the association of BIG proteins with the trans-Golgi network (TGN) (Mansour *et al*. 1999; Yamaji *et al*. 2000; Shinotsuka *et al*. 2002a,b; Zhao *et al*. 2002), in addition, the association with recycling endosomes was also demonstrated (Shin *et al*. 2004; Shen *et al*. 2006). BIG1 and BIG2 proteins revealed considerable similarities to each other in the sequence and domain organization (Mouratou *et al*. 2005), however, it is still not clear whether both proteins play redundant or distinct role in the control of membrane trafficking. BIG1 but not BIG2 is necessary for β1 integrin glycosylation by Golgi enzymes (Shen *et al.,* 2007). On the other hand the knockdown of BIG1 and BIG2 by RNAi in cell culture indicated that BIG1 and BIG2 play redundant role in trafficking between *trans*-Golgi network (TGN) and endosomes (Ishizaki *et al*. 2008). In mouse BIG1 and BIG2 are encoded by *Arfgef1* and *Arfgef2* genes, located at chromosome 1 and 2, respectively. The *Arfgef2* gene is brightly expressed in different tissues (Togawa *et al*. 1999)

To analyse the role of this gene in mouse embryonic development we have identified a gene-trap line with vector integration in the *Arfgef2* gene. Our data indicate that murine zygotic copies of the *Arfgef2* gene are activated in 4-cell stage embryos and that this activation is essential for early embryonic development. Moreover, our results suggest that *Arfgef2* function can not be compensated by *Arfgef1*.

Results

Characterization of the Arfgef2 *gene-trap mouse line*

Here we report the characterization of a gene-trap mouse line in which the gene trap vector was integrated in mouse *Arfgef2* gene. To identify the trapped gene a 5' rapid amplification of cDNA ends (RACE) analysis from known vector sequences was performed. A 900 bp fusion transcript was amplified in brain RNA isolated from a heterozygous male. Sequence analysis of the RACE PCR product revealed that flanking sequences of the gene trap vector derived from the mouse *Arfgef2* gene (Gene ID: 99371). Sequence alignment suggested that the integration of the gene-trap construct occurred downstream of exon 7 of the *Arfgef2* gene. To prove the integration on genomic level a genomic phage library of a heterozygous mouse was generated and screened using a β-galactosidase gene specific probe. Sequence analysis of identified clones demonstrated that the gene-trap vector was integrated in intron 7 of the *Arfgef2* gene (Fig. 1A).

The gene trap vector contains a polyadenylation recognition motif just after the LacZ reporter gene indicating that a putative fusion transcript lacks part of the *Arfgef2* gene, including sequences encoding for the sec7 domain (Fig. 1B). It can be assumed that such truncated transcripts do not encode for functional ARFGEF2 polypeptides. The deduced protein (if any produced) encoded by the trapped transcript will be truncated up-

