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p38 and a p38-Interacting Protein Are Critical for Downregulation of E-Cadherin during Mouse Gastrulation

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SUMMARY

During vertebrate gastrulation, an epithelial to mesenchymal transition (EMT) is necessary for migration of mesoderm from the primitive streak. We demonstrate that p38 MAP kinase and a p38-interacting protein (p38IP) are critically required for downregulation of E-cadherin during gastrulation. In an ENU-mutagenesis screen we identified the *droopy eye* (*drey*) mutation, which affects splicing of *p38IP*. $p38IP^{drey}$ mutant embryos display incompletely penetrant defects in neural tube closure, eye development, and gastrulation. A stronger allele ($p38IP^{RRK}$) exhibits gastrulation defects in which mesoderm migration is defective due to deficiency in E-cadherin protein downregulation in the primitive streak. We show that p38IP binds directly to p38 and is required for p38 activation in vivo. Moreover, both p38 and p38IP are required for E-cadherin downregulation during gastrulation. Finally, p38 regulates E-cadherin protein expression downstream from NCK-interacting kinase (NIK) and independently of the regulation of transcription by Fibroblast Growth Factor (Fgf) signaling and Snail.

INTRODUCTION

Gastrulation in the mouse embryo begins at embryonic day 6.25 (E6.25) and results in the formation of the three definitive germ layers and establishment of the embryonic body plan (reviewed in Tam and Behringer, 1997). During gastrulation, the epiblast is a cup-like structure nestled within the visceral endoderm (Figure 3I). Mesoderm cells migrate through the primitive streak in the posterior region of the epiblast and undergo an epithelial to mesenchymal transition (EMT) and migrate away from the primitive streak. These morphogenic movements are mediated by regulated changes in cell adhesion (reviewed in Shook and Keller, 2003).

Fibroblast Growth Factor (Fgf) signaling in the primitive streak regulates EMT during mouse gastrulation. *Fgf8* and *Fgfr1* mutant embryos display gastrulation defects in which mesoderm fails to migrate away from the streak (Deng et al., 1994; Sun et al., 1999; Yamaguchi et al., 1994). Significantly, *Fgfr1* mutant embryos fail to express *Snail* and to downregulate expression of *E-cadherin* (Ciruna and Rossant, 2001). Snail acts as a transcriptional repressor to inhibit *E-cadherin* expression (Batlle et al., 2000; Cano et al., 2000), and in *Snail* mutant embryos *E-cadherin* is not downregulated (Carver et al., 2001). These data suggest a molecular pathway where Fgf signaling, through regulation of *Snail* expression, downregulates *E-cadherin* expression to promote EMT during gastrulation.

Targeted disruption of *NCK- interacting kinase/Map4k4* (*NIK*) results in a similar phenotype to *Fgfr1* mutant embryos in which mesoderm accumulates in the primitive streak (Deng et al., 1994; Xue et al., 2001; Yamaguchi et al., 1994). In contrast to *Fgfr1* mutants, *NIK* mutant embryos express molecular markers of mesoderm development, indicating that mesoderm is properly specified although it does not migrate. While the signal transduction pathway(s) downstream of Fgf and NIK in the gastrulating embryo have not been elucidated, both can activate MAPK cascades (reviewed in Bottcher and Niehrs, 2005; Buday et al., 2002). MAPKs are a family of conserved serine/threonine protein kinases that function in kinase cascades, resulting in phosphorylation and activation of transcription factors and/or additional kinases (reviewed in Chang and Karin, 2001; New and Han, 1998). In mice, the p38 group

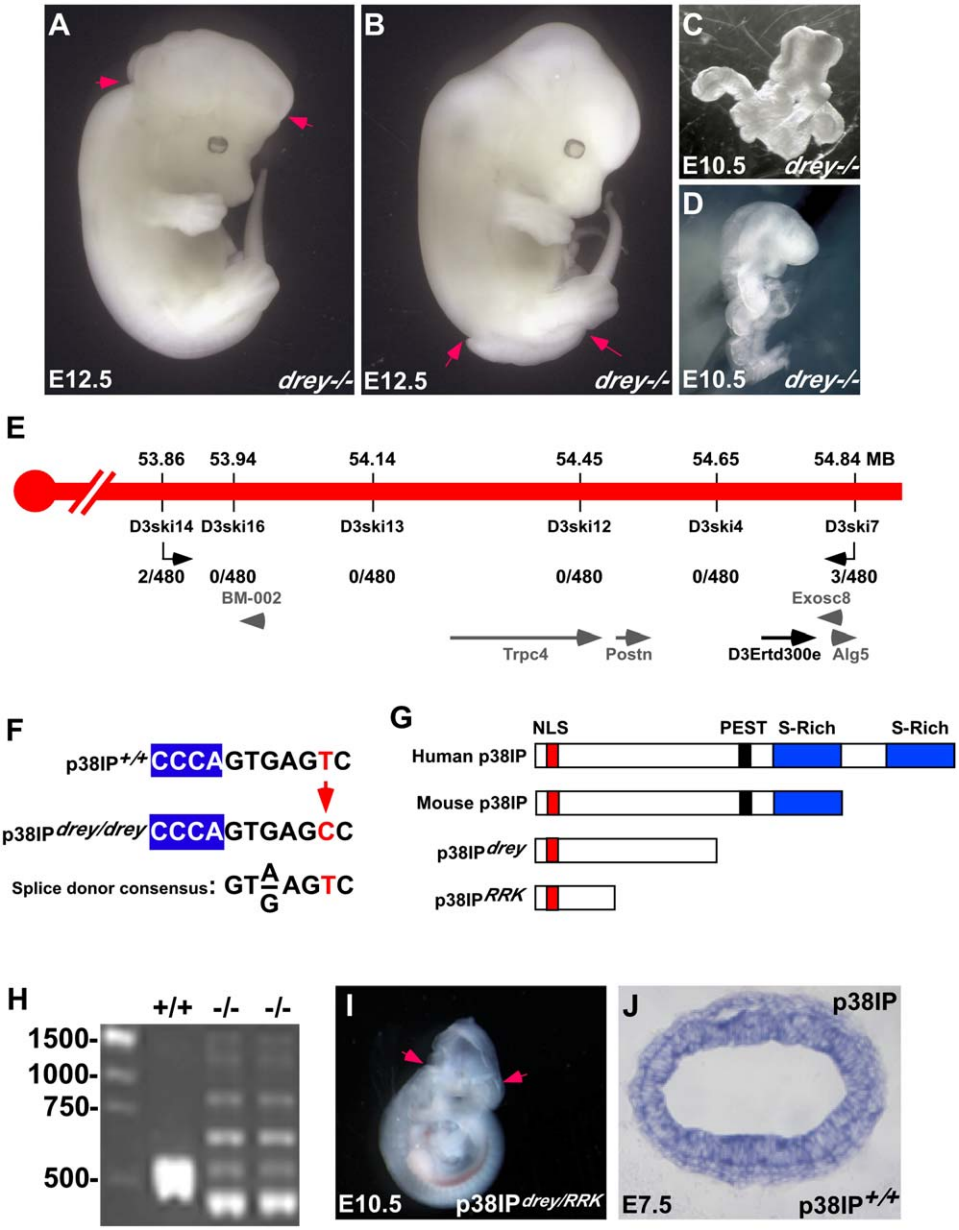

Figure 1. The *drey* Mouse Mutation Disrupts a p38-Interacting Protein

(A–D) *drey*$^{-/-}$ mutant embryos exhibit a range of phenotypes. (A) E12.5 mutant embryo with exencephaly (between red arrows) and the "droopy eye" phenotype. (B) E12.5 mutant with spina bifida (between red arrows). (C and D) E10.5 mutant embryos demonstrating defects in mesoderm development. (C) Embryo exhibits growth retardation, failure to turn, exencephaly, and various mesoderm defects including lack of somites. (D) Embryo exhibits growth retardation, posterior truncations, and an open neural tube.

