Essential role for Abi1 in embryonic survival and WAVE2 complex integrity

Patrycja M. Dubielecka^{a,1}, Kathrin I. Ladwein^{b,1}, Xiaoling Xiong^a, Isabelle Migeotte^c, Anna Chorzalska^a, Kathryn V. Anderson^c, Janet A. Sawicki^d, Klemens Rottner^{b,e}, Theresia E. Stradal^{b,f}, and Leszek Kotula^{a,2}

^aLaboratory of Cell Signaling, New York Blood Center, New York, NY 10065; ^bHelmholtz Centre for Infection Research, D-38124 Braunschweig, Germany; ^cDevelopmental Biology Program, Sloan-Kettering Institute, New York, NY 10065; ^dLankenau Institute for Medical Research, Wynnewood, PA 19096; ^eInstitute of Genetics, University of Bonn, 53117 Bonn, Germany; and ^fInstitute for Molecular Cell Biology, University of Münster, D-48149 Münster, Germany

Edited* by Hilary Koprowski, Thomas Jefferson University, Philadelphia, PA, and approved March 15, 2011 (received for review November 11, 2010)

Abi1 interactor 1 (**Abi1**) plays a critical function in actin cytoskeleton dynamics through participation in the **WAVE2** complex. To gain a better understanding of the specific role of **Abi1**, we generated a conditional **Abi1**-KO mouse model and MEFs lacking **Abi1** expression. **Abi1**-KO cells displayed defective regulation of the actin cytoskeleton, and this dysregulation was ascribed to altered activity of the **WAVE2** complex. Changes in motility of **Abi1**-KO cells were manifested by a decreased migration rate and distance but increased directional persistence. Although these phenotypes did not correlate with peripheral ruffling, which was unaffected, **Abi1**-KO cells exhibited decreased dorsal ruffling. Western blotting analysis of **Abi1**-KO cell lysates indicated reduced levels of the **WAVE** complex components **WAVE1** and **WAVE2**, **Nap1**, and **Sra-1/PIR121**. Although relative **Abi2** levels were more than doubled in **Abi1**-KO cells, the absolute **Abi2** expression in these cells amounted only to a fifth of **Abi1** levels in the control cell line. This finding suggests that the presence of **Abi1** is critical for the integrity and stability of **WAVE** complex and that **Abi2** levels are not sufficiently increased to compensate fully for the loss of **Abi1** in KO cells and to restore the integrity and function of the **WAVE** complex. The essential function of **Abi1** in **WAVE** complexes and their regulation might explain the observed embryonic lethality of **Abi1**-deficient embryos, which survived until approximately embryonic day 11.5 and displayed malformations in the developing heart and brain. Cells lacking **Abi1** and the conditional **Abi1**-KO mouse will serve as critical models for defining **Abi1** function.

cell motility | lamellipodium | Rac | Arp2/3-complex | Hssh3bp1

Essential physiological processes such as cell migration, adhesion, and endocytosis are dependent on the coordinated remodeling of the actin cytoskeleton, but the complexity of the mechanisms at play is only beginning to be appreciated (1, 2). Nucleation of actin filaments is catalyzed by different types of protein complexes, the most prominent of which include the Arp2/3 complex and members of the formin family of proteins (3). The Arp2/3 complex is regulated by additional proteins called “nucleation-promoting factors” (NPFs). The most prominent NPFs include the WASP and WAVE subfamilies (4), not only because they constitute key signaling nodes that connect activation signals from the Rho family of small GTPases with the actin polymerization machineries but also because the N-WASP and Arp2/3 complexes have emerged as frequent targets for viral or bacterial pathogens that usurp the host actin cytoskeleton for their needs (5).

Abi1 interactor 1 (**Abi1**) is one of the major regulators of actin cytoskeleton reorganization through participation in several multiprotein complexes that either associate with actin filaments through spectrin (6) or Eps8 (7) or that regulate the dynamics of actin polymerization more indirectly, for instance through regulation of small GTPase activities of the Rho family (4, 8). Rho GTPases are modulated by complexes of **Abi1** with Eps8–Sos1 (9, 10) and downstream actin polymerization e.g. by N-WASP (11) and **WAVE** complex (12–14). The ubiquitous **WAVE** complex, comprising the direct Rac interactor **Sra-1/PIR121**, its binding partner **Nap1**, plus **Abi1**, **HSPC300/Brick1**, and **WAVE2**, is considered to be the essential functional output module linking Rac1