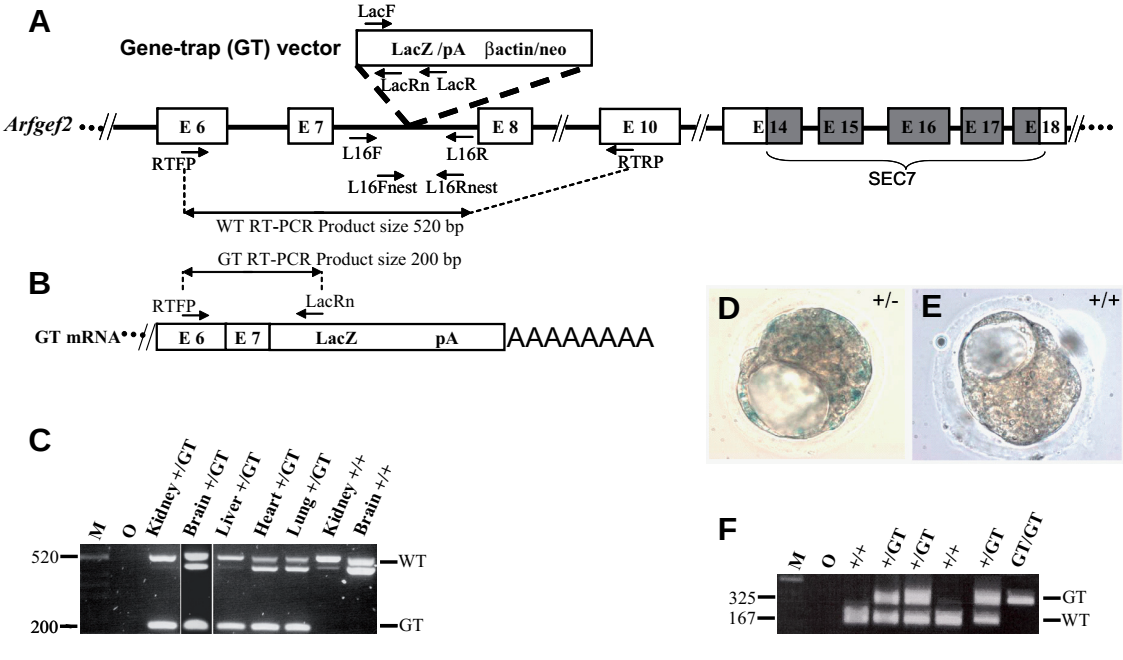


Fig. 1. Characterization of the gene-trap vector integration site. *Gene-trap vector integration had occurred after the exon 7 of the mouse* Arfgef2 *gene* **(A)**, *thus the resulting fusion transcript* **(B)** *lacks sequences encoding the sec7 domain (resulted protein is truncated after amino acid in position 309). Positions of primers used for expression analyses are indicated.* **(C)** *In RT-PCR analysis a 200 bp fusion transcript was detected in all tissues isolated from heterozygous animal (+/-). In addition two approximately 500 bp amplification products were observed, derived from the wild type* Arfgef2 *allele. In wild type control RNA no fusion transcript could be amplified, whereas both wild type transcript variants were present. The expression of the reporter protein lacZ was monitored using β-galactosidase staining in heterozygous blastocysts* **(D)**. *Positive staining was observed in both, the inner cell mass and in cells of the trophectoderm.* **(E)** *No staining in wild type blastocysts was observed.* **(F)** *Heterozygous animals were crossbred together and embryos at different developmental stages were genotyped using PCR strategy (2-cell stage embryos genotyping is given as an example on this figure). Positions of primers and expected product size are given in A. The 167 bp product represents the wild type allele (WT) while the 325 bp fragment was amplified from the gene-trap allele (GT). M – molecular-mass standard, +/+ - wild type, +/GT – heterozygous and GT/GT – homozygous gene trap animal.*

stream the sec7 domain, after the amino acid 309. Therefore, we analysed the expression pattern of the *Arfgef2*-gene in wild type and heterozygous animals by RT-PCR. The primer combination RTFP and RTRP (Fig. 1A) was used to amplify a 520 bp product representing the wild type allele. For transcripts derived from the trapped allele the primer pair RTFP and LacRn (Fig. 1B) was utilized to amplify a 200 bp product. This analysis revealed that transcripts of the wild type allele as well as of the trapped allele could be amplified in RNA from kidney, brain, liver, lung and heart tissue isolated from a heterozygous mouse while in the RNA from a wild type control only the 520 bp fragment was detected (Fig. 1C). Moreover, RT-PCR analysis demonstrated alternative splicing of the'*Arfgef2* gene. In addition to the 520 bp PCR product a second *Arfgef2* cDNA fragment was amplified lacking sequences of exon 7 (Fig. 1C lower WT band).

Expression of the trapped gene was also investigated using β-galactosidase staining. Reporter gene activity could be detected in adult tissues (data not shown) and in different embryonic developmental stages. In blastocysts inner cell mass as well as trophoectoderm cells were stained (Fig. 1D). No staining was observed in wild type control (Fig. 1E).

RT-PCR results proved that integration of the gene trap vector construct into intron 7 of the *Arfgef2* gene generated a fusion transcript and resulted in inactivation of the function of the trapped *Arfgef2* allele. Animals heterozygous for the integration of the gene trap vector were fertile and did not demonstrate any obvious malformation. However, genotype analyses of more than 200 offspring from heterozygous breeding detected no mice homozygous for the integration. This result indicates that homozygous inactivation of the *Arfgef2* gene leads to embryonic lethality. To determine the time of embryonic lethality embryos were genotyped by PCR. First we analysed embryos after the implantation stage, however, we could not detect any *Arfgef2* deficient embryos (data not shown). Then fertilized oocytes were isolated and cultured *in vitro* up to the blastocyst stage. Also no *Arfgef2* deficient embryos could be detected in 4 cell, morula or blastocyte stages. In contrast, in the syngamy and 2-cell stages homozygous *Arfgef2* deficient embryos were detected, although some of them demonstrated progressive fragmentation of the cytoplasm (Fig. 1F, Table 1). It should be pointed that the ratio of homozygous, heterozygous and wild type embryos did not correspond to expected Mendelian ratio because PCR genotyping failed by some embryos.