(E) Genetic map of *drey* interval on mouse chromosome 3. The number of recombination events over number of opportunities for recombination is indicated for each polymorphic marker. Markers D3ski16, D3ski13, D3ski12, and D3ski4 never separated from the *drey* phenotypes. Within this interval are six transcription units: BM02 (BM-002/ubiquitin folding modifier), Trpc4 (receptor-activated cation channel), Postn (periostin precursor [PN]/osteoblast-specific factor 3[OSF2]), D3Ertd300e (p38-Interacting Protein), Exosc8 (exosome complex exonuclease or RRP43/ribosomal processing protein 43), and Alg5 (Dolichyl-phosphate β-glucosyltransferase).

(F) The *drey* ENU-induced mutation results in a T to C transition (red) in the splice donor consensus sequence following exon 14 (blue box) in D3Ertd300e encoding a p38-interacting protein (p38IP).

(G) Predicted protein motifs in p38IP: a nuclear localization sequence (NLS, red), PEST sequence (black), and Serine-rich regions (S-Rich, blue). The human and mouse transcripts have 2202 bp and 1590 bp open reading frames that encode 733 and 530 amino acid proteins, respectively. The p38IP^{drey} and p38IP^{RRK} mutations result in truncated proteins at 317 and 133 amino acids, respectively.

is represented by four genes: *p38α/MAPK14, p38β/MAPK11, p38δ/MAPK13*, and *p38γ/MAPK12*. Deletion of either the *p38γ* or *p38β* genes does not result in any detectable developmental phenotype, while a deletion of the *p38δ* gene has not been reported (reviewed in Kuida and Boucher, 2004). $p38\alpha^{-/-}$ embryos die during midgestation due to a placental defect (Adams et al., 2000; Mudgett et al., 2000). However, treatment of early mouse embryos with the drug SB203580 to inhibit both p38α and p38β, but not p38δ or p38γ, suggests that both p38α and p38β are required for preimplantation development (Maekawa et al., 2005; Natale et al., 2004). These experiments also suggest that double mutant embryos would not likely survive preimplantation development, and alternative approaches are needed to determine the full range of activities of the p38 family during development.

Using a forward genetic screen, we have identified a series of ENU-induced mutations that disrupt neural tube closure or gastrulation in the mouse embryo (Garcia-Garcia et al., 2005; Kasarskis et al., 1998; Zohn et al., 2005). Here we describe an ENU-induced splicing mutation in the *droopy eye* (*drey*) line that results in a variety of incompletely penetrant phenotypes including defects in neural tube closure and gastrulation. Strikingly, a stronger allele results in highly penetrant gastrulation defects, in particular defects in migration of mesoderm from the primitive streak. We reveal that *drey* encodes a p38-interacting protein (p38IP) and is required for p38 activation in vivo. Furthermore, we demonstrate that p38 activation is required for downregulation of E-cadherin protein. Finally we present evidence that p38 acts downstream from NIK, in a parallel pathway to Fgfs and Snail, to ensure a robust EMT by regulating expression of the E-cadherin protein and transcript, respectively.

RESULTS

Neural Tube, Eye, and Mesoderm Defects in *drey* Embryos

As part of our ongoing ENU-mutagenesis screen, we identified the mutant mouse line *droopy eye* (*drey*). Mutant embryos exhibit a variety of incompletely penetrant defects including expansion of the retinal-pigmented epithelium over the dorsal half of the eye (present in 116/147 embryos analyzed; 79%) and neural tube closure defects consisting of spina bifida (21/147; 14%) and exencephaly (81/147; 55%; Figures 1A and 1B). A small percentage of embryos (4/147; 3%) show severe defects in mesoderm development including a malformed allantois, somite defects, and posterior mesoderm truncations (Figures 1C and 1D). Conversely, some *drey* mutant embryos (7/31; 23%) do not exhibit any detectible phenotypes, and in a few rare cases, *drey* mutant animals survive postnatally (4/92; 4%) and could reproduce (1/92; 1%).

drey Encodes a p38-Interacting Protein

Using meiotic recombination mapping, the *drey* mutation mapped to a 1 Mb region on mouse chromosome 3, which contains six predicted transcripts including the *D3Ertd300e* transcript (Figure 1E; NCBI accession #BC052702). *D3Ertd300e* is annotated as a p38-interacting protein based on identification of the human homolog in a yeast two-hybrid screen using p38α as bait (Y.L. and J.H., unpublished data; NCBI accession #AF093250). Furthermore, we demonstrate that D3Ertd300e interacts with p38 MAPK and is required for its activity in vivo (see below and Figure 2), thus we rename *D3Ertd300e: p38-interacting protein* (*p38IP*). There is very little known about the *p38IP* gene and there are no apparent studies of p38IP function. Human *p38IP/C13orf19* was found to have altered expression in prostate cancers relative to normal prostate tissue (Schmidt et al., 2001, 2005) and to be expressed in human hematopoietic stem cells (Gomes et al., 2002). Since p38 MAP Kinase has been shown to regulate a variety of biological processes such as apoptosis, proliferation, and gene expression (reviewed in New and Han, 1998), *p38IP* was an interesting candidate to test as the responsible gene for a regulator of neural tube closure. Upon sequencing *drey* genomic DNA around the splice junctions of the predicted *p38IP* transcript, a T to C transition was identified in the splice donor consensus sequence at the end of exon 14 (Figure 1F). The p38IP protein contains a classical nuclear localization sequence, a PEST sequence, and a serine-rich region at the C terminus (Figure 1G).

To determine if the mutation disrupts *p38IP* splicing, an RT-PCR assay was performed using primers flanking exon 14 (Figure 1H). Amplification of wild-type cDNA resulted in a single 500 bp PCR product, whereas mutant cDNA produced multiple products including a 500 bp product at low abundance, indicating that a small proportion of transcripts are spliced normally in mutant embryos. Sequencing of the aberrantly spliced transcripts indicates that they encode disrupted proteins with premature stop codons encountered at either 951 or 948 bp. The small proportion of normal transcripts produced likely accounts for the incomplete penetrance of phenotypes observed in *drey* mutant embryos.