activation to actin polymerization that drives lamellipodia protrusion at the plasma membrane (13, 14). **WAVE1** and **WAVE3** were proposed to have roles analogous to **WAVE2** in the complex (8). Although the function of **WAVE3** is less defined, the critical role of the other two **WAVEs** in dorsal ruffle formation was demonstrated (15, 16). Mechanistically, it is proposed that native **WAVE2** complex is inactive (17–19) but is activated at the membrane by simultaneous interaction with prenylated Rac-GTP and acidic phospholipids as well as by serine/threonine phosphorylation (20). Rac1 does not interact directly with **WAVE** but instead binds to the **Nap1**-binding protein **Sra-1** (12) and to **Abi1** (21). The **Abi1**-mediated tyrosine phosphorylation of **WAVE2** by **Abi1** regulates **WAVE2** complex activity either by regulating conformation of the complex, as suggested (22), or by regulating interactions among the complex components or with other cellular targets (10, 23, 24). The interaction of **Abi1** with p85 (24) might modulate PI3 kinase activity (10); this activity would be consistent with coincident modulation of **WAVE** complex activity by its interaction with PIP3, or PIP3-mediated membrane recruitment of **WAVE2** (25).

The *Xenopus laevis* homolog of **Abi1**, *xlan4*, was demonstrated to be critical for CNS development, and **Abi2** might be similarly required in mice (26). In mice **Abi2** has regulatory functions in cell–cell adhesion, cell migration, and tissue morphogenesis (27). **Abi2**-KO mice exhibit a severe memory loss thought to be caused by deficiencies in dendritic spine and adherens junction formation and abnormal cell–cell communication (27). A role for **Abi** in regulation of the cytoskeleton and in cytokinesis has been confirmed in *Dictyostelium* (28). *Dictyostelium* has only one **Abi** gene; thus it offers no specific conclusion for roles of mammalian **Abi1** or **Abi2**. Moreover, numerous studies have concluded that **Abi** proteins have overlapping functions in actin cytoskeleton regulation (29, 30). Studies using conventional **Abi1** (31) and **WAVE2** (16) mutant alleles suggested a critical role of **Abi1** in embryonic development, thus suggesting that a conditional allele would provide more definitive information about **Abi1**, including its function in adult organs.

Here we present the initial characterization of MEFs isolated from a conditional **Abi1**-KO mouse. Biochemical analyses of **Abi1**-KO cells indicated that the presence of **Abi1** is critical for **WAVE2** complex integrity. As a physiological readout of **Abi1** gene disruption, we observed impairment of local actin polymerization processes leading to decreased dorsal ruffle formation and cell migration. We also found that **Abi1** is essential for embryonic development, because **Abi1**-deficient embryos survived only until embryonic day 11.5 (E11.5) and displayed malformations in the developing heart and brain.

Author contributions: P.M.D., K.V.A., J.A.S., K.R., T.E.S., and L.K. designed research; P.M.D., K.I.L., X.X., I.M., A.C., and L.K. performed research; P.M.D., K.R., T.E.S., and L.K. contributed new reagents/analytic tools; P.M.D., K.I.L., X.X., I.M., K.V.A., J.A.S., K.R., T.E.S., and L.K. analyzed data; and P.M.D., K.R., T.E.S., and L.K. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

¹P.M.D. and K.I.L. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: lkotula@gmail.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016811108/-DCSupplemental.

Fig. 2. Characterization of Abi1-KO MEF cell lines. (A) Cre-recombinase-mediated exon 1 deletion of *Abi1* in MEF cell lines. DNA from MEF cells was genotyped with primers DL75' and Neogene13' (*SI Materials and Methods* and Fig. S1). Genotyping of MEF cell lines based on the PCR result is indicated below the panel. Floxed, *Abi1*-floxed allele; deleted, exon 1-deleted allele; *Abi1* (fl/fl), genotype homozygous for the floxed allele; *Abi1* (del/del), genotype homozygous for the deleted allele; *Abi1* (fl/del), heterozygous genotype. Note that in cell line #8 clone d3 has both alleles, indicating that in this cell line Cre-recombinase failed to recombine on one allele; hence the heterozygous genotype, *Abi1* (fl/del), is observed. Primer sequences are listed in *SI Materials and Methods*. (B) Southern blot analysis of MEF cell genomic DNA. MEF cell genomic DNA was digested with *Afl*III enzyme and hybridized with the 543-bp PB1/2 probe. Expected sizes were 4.15 kb for wild type; 3.65 kb for the floxed allele (neomycin cassette deleted; *loxP*⁺/*neo*⁻); and 4.84 kb for the deleted allele [exon 1 and neomycin cassette deleted; *loxP*⁺/*neo*⁻ (Ex1del)]. (C) Cre-recombinase-mediated loss of *Abi1* expression in MEF #3 cell line subclones. Western blot analysis of *Abi1* expression in parental MEF #3 *Abi1* (fl/fl) and in exon 1-deleted *Abi1* (del/del) MEF cell lines. Cell lysates of the indicated cell lines were blotted with antibody 7B6 (23). Clones #3-1 through #3-11 are subclones of the parental MEF#3 obtained following transient Cre-recombinase expression. *Abi1*(+/+) MEF WT, mouse embryonic cells expressing the wild-type *Abi1* gene. Protein-stained gel of the samples used for Western blot was used as loading control.