This finding indicates that disruption of the *Arfgef2* gene function by the gene-trap approach causes embryonic lethality at first stages of embryonic development.

Brefeldin A treatment inhibits embryonic cell division

It has been demonstrated that ARFGEF2 is inhibited by brefeldin A (BFA) (Togawa *et al*. 1999), therefore we tested whether BFA treatment inhibits early embryonic development in mouse. Wild type female mice were superovulated and mated with wild type males. Females positive for a vaginal plug were sacrificed and fertilized oocytes were isolated. Using light microscopy isolated fertilized oocytes were tested for the presence of two pronuclei and then cultured in the medium containing BFA. As controls fertilized oocytes were cultured in medium alone and in medium added with methanol because BFA was solved in methanol. At BFA concentration of 10 μg/ml (35 μM) we observed an inhibition of embryonic cell division, but embryos were still alive, because after removing BFA the effect was reversible. A total of 70 fertilized oocytes were used in each group. After 24 hours of incubation with BFA embryos were washed, counted and moved to the medium without BFA. As demonstrated in table 2 BFA inhibited significantly ($p<0.01$) the first embryonic division and no embryo reached the 4-cell stage. After transferring oocytes to culture medium (without BFA) almost all embryos restored the ability to divide (44 out of 46) but they were delayed in embryonic development by 1 day as compared to the control embryos (incubated from the beginning without BFA). By using higher BFA concentration complete inhibition of embryonic division was induced, however, this effect was not reversible and most of embryos died (data not shown). These results support the assumption that disruption of the function of *Arfgef2* (BIG2) leads to an arrest of the embryonic development at the very early stage.

Arfgef2 *undergoes alternative splicing*

RT-PCR analyses of the *Arfgef2* in heterozygous gene-trap or wild type mice have identified two *Arfgef2* transcript variants (Fig. 1C). This prompted us to analyze putative alternative splicing of this gene. The genomic organization of the mouse *Arfgef2* gene was obtained from the ENSEMBL database (www.ensembl.org) and series of primers were designed to cover the complete mRNA of this gene (supplementary table 1D). Besides the splicing variant without exon 7 no additional alternative splicing products were observed using RNA extracted from adult brain (Fig. 2A) or kidney (data not shown). Complete transcript was designated as *Arfgef2a* while transcript missing exon 7 was denoted as *Arfgef2b*. The exon 7 spans 69 bp and encodes for 23 amino acids, thus alternative splicing of exon 7 preserves the reading frame in the *Arfgef2b* transcript variant. Using different computer programs (http://smart.embl-heidelberg.de, Schultz *et al*., 1998) no known domain could be predicted within the 23 amino acids encoded by exon 7.

Next, the alternative splicing of exon 7 was analysed in different tissues. Primers located in exons 6 and 10 were used to amplify both transcript variants. In all analysed tissues both transcript variants could be detected. However, different intensities of the amplification products of *Arfgef2a* and *Arfgef2b* were observed (Fig. 2B). In RNA isolated from eye and brain both variants displayed approximately same intensity. In contrast, in kidney, liver, spleen, ovary and testis *Arfgef2a* seemed to be more

TABLE 1

GENOTYPING OF EMBRYOS OBTAINED FROM THE BREEDING OF HETEROZYGOTE MALE WITH HETEROZYGOTE FEMALE

	Embryo's genotype				
Development	**WT/WT**	**WT/GT**	**GT/GT**	**unknown**	**Total**
Syngamy degraded	0	1	8	28	37
Syngamy	21	23	5	42	91
2-cell degraded	4	2	3	8	17
2-cell	85	107	7	35	234
4-cell degraded	1	0	2	2	5
4-cell	93	101	0	42	236
8-cell	10	18	0	3	31
Morula	22	34	0	23	79
Blastocyst	31	62	0	23	116