To confirm that mutation of *p38IP* is responsible for the developmental defects in $p38IP^{drey}$ mutants, a second allele of *p38IP* was obtained from the BayGenomics gene-trap resource and used in a complementation test cross. ES cell clone RRK304 (designated $p38IP^{RRK}$) contains an insertion in the seventh intron of the *p38IP* gene, which is

(H) Splicing of p38IP transcript in $p38IP^{+/+}$ (+/+) and $p38IP^{drey/drey}$ (−/−) embryos. Some transcripts are spliced normally in $p38IP^{drey/drey}$ embryos whereas the majority of transcripts use alternate splice donor sequences.
(I) The $p38IP^{RRK}$ gene trap allele fails to complement $p38IP^{drey}$ as $p38IP^{drey/RRK}$ embryos at E10.5 exhibit exencephaly (between red arrows, I). p38IP mRNA is expressed ubiquitously in E7.5 embryos (J).

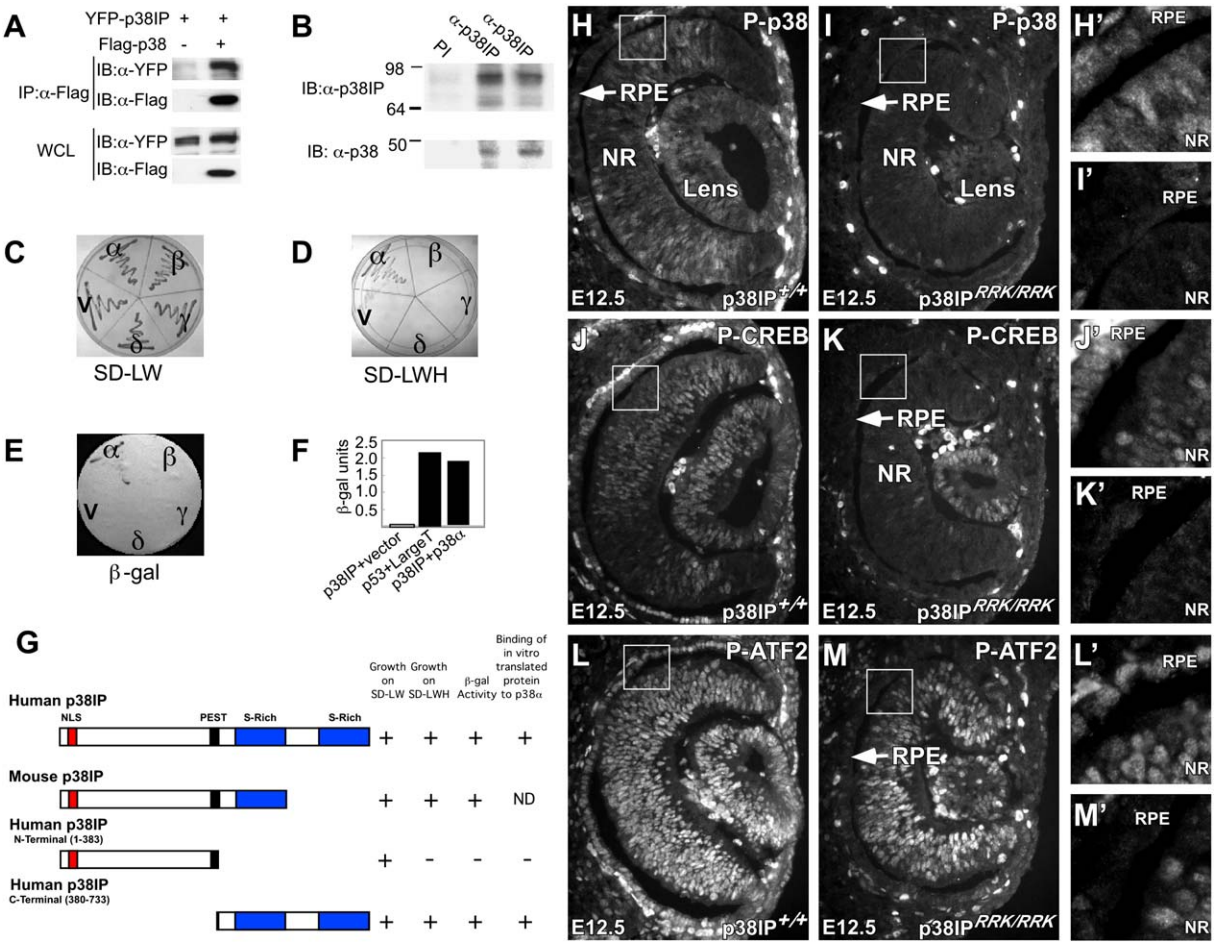

Figure 2. p38IP Interacts with p38α and Is Required for p38 Activation In Vivo

(A) Flag-p38α was coexpressed with YFP-p38IP in 293T cells. Immunoprecipitation was performed with anti-Flag (α-Flag) M2 agarose beads 24 hr after transfection, and the immunoprecipitates and cell lysates (WCL) analyzed by Western blot using anti-Flag (α-Flag) and anti-YFP (α-YFP) antibodies.

(B) 293T cells were lysed and immunoprecipitated with either preimmune serum (PI) or anti-p38IP antibody (α-p38IP) from two different His-p38IP immunized rabbits. The immunoprecipitates were analyzed by Western blot using anti-p38IP (α-p38IP) and anti-p38 (α-p38) antibodies.

(C) HF7c yeast cells were cotransfected with pGAD424-p38IP and pGBT9 (marked as V), pGBT9-p38α(AF) (α), pGBT9-p38β(AF) (β), pGBT9-p38γ(AF) (γ), or pGBT9-p38δ(AF) (δ). The cells were plated on minus leucine and tryptophan medium (SD-LW) to select cells cotransfected with pGAD424 and pGBT vectors.

(D) The same as (C) except medium lacking additional histidine (SD-LWH) was used to select for interaction.

(E) β-galactosidase colony-lift assay of the cells shown in (C).

(F) β-galactosidase activity in cells cotransfected with pGAD424-p38IP and pGBT9 (vector), pGAD424-p53 and pGBT9 Large T (p53/Large T), or pGAD424-p38IP and pGBT9 p38α(AF).

(G) AH109 yeast cells were cotransfected with pGAD424-p38IP(human), pGAD424-p38IP(mouse), pGAD424-N-terminal-p38IP(1–383) or pGAD424-C-terminal p38IP(380–733), and pGBT9 or pGBT9-p38α(AF) and plated on medium as above. Interaction was determined by growth on medium lacking additional histidine (SD-LWH), β-galactosidase activity, and by coimmunoprecipitation of in vitro translated p38IP(human), N-terminal p38IP(1–383), and C-terminal p38IP(380–733) with p38α (ND = not determined).