the relatively low abundance of the predominantly neuronal-specific WAVE3 (34) in these cells.

Increased Levels of Abi2 Expression in an Abi1-KO Cell Line Reached Only 22% of the Endogenous Abi1 Level in the Control Floxed Cell Line. To determine the absolute concentration of *Abi1* proteins in cell lysates, predetermined amounts of purified recombinant *Abi1* or *Abi2* protein standards were added into MEF cell lysate sam-

ples from *Abi1*-KO and *Abi1* floxed (fl/fl) control cell lines. The comparative evaluation of the control recombinant *Abi1* and *Abi2* (Fig. S5) amounts vs. their endogenous levels, as detected by respective antibodies, allowed determination of the absolute amount of endogenous *Abi1* or *Abi2* proteins. Standard curves were generated by plotting the band intensities against the concentrations of control recombinant *Abi* GST-tagged proteins (~80 kDa) (Fig. S6). Based on the standard curves, we calculated that, although the relative intensity of *Abi2* was about two times higher in *Abi1*-KO cells than in control cells, the difference in the absolute amount of endogenous *Abi2* vs. *Abi1* in control cells was less than 10%: 0.914 ± 0.096 ng *Abi2* vs. 11.88 ± 0.55 ng *Abi1* per microgram of total protein in the sample. This evaluation indicated that despite enhancement of *Abi2* expression in *Abi1*-KO cells, its level did not exceed 22% of the endogenous *Abi1* in control cells. Thus, it seems likely that the failure to up-regulate *Abi2* sufficiently is one of the reasons *Abi2* does not reconstitute the WAVE complex in *Abi1*-KO cell lines.

Defect in Formation of PDGF-Induced Dorsal Ruffles in Abi1-KO MEF Cells. *Abi1*, as the critical component of WAVE2 complex, is known to be involved in actin reorganization resulting in lamellipodia and in actin-rich peripheral and dorsal ruffle formation upon growth factor stimulation (9, 30, 35). Therefore, we asked whether any defects in these structures were observed in cells lacking expression of a functional *Abi1* gene. We found that upon PDGF treatment, both *Abi1* control and *Abi1*-deficient cells displayed peripheral as well as dorsal ruffle formation, as assessed by Alexa Fluor 594-phalloidin staining, and WAVE2 was localized to these structures, as expected, although it was less prominent on average in *Abi1*-deficient clones (Fig. 4A). *Abi1* was highly enriched in dorsal ruffles and localized at the tips of ruffles and in the cell periphery in *Abi1* control cells but could not be detected in the *Abi1*-KO cell lines (Fig. 4A). Evaluation of the number and topology of dorsal ruffles revealed an ~50% decrease in the formation (Fig. 4B and C) and lower apparent prominence of these structures in *Abi1*-KO cells (Fig. 4D). This observation indicates an important role of *Abi1* in the formation of dorsal ruffles. However, despite the reported critical role of the WAVE2 complex in peripheral ruffle formation (15, 36) and the observed decrease in WAVE2 complex levels, PDGF-induced peripheral ruffling was not affected in *Abi1*-KO cells (Fig. 4B and C). Moreover, neither the expression levels of Eps8 and Sos1 nor total Rac activation levels in response to PDGF correlated with the loss of *Abi1*, indicating that defective Rac activation downstream of the Eps8/*Abi1*/Sos1 complex does not cause the observed dorsal ruffling phenotype (Fig. S4B and C).