abundant, whereas in RNA from heart, lung and muscle tissues preferentially the *Arfgef2b* was detected. The splicing pattern was also analysed during embryonic development (Fig. 2C). Both transcript variants were detected from unfertilized oocyte up to embryonic day 18.5. At 4-cell stage both variants were of the same intensity, whereas in more advance stages of embryonic development as well as in embryonic stem cells (ESC) the *Arfgef2a* variant was more abundant. The expression in testis was analysed in more detail to clarify whether stronger *Arfgef2a* expression in the testis is due to germ cells or somatic tissue. RNA isolated from adult testes of mutant mouse strains with spermatogenesis arrested at different stages was analyzed by RT-PCR: *W/W^v* mutants have no germ cells in the testis, while in *Tfm* mice primary spermatocytes can be detected. In the *olt/olt* mutant spermiogenesis is affected after step 13 and in *qk/qk* mice elongated spermatids can be found (step 16, Lyon and Searle, 1989; Chubb, 1992). Moreover, *Arfgef2* expression was analyzed in germ stem cells (maGSCs, Guan *et al.*, 2006). Similar expression patterns were observed in maGSCs and in *W/W^v* mutants indicating that the stronger expression of the *Arfgef2a* variant in the testis is due to somatic as well as germ cells (Fig. 2C).

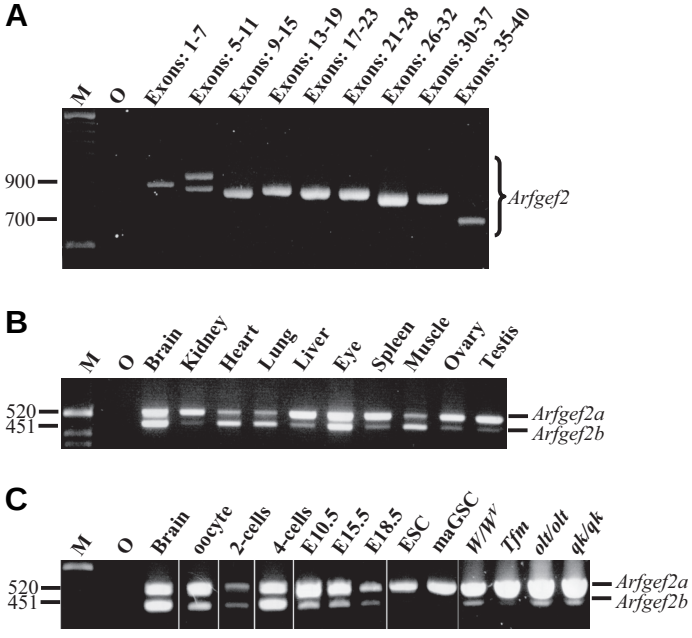


Fig. 2. Analyses of alternative splicing of the *Arfgef2* gene. *Series of primers were designed to cover whole mRNA of the* Arfgef2 *gene (accession number in the ENSEMBL data base ENSMUST00000099078). RNA was isolated from tissues of 129Sv mice. RT-PCR analysis revealed that two alternative spliced products of the* Arfgef2 *gene could be amplified with primers located in exons 5 and 11* **(A)**. *Sequence analysis revealed that the larger transcript (*Arfgef2a*) contains exons 5 to 11 while the shorter transcript (*Arfgef2b*) lacks sequences derived from exon 7.* **(B)** *RT-PCR was used to investigate alternative splicing in different tissues. Results suggest a tissue specific expression pattern of both transcript variants. In contrast to brain and eye where similar intensity of both variants were observed, in kidney, liver, spleen, ovary and testis the* Arfgef2a *is predominantly expressed whereas in heart, lung and muscle* Arfgef2b *is expressed at higher level.* **(C)** *The alternative splicing could also be visualized during mouse embryonic development, in germ cells (maGSC), embryonic stem cells (ESC) and in the testis from different mutants. Abbreviations: maGSC, multipotent adult germline stem cells; ESC, embryonic stem cells; Tfm, testicular feminization; olt/olt, oligotriche, qk/qk, quaking; W/W^v, white spotting.*

TABLE 2

BFA TREATMENT OF FERTILIZED OOCYTES

A

	Day E0,5	Day E1,5					
	isolated 1-cell	degraded	1-cell	2-cell	4-cell	sum 2/4-cel (%)	sum all
M16	**70**	11	16	42	1	**43 (61%)**	**70**
M16 + MeOH	**70**	6	15	44	3	**47 (69%)**	**68**
M16 +BFA	**70**	22	27	19	0	**19 (28 %)***	**68**

Embryos were moved into M16 without BFA

B

	Day E3,5						
	degraded	1-cell	2-cell	4-cell	8-cell	mor/blast	sum
from M16	6	11	14	23	6	5	**65**
from M16 + MeOH	10	12	14	16	9	7	**68**
from M16 +BFA	21	2	24	17	3	0	**67**

(A) Fertilized oocytes were cultured in medium containing BFA (35 mM, row M16+BFA). As controls fertilized oocytes were cultured in medium alone (row M16) and in medium added with MeOH (row M16+MeOH). After 24 hours of incubation with BFA embryos were washed, counted, moved to the medium without BFA and cultured for further two days **(B)**. *- the number of 2/4-cell stage embryos cultured in medium with BFA was significantly reduced as compare with those cultured in M16 or M16 + MeOH, p<0.01.