(H–M) Analysis of phosphorylation of p38, ATF2, and CREB in the eye of E12.5 wild-type (H, J, and L) and mutant (I, K, and M) embryos. (H′)–(M′) shows higher magnification of boxed region in (H)–(M). Phosphorylated p38 is detected in the retinal-pigmented epithelium (RPE), neural retina (NR), and the lens of wild-type embryos (H and H′), whereas it is only detected in a few scattered cells in the p38IP$^{drey/drey}$ eye (I and I′). Phosphorylated CREB is detected in RPE, NR, and lens of wild-type eyes (J and J′) but is not activated in RPE or NR of the p38IP$^{drey/drey}$ eye (K and K′). Phosphorylated-ATF2 is detected in the RPE, NR, and lens of wild-type (L and L′) but is downregulated in the RPE of the p38IP$^{drey/drey}$ eye (M and M′).

predicted to fuse the first 133 amino acids of p38IP to a β-galactosidase and neomycin phosphotransferase cassette. To determine the fidelity of splicing of the genetrap cassette into the *p38IP* transcript, RT-PCR using primers that flank exons 7 and 8 was performed. The absence of any detectable normal transcript in *p38IP*$^{RRK/RRK}$ mutant embryos suggests that this allele is likely a null or a severe hypomorph (data not shown). Similar to *p38IP*$^{drey/drey}$

mutant embryos, transheterozygous *p38IP*$^{drey/RRK}$ mutant embryos exhibit incompletely penetrant phenotypes that range from gastrulation and neural tube defects to morphologically normal embryos (Figure 1I and data not shown). This genetic experiment confirms that the mutation in *p38IP* is responsible for the *drey* phenotypes.

To determine if *p38IP* is expressed in a pattern that is consistent with a role in regulation of gastrulation or neurulation, *p38IP* expression was examined by in situ hybridization using an antisense RNA probe against the *p38IP* transcript in wild-type embryos and LacZ in p38IP$^{RRK/+}$ embryos. These experiments reveal that the *p38IP* transcript is expressed ubiquitously at all stages examined (E7.5–E12.5; Figure 1J and data not shown).

p38IP Is Required for Activation of p38 In Vivo

As mentioned above, we identified the human homolog of p38IP in a yeast two-hybrid screen using p38α as bait. Six colonies representing three different genes were identified out of 2×10^7 colonies screened. Two of the proteins were known p38α interactors: the p38α substrate MK2 and Pax6 (Mikkola et al., 1999; Stokoe et al., 1992). One undescribed 353 amino acid peptide was identified, and because there was no information available about the gene, we named it p38-Interacting Protein (p38IP) (Y.L. and J.H., unpublished data; NCBI accession #AF093250). To confirm the interaction, we tested the ability of p38IP to interact with p38α in coimmunoprecipitation assays. When YFP-tagged p38IP and Flag-tagged p38α are coexpressed in 293T cells, p38IP associates with p38α (Figure 2A). Interaction between endogenous p38IP and p38α is also detected following immunoprecipitation of untransfected 293T cells (Figure 2B). To determine the specificity of p38IP binding to p38, p38 isoforms with the TGY dual phosphorylation site changed to AGF were used in a yeast two-hybrid interaction assay (Figures 2C–2F). Cells cotransfected with p38IP and p38α are able to grow on SD-LWH media and induce β-galactosidase activity, indicating an interaction, whereas p38IP does not interact by these assays in yeast with p38β, p38δ, p38γ, or vector control pGBT9 (Figures 2C–2E). Coexpression of p38IP and p38α is able to significantly induce β-galactosidase activity comparable to that of p53 and large T antigen (Figure 2F).

In comparison to human p38IP, mouse p38IP ends at amino acid 530, resulting in a single N-terminal Serine-rich domain. To determine if the mouse p38IP also interacts with p38, we tested binding in a yeast two-hybrid assay. Identical to human p38IP, mouse p38IP only interacted with the p38α isoform when scored for growth on medium lacking histidine (SD-LWH) and induction of β-galactosidase (Figure 2G and data not shown). To determine if the truncated p38IP proteins that are potentially produced in p38IP^{drey} and p38IP^{RRK} mutant mice (Figure 1G) can bind to p38α, we mapped the binding domain in the p38IP protein. Yeast coexpressing N-terminal p38IP (1–383) and p38α are not able to grow on SD-LWH media or induce the β-galactosidase reporter, indicating that p38IP does not interact with this fragment. In contrast, cells cotransfected with C-terminal p38IP (380–733) and p38α are able to grow on SD-LWH media and induce β-galactosidase, indicating that the p38α interaction domain is in the C-terminal region of the protein. The interaction domain also was confirmed in a coimmunoprecipitation assay using in vitro translated proteins (data summarized in Figure 2G). These data reveal that the truncated proteins potentially produced by the two mutant alleles of p38IP do not bind p38α.

To determine the in vivo relevance of the interaction between p38IP and p38, we examined whether p38 activity was misregulated in the *drey* mutant mouse. The activation of p38 and its downstream substrates CREB and ATF2 were examined using phospho-specific antibodies in wild-type and *drey* mutant eyes, an organ that is affected by p38IP truncation. In the wild-type E12.5 eye, p38, CREB, and ATF2 are phosphorylated in the retinal-pigmented epithelium (RPE; p38: 53%, CREB: 67%, ATF2: 89% of cells), neural retina (NR; p38: 69%, CREB: 57%, ATF2: 79% of cells), and lens (p38: 44%, CREB: 46%, ATF2: 74% of cells; Figures 2H, 2J, and 2L). In contrast, in the *p38IP*$^{drey/drey}$ mutant eye, phosphorylated p38 is only detected at low levels in a few cells scattered throughout the eye (RPE: 1%; NR: 6%; lens: 7% of cells; Figure 2I). The phosphorylation of p38 substrates is not detected in specific cell layers in *p38IP*$^{drey/drey}$ mutant eyes. Phosphorylated CREB is detected in the lens of *p38IP*$^{drey/drey}$ eyes (53%) but is greatly reduced in the RPE (4%) and neural retina (4%; Figure 2K). While phosphorylated ATF2 is detected in the neural retina (88%) and lens (75%) of *p38IP*$^{drey/drey}$ eyes, it is significantly reduced in the RPE (9%; Figure 2M). These data reveal that p38IP interacts with p38α, and in mutant embryonic tissues in which truncated p38IP cannot bind p38α, activation of p38 and downstream substrates is impaired in vivo.

p38IP Is Required for Development of Mesoderm

To investigate further the role of p38IP in embryogenesis, we examined mutant phenotypes in mouse embryos homozygous for the more severe *p38IP*^{RRK} allele (Figure 3). E9.5 and E10.5 mutant embryos exhibit multiple developmental defects consistent with abnormalities in development of mesoderm (Figures 3A–3D). Yolk sac membranes are wrinkled and poorly vascularized, and mutants develop a malformed allantois that fails to fuse to the chorion. Mutant embryos are necrotic, developmentally delayed, exhibit misshapen head folds and exencephaly, and fail to form somites or form only a few anterior somites.