Cells Lacking Abi1 Exhibit Cell-Motility Defects. *Abi1* has been implicated as a regulator of actin cytoskeleton-dependent cell motility, mainly as an integral component of the WAVE2 complex. Therefore, we examined *Abi1*-KO MEF cell lines in a series of motility assays (Fig. 5). Random cell motility was recorded by video microscopy and was analyzed using the cell-tracking tool (*SI Materials and Methods*). Control cells migrated an average of 390 μ m, whereas KO cells migrated an average of 260 μ m. The

Fig. 4. Cell morphologies of Abi1-KO cell lines. (A and B) Localization of Abi1 and WAVE2 in control and Abi1-deficient MEF cells after PDGF stimulation. Control Abi1 (fl/fl) and Abi1 (del/del) cell lines were grown on glass coverslips, serum-starved, and treated with PDGF. Cells then were immunostained with antibodies to Abi1 [anti-Abi1 (4E2)], WAVE2 (#1735), and phalloidin as described in [SI Materials and Methods](#). Abi1 and WAVE2 localized to circular dorsal and peripheral ruffles of precursor cells but were absent (Abi1) or weaker (WAVE2) in these structures in KO cells. (C) Quantification of dorsal and peripheral ruffling. (Left) MEF control (fl/fl), and Abi1-KO cells (del/del) were plated on glass coverslips and serum-starved overnight. Cells were stimulated with PDGF, fixed, and stained with Alexa Fluor 594-conjugated phalloidin to detect F-actin. (Right) The percentages of cells with different cell morphologies in response to PDGF treatment were quantified in the indicated MEF cell lines. Note the reduction of cells displaying dorsal (Upper) but not peripheral (Lower) ruffles in independent Abi1-null clones (c1, c2, d1, d2) compared with their respective control Abi1 (fl/fl) cells (#3 or #8), as indicated. Categories of cell morphologies for quantification were as follows: with ruffles, without ruffles, or with ambiguous morphology. At least 100 cells were analyzed and categorized for each condition. Columns show the percentage of cells of the respective morphology displayed as arithmetic means $\pm$ SEM of at least three independent experiments. (D) F-actin staining in PDGF-induced dorsal ruffles in control Abi1 (fl/fl) cells and in Abi1-KO MEF cells [Abi1 (del/del)]. F-actin staining of ruffles revealed different topology and apparent reduction of F-actin in dorsal ruffles in Abi1-KO cells vs. the control cell line. Images were taken from two focal planes, A (Left) and B (Right).

migration rate of control cells was $0.54 \mu\text{m}/\text{min}$, compared with $0.36 \mu\text{m}/\text{min}$ for KO cells. However, Abi1-KO cells exhibited increased directional persistence. The net distance vs. true distance ratio was 0.25 for control cells vs. 0.37 for KO cells (arithmetic means, $P < 0.0001$ for all comparisons). Thus, migration rate and distance were reduced, but directional persistence was increased in Abi1-KO MEF cells compared with control cells. In the wound-healing assay, a statistically significant impairment of wound closure by cells lacking Abi1 was observed: $3 \mu\text{m}^2/\text{min}$ by control cells vs. $2.39 \mu\text{m}^2/\text{min}$ by KO cells (Fig. 6 and [Movies S1, S2, and S3](#)). Taken together, these results indicate that Abi1 removal caused reduced wound-closure rates, probably because of the impairment in cell migration.

Discussion

Here we report the production and initial phenotypic characterization of MEF cells lacking a functional *Abi1* gene. Among other strategies used to generate *Abi1*-KO animals and cell lines, tar-

geting of exon 1 has proven successful. Following Cre-mediated recombination, MEF cells lacking *Abi1* mRNA and protein expression were obtained. In this study, we focused on the characterization of WAVE2 complex-dependent processes, knowing that participation in the complex is a critical aspect of *Abi1* function.

Abi1-KO MEF cells are viable and exhibit no apparent loss of ability to form lamellipodia, stress fibers, or peripheral ruffles. However, Abi1-KO cells displayed decreased efficiency of PDGF-induced dorsal ruffling formation. In addition, dorsal ruffles formed in Abi1-KO cells were much less prominent and displayed less intense F-actin staining compared with control cell lines. Because the levels of the Eps8/Sos1 complex did not correlate with defective dorsal ruffling, we assume that this phenotype is a consequence of the significant instability of WAVE2 complex in the absence of Abi1. The levels of WAVE2 complex components WAVE2, Sra-1/PIR121, and Nap1 were significantly lower in cells lacking Abi1. These data are consistent with previous siRNA knockdown experiments showing that upon down-