Zygotic Arfgef2 *gene is activated at 4-cell stage*

The lethality of *Arfgef2*-deficient embryos during the first steps of development indicated that the ARFGEF2 protein encoded by the embryonic genome acts very early in mouse embryonic development. *Arfgef2* mRNA can be detected in unfertilized oocyte (Fig. 2C) and in different embryonic stages. To determine the stage of embryonic development in which the embryonic copy of the *Arfgef2* gene is activated, the SNP data base (http://www.ncbi.nlm.nih.gov/SNP) was explored to indentify polymorphic markers within the *Arfgef2* cDNA sequence. Two SNPs differentiating 129/Sv and C57Bl strains were identified in exon 20 and 21 of *Arfgef2* (Fig. 3A). For the test five 129/Sv females were mated with five C57Bl males and vice versa (Fig. 3B). Two-cell embryos were isolated from vaginal plug positive females. From approximately 40 embryos the mRNA was directly prepared, while the rest of the 2-cell embryos were cultured in M16 medium until 4-cell and 8-cell stages. Approximately thirty 4-cell stage and thirty 8-cell stage embryos were collected for RNA isolation. After reverse transcription the cDNA was used as a template in a PCR reaction with primers L16AGCTFP and L16AGCTRP (Fig. 3A, supplementary table 1E). PCR products were purified and cloned into pGEM-Teasy vector and analysed by sequencing. Because it was not possible to discriminate between maternal RNA storage and *Arfgef2* transcripts derived from the maternal allele, only the activation of the paternal *Arfgef2* allele could be determined. Thirty-two clones were sequenced derived from the 2-cell stage embryos and all represented the maternal SNP variant. In contrast, out of 17 clones from 4-cell stage embryos 13 represented the maternal SNP transcript and 4 clones revealed the paternal SNP sequence. From the 8-cell stage 14 sequences were analysed and 6 clones represented the maternal transcript while 8 sequences harbour the paternal SNP (Fig. 3C). This result indicates

that the activation of the paternal *Arfgef2* allele occurs at the 4-cell stage in the mouse.

Discussion

We have analysed a mouse gene trap line in which the function of the *Arfgef2* gene encoding BIG2 was affected. The insertion of the gene trap vector occurred in intron 7 of the *Arfgef2* gene resulting in a putative truncated gene product missing essential domains of the encoded BIG2 polypeptide. *Arfgef2* transcripts were detected in all investigated tissues of wild type mouse suggesting a ubiquitous expression of this gene. Moreover, alternative *Arfgef2* transcripts missing exon 7 were identified, although the physiological relevance of the splice variant is unknown. Also in human alternatively spliced variants were reported for *ARFGEF1*, *ARFGEF2* and *GBF1* (Claude *et al*. 2003; Mouratou *et al*. 2005). However, for the *ARFGEF2* gene the alternative splicing affects exon 35 (Mouratou *et al*. 2005). *Arfgef2* transcripts were detected in oocytes indicating maternal storage and putative function of *Arfgef2* in first steps of zygotic development. In mammals maternal mRNA deposited in the oocyte are degraded shortly after fertilization and can control only first few cell divisions (Thompson *et al*. 1998). Zygotic genome activation (ZGA) occurs very early during embryonic development. In the mouse, a minor burst of ZGA toward the end of the one-cell stage is followed by a major burst during the two-cell to four-cell stages and then the mid-preimplantation gene activation (MGA) occurs (Latham *et al*. 1992; Vernet *et al*. 1992; Aoki *et al*. 1997; Thompson *et al*. 1998; Schultz, 2002; Hamatani *et al*. 2006). The *Arfgef2* zygotic activation belongs to the major ZGA at 4-cell stage. The *Arfgef2* mRNA stockpile deposited in oocyte might explain the first embryonic division in some *Arfgef2*$^{GT/GT}$ homozygous embryos. However, the lack of 4-cell mutant embryos indicates fast wastage of the stored *Arfgef2* mRNA.