These widespread abnormalities in development of mesodermally derived tissues could originate during gastrulation when mesoderm is specified and migrates. At gastrulation, *p38IP*$^{RRK/RRK}$ mutant embryos contain a mass of cells on the posterior side (Figures 3E and 3F). Histological analysis reveals that a significant proportion of the mesoderm failed to migrate away from the primitive streak (Figures 3G and 3H). Mesoderm migration defects were consistently observed although the extent varied between mutants.

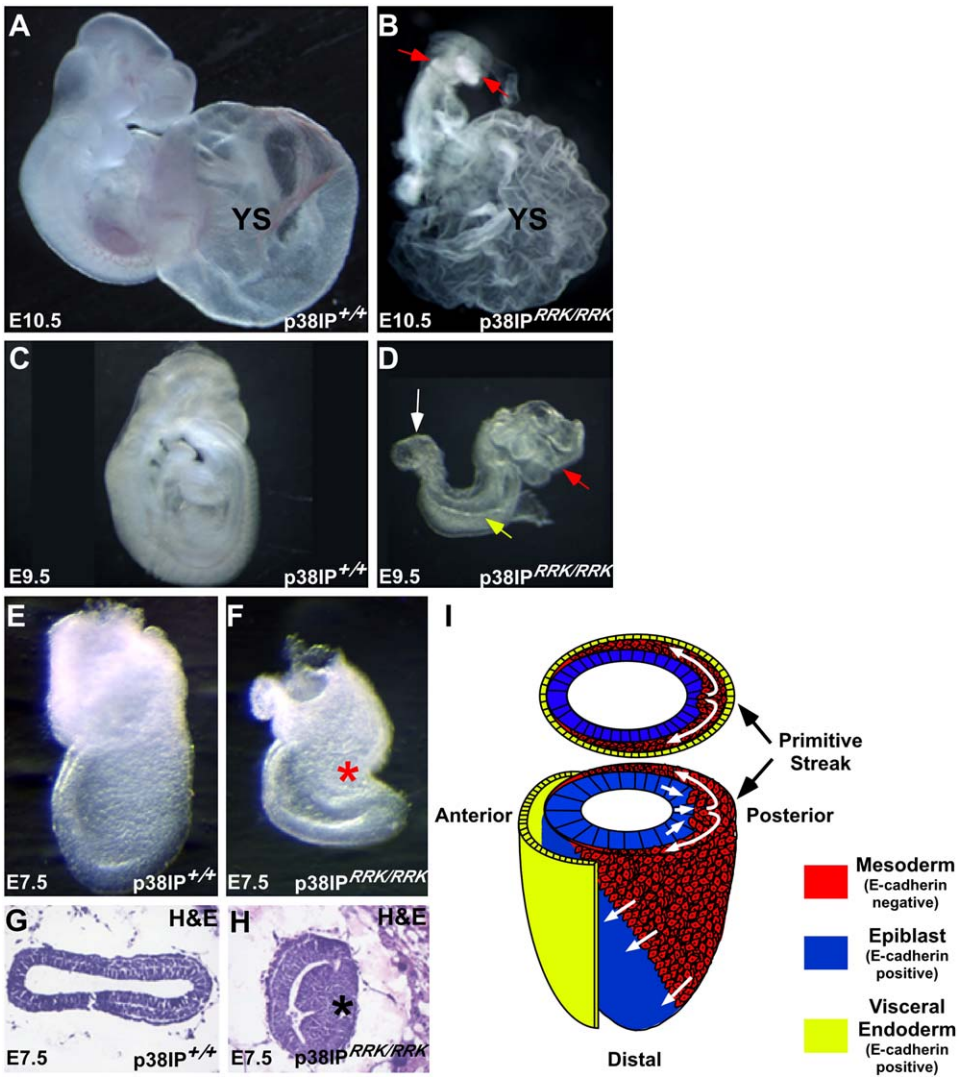

Figure 3. Defects in Mesoderm Development and Migration in p38IP$^{RRK/RRK}$ Mutant Embryos

At E10.5, p38IP$^{RRK/RRK}$ mutant embryos (B) exhibit exencephaly (between red arrows), are necrotic, are developmentally delayed, and exhibit defects in development of extraembryonic mesoderm such as malformed and avascular yolk sacs (YS) compared to sibling controls (A). E9.5 p38IP$^{RRK/RRK}$ mutant embryos (D) are developmentally delayed with enlarged head folds (red arrow) and exhibit multiple defects in mesoderm development, such as lack of somites (yellow arrow) and malformed allantois (white arrow), as compared with sibling controls (C). At E7.5, a mass of cells is observed accumulating in the primitive streak of p38IP$^{RRK/RRK}$ mutant embryos (red *, F) compared with controls (E). Hematoxylin and Eosin (H&E) staining of E7.5 p38IP$^{+/+}$ (G) and p38IP$^{RRK/RRK}$ mutant (H) embryos demonstrates that mesoderm cells accumulate in the mutant primitive streak (black *). (I) Schematic of gastrulation in the mouse embryo. The epiblast of the E7.5 gastrula stage mouse embryo is shaped like a cup nestled within a cup of visceral endoderm. Mesoderm is induced in the posterior region of the epiblast and undergoes an EMT to delaminate and migrate out of the streak. Completion of EMT is dependent upon downregulation of E-cadherin. Once the mesoderm has exited the streak, it migrates anteriorly between the epiblast and visceral endoderm layers. Primitive streak to the right in (E)–(I).

This phenotype is strikingly similar to that of *Fgfr1* and *Fgf8* mutant embryos (Deng et al., 1994; Sun et al., 1999; Yamaguchi et al., 1994). *Fgfr1* is required for the expression of a series of mesoderm-specific markers such as *Sprouty2, Tbx6, Brachyury*, and *Lim1*; moreover, *Fgfr1* is required for the expression of *Snail*, which mediates downregulation of the transcript for the E-cadherin cell adhesion protein to promote EMT (Ciruna and Rossant, 2001). However, in contrast to *Fgf* mutants, *Sprouty2, Tbx6*, and *Brachyury* are correctly expressed in *p38IP$^{RRK/RRK}$* mutant embryos (Figures 4A–4F). In wild-type embryos, *Lim1* is expressed at higher levels in cells as they migrate away from the primitive streak (Barnes et al., 1994). In mutant embryos, *Lim1* is expressed, although in a more limited range (Figures 4G and 4H), suggesting that cells are turning on the mesenchymal developmental program but nevertheless fail to properly migrate away from the streak.