Fig. 5. Impaired motility of MEFs lacking Abi1. The parameters of random cell motility, migration distance, rate, and directional persistence were evaluated in Abi1-null cell lines (c1 and c2) and Abi1 floxed (fl/fl) cells. Cells were seeded under the same conditions and monitored by time-lapse microscopy. Different parameters of cell motility, as indicated above graphs ($n = 40$ movies per parameter/cell line) were evaluated using ImageJ. Directional persistence was determined as the ratio of total distance traveled over total length of the migration path. Data are presented as $\pm$ SEM, based on four independent experiments.

regulation of individual subunits, the remaining components of the WAVE2 complex are coordinately decreased (11, 37–39). Our results demonstrate that lack of Abi1 caused significant instability of the WAVE2 complex, thus confirming the critical role of Abi1 in the complex function.

In addition to WAVE2, we also observed a decrease in WAVE1 levels, suggesting that Abi1 might be incorporated into complexes with WAVE1, as shown previously (20), and not only

with WAVE2. Although WAVE1 originally had been ascribed a role in dorsal but not peripheral ruffle formation (15), this conclusion was contradicted more recently, because WAVE1 KO cells were observed to form multiple dorsal ruffles (36). Likewise, both WAVE1 and WAVE2 previously had been observed to accumulate at the tips of lamellipodia and peripheral ruffles (14, 40), thus indicating that potential functional differences between WAVE1 and WAVE2 are not caused by their differential functions in the formation of peripheral versus dorsal ruffles. Because Abi1-null fibroblasts have reduced expression of both WAVE2 and WAVE1, we speculate that the observed defect in dorsal but not peripheral ruffling simply reflects a higher sensitivity of dorsal ruffles to abrogation of WAVE complex activity rather than differential subcellular functions of WAVE1 versus WAVE2. This hypothesis is consistent with lower levels of total WAVE1/2/3 proteins in Abi1-null fibroblast clones compared with control cells. Consequently, lower WAVE levels cause lower output into Arp2/3 complex activity and F-actin polymerization, as indeed was observed (Fig. 4D). Other consequences of reduced WAVE complex function and Abi1 deficiency are decreased random and directed cellular motility.

Although Abi2 expression was more than doubled in cells lacking Abi1, this response did not compensate for the loss of Abi1 in the apparent loss of integrity of WAVE2 complex, nor did it compensate for loss of Abi1 function pertaining to dorsal ruffling and cellular motility, probably because the amount of Abi2 is too low (demonstrated here to be less than 10% of Abi1 in Abi1-expressing control cells). Nevertheless, the increased amounts of Abi2 and concomitant increase of WAVE3 may well contribute to the relative modesty of phenotypes observed in Abi1-deficient cells.

Abi1 or Abi2 can be incorporated interchangeably into WAVE2 complex. For example, in leukocytes, Abi1 was found in the WAVE2 complex and Abi2 in WAVE1 complex, whereas both Abi1 and Abi2 were present in WAVE2 complex in A431 cells (20). The basis for specificity of Abi protein incorporation into WAVE1 or WAVE2 complex is not known, although, based on high sequence conservation of binding sites, the affinities of the Abi proteins for WAVE proteins are expected to be similar (20, 22, 31). One explanation might be that Abi1 and Abi2 exhibit different patterns of threonine and serine phosphorylation (20), which is required for full activation of WAVE2 complex by Rac and acidic phospholipids (20). Nonetheless, concurrent down-regulation of WAVE1 and WAVE2 in Abi1-KO cells suggests that Abi1 incorporates into both WAVE2 and WAVE1 complexes or, alternatively, that reduced stability of subunits shared by both WAVE1- and WAVE2-containing complexes, such as Sra-1/PIR121 or Nap1, indirectly down-regulates WAVE1 expression. We cannot distinguish between these possibilities at present. Finally, the apparent dysregulation of WAVE3 expression also will have to be explored in the future.

We propose that loss of WAVE2 complex function and dysregulation of WAVE1 and WAVE3 caused by the lack of Abi1 might explain the early embryonic death of *Abi1*-null embryos. A nonoverlapping function of Abi proteins in embryonic development is indicated by the fact that Abi2 deficiency is not

Fig. 6. Wound-healing migration assay. Cells were seeded under identical conditions. After formation of a confluent monolayer, a wound was produced by scratching with a fine pipette tip. Rate of wound closure was determined by measuring the area remaining uncovered by cells per unit of time. Data represent means $\pm$ SEM of four independent assays of Abi1-KO cell lines (c1 and c2) and control (fl/fl) MEF cells.