In contrast to the mouse phenotype, mutations in either of two genes, *Filamin A (FLNA)* or *ADP-ribosylation factor guanine exchange factor 2 (ARFGEF2)* were described as the underlying causes of periventricular heterotopia in human (Fox *et al*. 1998; Sheen *et al*. 2004; de Wit *et al*. 2009). In three families autosomal recessive inherited mutations have been identified in the *ARFGEF2* gene. In one family a child with inherited double frame shift mutation followed by premature stop codon was reported. The duplication c.2031-2038 was located within the Sec7 domain and the deletion c.3798-3802 after the sequence encoding for this domain (de Wit *et al*. 2009). In one pedigree reported by Sheen *et al*. (2004) the 625G-A transition in exon 6 was found, which resulted in amino acid substitution E209K. Interestingly, in another pedigree from the same report a complex mutation in exon 3 with two nucleotide substitutions and homozygous single base deletion was found in an affected patient resulting in a predicted premature protein truncation upstream of the Sec7 domain (Sheen *et al*. 2004). Although, the predicted truncated protein lacks the Sec7 domain, similar as it could be suggested for the gene trap insertion, different phenotypes resulted in human and mouse. This observation could reflect different mechanisms controlling the GDP to GTP exchange in human and rodents. It has been demonstrated that Tyrphostin AG1478 disperses *cis*-Golgi network by inhibiting GBF1 in different human cells whereas rodent cells seems to be resistant to AG1478 (Pan *et al*. 2008). Moreover, activities of other proteins that are controlling the GDP to GTP exchange may be regulated in different ways in human and rodents. This can explain the distinct consequences of BIG2 inactivation in human and mouse. It should be also noted that in human the molecular characterization of *ARFGEF2* function is widely restricted to its role in neuron migration (Guerrini *et al*. 2008; Spalice *et al*. 2009) while BIG2 role in early human embryonic development still remains unknown. The gene trap mutation shows an autosomal recessive mode of inheritance. The heterozygous animals appeared normal therefore, the semi-dominant model seems to be unlikely,

A

Arfgef2 part of cDNA sequence (position 2891-3168):

L16AGCTFP

ATTGGCCTGCAGAACTGTGATGACACGGAGGTGGCCTCCCTGTGTTTG

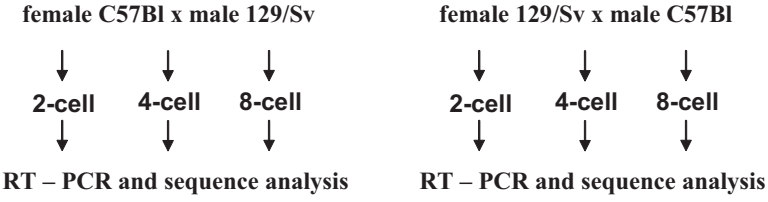
GAAGGCATCAGGTGTGCAGTCCGCATTGCCTGCATCTTTGG**R**ATGCA
GCTGGAACGAGATGCCTATGTTCAGGCCCTTGCTCGCTTTTCCCTGCTG
ACAGCCAGCTCCAGCATCACAGAAATGAAGCAGAAAAACATCGACAC

CATAAAGACACTCATCACTGTGGCTCACAC**Y**GATGGCAACTACCTTG
GCAACTCCTGGCATGAGATCTTGAAGTGCATCAGCCAGCT

L16AGCTRP

Arfgef2 SNPs:
rs29577446 Chr2:166554884f **R=A/G** C57Bl – A, 129/Sv – G
rs27295574 Chr2:166556023f **Y=C/T** C57Bl – C, 129/Sv – T

B



C

Arfgef2 allele expression

Number of clones analyzed	Embryonic stage		
	2-cell	4-cell	8-cell
Total	32	17	14
Maternal allel	32	13	6
Paternal allel	0	4	8

Fig. 3. *Arfgef2* expression during early embryonic development. *To ascertain the onset of* Arfgef2 *gene expression during embryonic development two single nucleotide polymorphisms (SNPs): rs29577446 and rs27295574 were identified in ENSEMBL database for differentiation between* Arfgef2 *transcripts derived from C57Bl6 and 129/Sv mouse strains* **(A)**. *C57Bl females were bred with 129/Sv males and vice versa 129/Sv females with C57Bl males* **(B)**. *Two-cell, 4-cell and 8-cell embryos were isolated.* Arfgef2 *transcripts were amplified by RT-PCR with primers L16AGCTFP and L16AGCTRP (marked as underlined sequence), cloned and sequenced.* **(C)** *In 2-cell embryos no paternal allele expression could be identified, whereas in RNA from 4-cell and 8-cell embryos* Arfgef2 *transcripts derived from both, maternal and paternal alleles were found.*

although we can not exclude it completely. Embryonic lethality of homozygous mice suggests a los-of-function mode of action by the gene trap insertion.