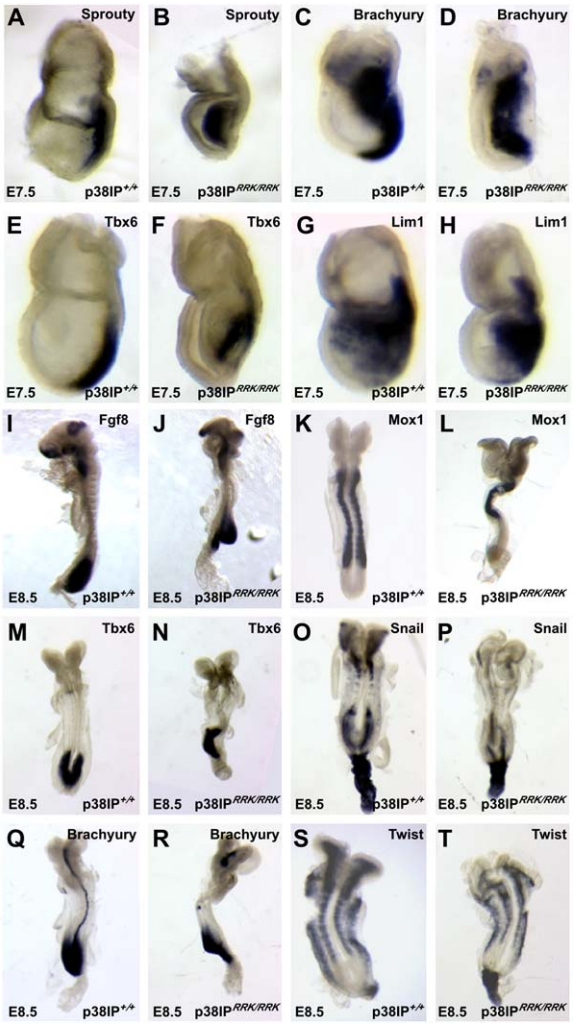

Figure 4. Mesoderm Is Properly Specified in p38IP$^{RRK/RRK}$ Mutant Embryos But Disorganized

Expression of molecular markers of mesoderm at E7.5: Sprouty2 (A and B), Brachyury (C and D), Tbx6 (E and F), and Lim1 (G and H) expression in p38IP$^{+/+}$ (A, C, E, and G) and p38IP$^{RRK/RRK}$ mutant (B, D, F, and H) embryos. Primitive streak to the right in (A)–(H). Expression of molecular markers at E8.5: Fgf8 (I and J), Mox1 (K and L), Tbx6 (M and N), Snail (O and P), Brachyury (Q and R), and Twist (S and T) in p38IP$^{+/+}$ (I, K, M, O, Q, and S) and p38IP$^{RRK/RRK}$ mutant (J, L, N, P, R, and T) embryos.

At E8.5, examination of molecular marker expression reveals that mesoderm is properly specified although disorganized in mutant embryos (Figures 4I–4T). Presomitic mesoderm is specified as evidenced by *Fgf8* and *Tbx6* expression (Figures 4I, 4J, 4M,and 4N). *Mox1*, a somite marker, is expressed in mutant embryos, although not organized into somitomeres (Figures 4K and 4L). Less severely affected mutant embryos do form some anterior somites as evident by expression of *Twist* (Figures 4S and 4T). Often the paraxial mesoderm is not divided into discrete left and right domains, likely due to defects in development of the midline. Examination of *Brachyury*, a midline axial mesoderm marker, shows that the midline is present in mutant embryos but is often severely disrupted and discontinuous (Figures 4Q and 4R). Less severely affected embryos, however, show division of *Snail* and *Twist* expression into discrete left and right domains, indicating a more normal midline (Figures 4O, 4P, 4S, and 4T). These results indicate that both axial and nonaxial mesoderm is specified in *p38IP$^{RRK/RRK}$* mutant embryos but disorganized.

p38IP Is Required for Downregulation of E-Cadherin

To determine if these defects in mesoderm migration are related to defects in p38 activation, p38 phosphorylation was determined in the primitive streak of E7.5 wild-type and mutant embryos. In wild-type embryos, phosphorylated p38 is detected ubiquitously in all germ layers (Figures 5A–5C). Despite the ubiquitous expression pattern of p38IP, in mutant embryos, phosphorylated p38 is not detected specifically in the primitive streak (between arrows) and in the mesoderm that fails to migrate away from the streak (arrowhead, Figures 5D–5F). Phosphorylated p38 is detected, however, in the rest of the embryo, including the few mesoderm cells that are able to migrate away from the primitive streak.

Fgfr1 regulates the expression of *Snail* transcript and the Snail protein represses *E-cadherin* transcription to promote EMT (Ciruna and Rossant, 2001). To determine if this pathway is disrupted in p38IP$^{RRK/RRK}$ mutant embryos, the expression of *Snail* and *E-cadherin* was examined during gastrulation. *Snail* is expressed at normal levels in the primitive streak of mutant embryos, indicating that p38IP is not required for regulation of *Snail* expression (Figures 5G and 5J). Consistent with the expression of Snail, which acts as a transcriptional repressor at the *E-cadherin* promoter, the *E-cadherin* transcript is downregulated in the mesoderm of mutant embryos, although these cells fail to migrate (Figures 5H and 5L).

The E-cadherin protein is localized to the adherence junctions of cells in the epithelium, and the *E-cadherin* transcript gets rapidly downregulated as cells exit the epithelial cell layer (Figure 5I). Yet, in the cells that have just exited the epithelial layer and downregulated the E-cadherin transcript, E-cadherin protein is still present at the junctions and gets downregulated as cells migrate away from the streak (Figure 5M). This observation suggests that E-cadherin protein expression is also regulated posttranscriptionally. To determine if p38IP is required for posttranscriptional regulation of E-cadherin, E-cadherin protein expression was examined in mutant embryos (Figures 5N and 5O). Strikingly, in mutant embryos, the E-cadherin protein remains localized to the junction of the cells that fail to migrate away from the primitive streak. These results raise the intriguing possibility that two pathways act independently to regulate E-cadherin in the primitive streak to allow mesoderm migration: Fgf/Snail acts at the transcriptional level to downregulate the *E-cadherin* transcript and p38IP acts to downregulate or destabilize the E-cadherin protein.

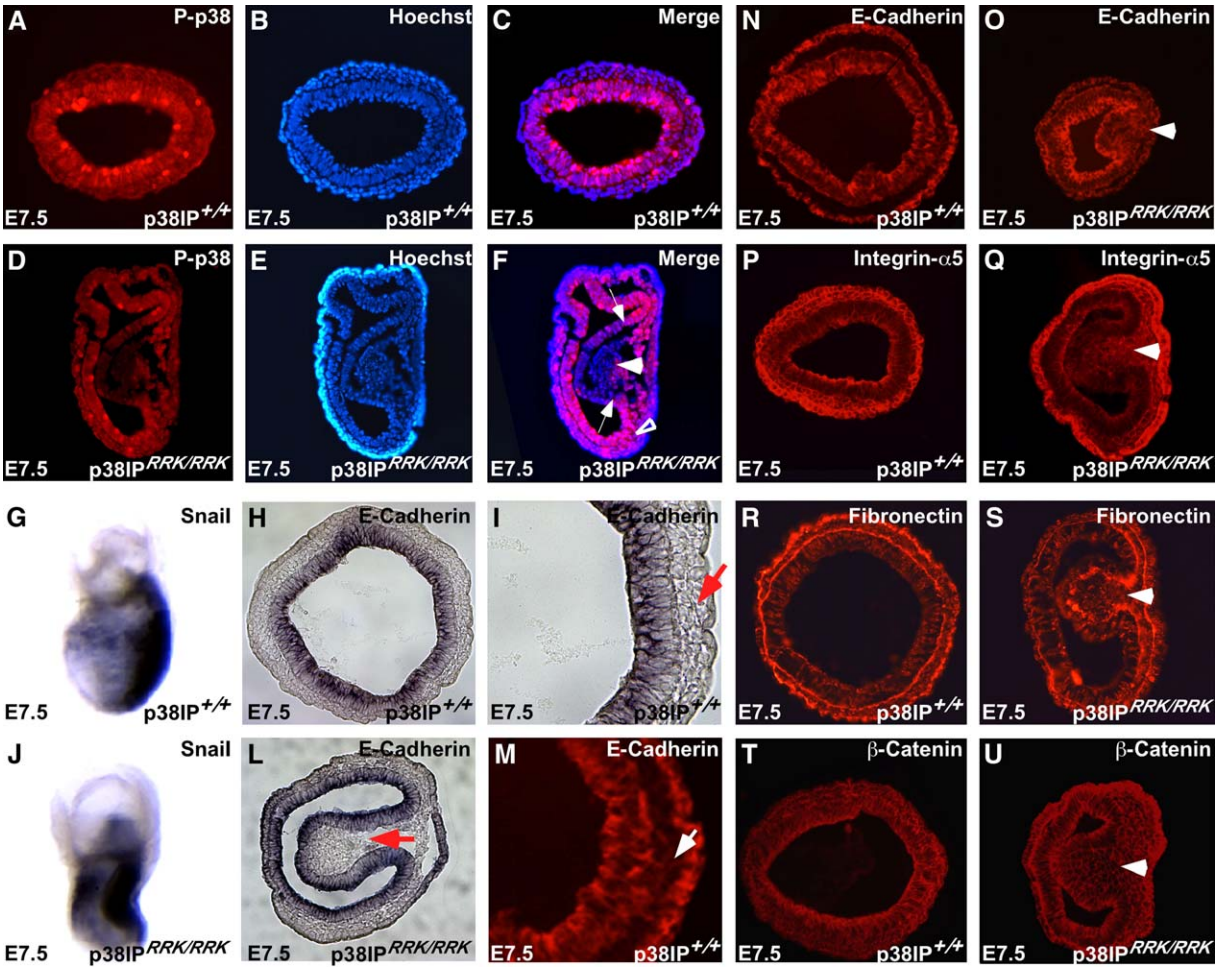

Figure 5. p38IP Is Required for Activation of p38 in the Primitive Streak and for Downregulation of E-Cadherin Protein Independently of Snail

(A–F) In E7.5 p38IP$^{+/+}$ embryos (A–C), p38 is activated throughout the embryo as revealed by immunofluorescence using anti-phospho-p38 antibody (P-p38). In p38IP$^{RRK/RRK}$ mutant embryos (D–F), p38 is not phosphorylated specifically in the primitive streak (between arrows in F) and mesoderm that fails to migrate away from the primitive streak (arrowhead in F). Open arrowhead indicates some of the few mesoderm cells that migrated away from the primitive streak and activated p38. (C) and (F) show overlay of P-p38 localization (A and D) and nuclei (B and E). Regions where p38 is activated are in pink while regions where p38 is not activated are blue.

Snail is expressed in the primitive streak of E7.5 p38IP$^{+/+}$ (G) and p38IP$^{RRK/RRK}$ mutant (J) embryos. As mesoderm leaves the primitive streak, E-cadherin expression is downregulated in p38IP$^{+/+}$ embryos at both the transcriptional (H and I) and protein (M and N) levels, whereas in p38IP$^{RRK/RRK}$ mutants the E-cadherin transcript is downregulated (L) but not the protein (O) in cells that fail to migrate away from the streak (arrowhead). Markers of EMT such as Integrin-α5 (P) and Fibronectin (R) are upregulated in mesoderm as it migrates away from the primitive streak of wild-type embryos. Integrin-α5 (Q) and Fibronectin (S) are upregulated normally (arrowhead) in cells that fail to migrate from the p38IP$^{RRK/RRK}$ mutant streak. β-catenin expression in wild-type (T) and p38IP$^{RRK/RRK}$ mutant (U) embryos highlights the epithelial and mesenchymal cell morphologies. E-cadherin-positive cells that fail to migrate away from the streak exhibit a mesenchymal morphology (arrowhead). Primitive streak is to the right in (A)–(U).

When cells undergo EMT, the expression levels of a number of cell adhesion markers become either up- or downregulated. To determine if p38IP is required for other aspects of EMT, additional EMT markers were examined in mutant embryos. Markers of mesoderm migration such as Integrin-α5 and Fibronectin are efficiently upregulated in mutant mesoderm that fails to migrate away from the primitive streak (Figures 5P–5S). In addition, as cells delaminate from the primitive streak, they change morphology as highlighted by staining with an anti-β-catenin antibody (Figures 5T and 5U). Taken together, these results suggest that while *p38IP*$^{RRK/RRK}$ mutant mesoderm undergoes a partial EMT, it fails to complete EMT and downregulate the E-cadherin protein.

To study further the requirement for p38IP in mesoderm migration, mesoderm from the primitive streak of E7.5 wild-type embryos or the cells that fail to migrate away from the primitive streak of mutant embryos were dissected and cultured on fibronectin-coated plates. Wild-type mesoderm migrates extensively away from the

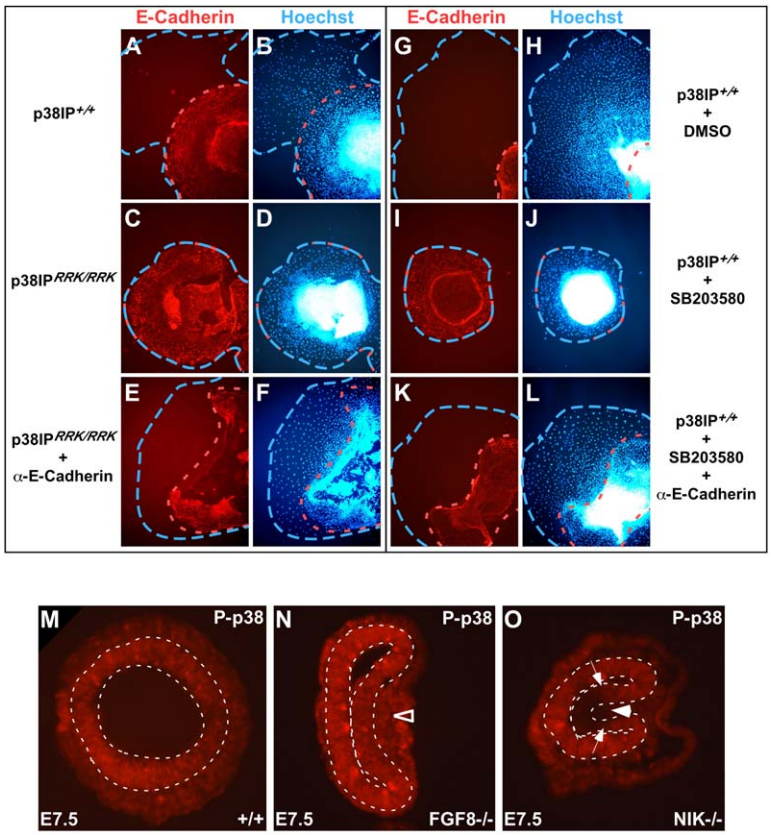

Figure 6. p38IP and p38 Are Required for Downregulation of E-Cadherin in a Fgf-Independent Pathway