Brefeldin A (BFA) has been used as an inhibitor of BIG proteins (Mansour *et al*. 1999; Togawa *et al*. 1999; Shin and Nakayama 2004). Interestingly, in molluscs the inhibition of protein processing and secretion by BFA treatment resulted in abnormal bilateral cleavage of 3D blastomere (Gonzales *et al*. 2007). The subsequent body plan of BFA-treated mollusc embryos was radial symmetric. In mice, blastocysts incubated with BFA resulted in reduced outgrowth and reduced fibronectin binding activity of trophoblast cells (Schultz *et al*. 1997). Even BFA concentration of 400 μM did not compromise blastocyst viability, since embryos exposed to this inhibitor retained their ability to outgrowth. In contrast, we have observed that in BFA concentrations more than 40 μM fertilized oocytes or 2-cell stage embryos died after 24 h of incubation (data not shown). This observation probably indicates that the very early embryonic development is particularly sensitive to the inhibition of protein trafficking. The cytotoxic character of BFA was also demonstrated on human Jurkat-T cell line (Guo *et al*. 1998) and PC 12 cells incubated with BFA over 24 hrs (Chen and Gao 2002). In *C. elegans* BFA disrupted the terminal phase of cytokinesis of the EMS blastomere (Skop *et al*. 2001). Blastomere cells start to divide symmetrically instead of asymmetrically and the furrow regresses. As a result of this event the EMS cell had two nuclei. The other blastomere P2 attempted to divide but has regressed (Skop *et al*. 2001). We could demonstrate that BFA can partially resemble the phenotype of *Arfgef2*$^{GT/GT}$ mutant embryonic development. One can argue that BFA inhibits not only BIG2 but also GBF1 and BIG1, however, it has been demonstrated that overexpression of GBF1 and BIGs have opposite effects on the sensitivity to BFA (Claude *et al*. 1999; Shinotsuka *et al*. 2002b; Manolea *et al*. 2008). Moreover, the phenotype of GBF1-depleted cells by using siRNA differed significantly from those observed in cells incubated with BFA (Szul *et al*. 2007). On the other hand the expression of a catalytically inactive mutant of BIG2 induces membrane tubulation similar to the effect of BFA treatment (Shin *et al*. 2004). BIG1 and BIG2 form heterodimers (Yamaji *et al*. 2000) therefore, we can not exclude that BFA inhibition of BIG1 might also influences cell division in early embryonic development, however, the observations that homozygous *Arfgef2*$^{GT/GT}$ embryos died at the 2/4 cell stage clearly indicates that BIG1 can not compensate the lack of BIG2 in mouse.

Taken together, we conclude that *Arfgef2* (BIG2) function is essential for early steps in mouse embryonic development and this function can not be compensated by *Arfgef1* (BIG1).

Materials and Methods

Generation of the Arfgef2 *gene trap mouse*

The gene trap line was generated in a large-scale gene trap-screening programme as described before (Adham *et al*. 2008). Briefly, the gene trap vector consists of a splice acceptor (SA) sequence, the encephalomyocarditis virus internal ribosome entry site (IRES) that directs the translation of a fusion protein with β-galactosidase followed by the simian virus 40 late poly (A) signal, next the beta-actin promoter was located controlling the activity of the neomycin-resistance gene. The generation of recombinant ES-cell line 2C98 was performed as described previously (Salminen *et al*. 1998). From chimeric males mated with NMRI females heterozygous progeny were obtained and genotyped by PCR using LacF, LacR primers localized in βGeo cassette (Chowdhury *et al*. 1997, Supplementary table 1A). To generate F2 animals heterozygous males and females were crossbred. To distinguish between wild type and gene trap allele a PCR reaction with primers L16F, L16R and LacR (Supplementary table 1A) was established. All animal experimentations were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Goettingen.

Embryo collection and genotyping

Heterozygous males and females were mated. The day of vaginal plug was counted as day E0.5. Fertilized oocytes (syngamy stage) were collected at day E0.5 and tested for pronuclear stages using light microscopy. Pronuclear stage embryos were incubated for 4-6 hours in M16 (Sigma) medium to allow the pronuclei to fuse (syngamy) and were than frozen until further analysis. Embryos at 2-cell stage were flushed out from oviduct at day E1.5. To avoid contamination with maternal cells, 2-cell stage embryos were cultivated in M16 medium (Sigma) at 37°C and 5% CO_2 until they developed to 4-cell, 8-cell, morula or blastocyst stages. In order to remove polar bodies, zona pellucida was dissolved in a drop of Tyrode's acid solution and zona-free embryos were transferred into the drop of M2 medium (Sigma). Next, embryos were incubated in the drop of trypsin (Trypsin/EDTA, PAN Biotech Germany) and the dissociation of polar bodies was controlled in light microscope. All embryos were collected individually in a PCR tube with 4 μl of water, boiled at 95°C for 10 min and subsequently frozen at -80°C for 15 min. This boiling/freezing step was repeated again and the DNA obtained from lysed embryos was used for PCR based genotyping. For the first PCR reaction 16 cycles for amplification were done and then 5 μl were used as template in a nested PCR with following primers: L16Fnest, L16Rnest and LacRn (Supplementary table 1A). The size of the amplified fragment was 167 bp for the wild type allele and 325 bp for the gene trap allele.

LacZ staining of blastocysts

Blastocysts were flushed out from the uterus at the day E4.5 with M2 medium (Sigma), rinsed with PBS and fixed for 10 min at 4°C with 0.25 % glutaraldehyde in PBS. After four washes in PBS, the embryos were incubated in the staining solution containing 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma Chemical), 1mM $MgCl_2$, 10 mM potassium ferricyanide and 10 mM potassium ferrocyanide in PBS. Incubation was carried out for 12h at 37°C. Stained embryos were analysed using an Olympus BX60 light microscope with software equipment.

5'RACE PCR

Cloning of the gene-trap fusion transcript was performed by using 5'RACE System (Version 2.0, GibcoBRL, Karlsruhe, Germany) according to the manufacturer's instruction. The cDNA was synthesized using the GSP1 primer and 1 μg of total brain RNA isolated from a heterozygous mouse. After the TdT tailing the cDNA was used as a template in PCR amplification with GSP2 and AAP (5'RACE System) primers. The first PCR product was diluted 10 times and used in second PCR amplification using GSP3 and AUAP (5'RACE system) primers. Primer's sequences are given in supplementary table 1B. The resulting PCR product was purified, cloned into the pGEM-Teasy vector (Promega, Mannheim, Germany) and sequenced.

Construction of genomic phage library

To determine the site of gene-trap vector integration, a genomic phage library was generated in the λDASH-II vector (Stratagene) using DNA of a heterozygous mouse as described previously (Nayernia *et al*. 2003). Using the β-galactosidase gene specific probe recombinant clones were identified and sequenced. Sequences flanking the gene

trap vector insertion were identified using the BLAST program (Altschul *et al*. 1990).

RT-PCR analysis

Total RNA (1μg) was reverse-transcribed with oligo(dT)-primers in a final volume of 20 μl using 200 units of *Superscript* reverse transcriptase (Invitrogen, Karlsruhe, Germany). PCR was carried out with 2 μl of cDNA, 10 pmol of forward and reverse primers each and 3 units of *Taq* polymerase. The primer combination RTFP and RTRP (Supplementary table 1C) generated two products (520 and 451 bp) representing the two wild type splice variants whereas by using primers RTFP and LacRn a 200 bp gene trap fusion transcript was amplified. Alternative splicing of the *Arfgef2* gene was determined by primers sets located in different exons of the gene (Supplementary table 1D) and RT-PCR condition as described above.

Brefeldin A treatment

Female mice were superovulated and mated with males. Vaginal plug positive females were sacrificed and oocytes were isolated from oviduct. Oocytes were monitored for the presence of two pronuclei under the light microscope and fertilized oocytes were cultivated in M16 medium (Sigma) containing brefeldin A (BFA, Sigma-Aldrich, Steinheim, Germany) solved in methanol. The cell culture was performed in 5% CO_2 at 37°C, as described previously (Schultz *et al*. 1997). As a control fertilized oocytes were cultivated in normal medium and in medium containing methanol. After 24 hours of BFA incubation embryos were washed, counted and transferred into the M16 medium without BFA and cultivated for further 48 hrs. Finally, embryos were analysed under the Olympus BX60 microscope. Results were pooled for embryos at 2 and 4 cell stage and 2 x 2 chi-square statistic was calculated to test the differences in frequency of 2/4 cell stage embryos in the group treated with BFA against frequency of 2/4 stage cell embryos from the group cultured in M16 or M16 with methanol. A p value <0.01 was considered as significant.

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