(A–L) Cultured primitive streak explants were costained with anti-E-cadherin antibody (A, C, E, G, I, and K) and the nuclear stain Hoechst to visualize the cells that no longer express E-cadherin (B, D, F, H, J, and L). Dotted red line indicates border of E-cadherin expression and dotted blue line indicates border of Hoechst staining. Wild-type mesoderm cells from primitive streak explants (A and B) downregulate E-cadherin expression and migrate extensively away from the explant, while cells from p38IP$^{RRK/RRK}$ mutant embryos (C and D) fail to downregulate E-cadherin and do not migrate away from the streak. Incubation of p38IP$^{RRK/RRK}$ mutant primitive streak with the anti-function E-cadherin antibody results in downregulation of E-cadherin expression and extensive migration of mesoderm away from the streak (E and F). Cells from primitive streak explants from E7.5 p38IP$^{+/+}$ embryos incubated with DMSO downregulate E-cadherin and migrate away from the explant (G and H), while cells from explants incubated with 20 μM p38 kinase inhibitor SB203580 do not downregulate E-cadherin or migrate (I and J), whereas coincubation with 20 μM SB203580 plus the anti-function E-cadherin antibody rescues EMT and mesoderm migration (K and L). Activation of p38 is impaired in the primitive streak of *NIK* but not *Fgf8* mutant embryos. Phospho-p38 expression in E7.5 wild-type (M), *Fgf8*$^{-/-}$ (N), and *NIK*$^{-/-}$ (O) mutant embryos. p38 is activated in cells that fail to migrate from the primitive streak in *Fgf8*$^{-/-}$ mutant embryos (open arrow in N). In *NIK*$^{-/-}$ mutant embryos (O), p38 is not phosphorylated specifically in the primitive streak (between arrows) and mesoderm that fails to migrate away from the primitive streak (arrowhead). White dotted line highlights epiblast. Primitive streak is to the right.

explant coincident with the downregulation of E-cadherin protein (Figures 6A and 6B and Burdsal et al., 1993). As seen in vivo, cells from the mutant primitive streak that failed to downregulate E-cadherin also failed to migrate when explanted (Figures 6C and 6D). To determine whether *p38IP*$^{RRK/RRK}$ mutant cells can migrate if E-cadherin function is blocked, explants were incubated with a function-perturbing anti-E-cadherin antibody. This resulted in both the downregulation of E-cadherin and extensive migration of mutant cells away from the explant (Figures 6E and 6F). These results suggest that *p38IP*$^{RRK/RRK}$ mutant cells likely remain in the primitive streak due to a deficiency in the downregulation of E-cadherin protein, but otherwise p38IP is not required for general cell migration.

p38 Activity Is Required for E-Cadherin Downregulation

p38IP$^{RRK/RRK}$ mutant embryos fail to activate p38 specifically in the primitive streak and the mesoderm that fails to downregulate E-cadherin (Figures 5F and 5O). To determine if the failure to downregulate E-cadherin in *p38IP*$^{RRK/RRK}$ mutant embryos is due to a failure to activate p38 in the primitive streak, explants from wild-type embryos were cultured in the presence of either a specific p38 kinase inhibitor or vehicle control (DMSO). The chemical inhibitor SB203580 targets both p38α and p38β but not p38δ or p38γ (Cuenda et al., 1995). In control explants incubated with DMSO, cells are capable of downregulating E-cadherin and migrating away from the explant (Figures 6G and 6H). In contrast, explants treated with 20 μM SB203580 did not downregulate E-cadherin and failed to migrate away from the explant (Figures 6I and 6J). This result indicates that p38 activity is required for downregulation of E-cadherin and EMT. To confirm this and to test whether p38 is also required for mesoderm migration after EMT, explants were incubated in 20 μM SB203580 and the function blocking anti-E-cadherin antibody (Figures 6K and 6L). Similar to *p38IP*$^{RRK/RRK}$ mutant explants, addition of anti- E-cadherin antibody rescued EMT and cell migration. These results indicate that both p38IP and p38 are required for downregulation of E-cadherin but not for mesoderm migration.

p38 Acts Downstream of NIK in Mesoderm Migration

To determine if Fgf signaling is required for p38 activation in the primitive streak, p38 phosphorylation was examined in *Fgf8* mutant embryos (Figures 6M and 6N). In contrast to *p38IP*$^{RRK/RRK}$ mutants (Figures 5D–5F), *Fgf8* mutant embryos showed robust activation of p38 in the primitive

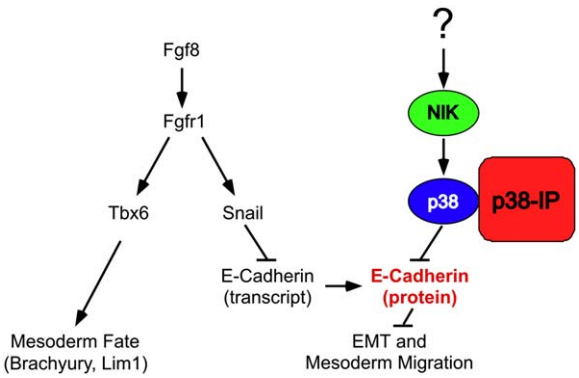

Figure 7. Model for Pathways Resulting in E-Cadherin Downregulation

Fgf signaling through Fgfr1 results in activation of two pathways regulating mesoderm cell fate (expression of Tbx6 and Brachyury) or mesoderm migration. To regulate mesoderm migration, Fgf signaling is required for expression of Snail, which downregulates expression of the E-cadherin transcript. In an independent pathway, NIK/Map4k4 is needed for activation of p38, and p38IP is also required for activation of p38 in vivo. p38 and p38IP activity are required for downregulation of the E-cadherin protein. Both the Fgf/Snail and NIK/p38/p38IP pathways are required to ensure a robust EMT and to allow mesoderm to migrate from the primitive streak.

streak and the cells that fail to migrate. The *Drosophila* homolog of *NIK*/*Map4k4*, *Misshapen* (*Msn*), requires p38 activity for some in vivo responses (Paricio et al., 1999), and the phenotype of $NIK^{-/-}$ embryos (Xue et al., 2001) is similar to that of $p38IP^{RRK/RRK}$, suggesting that they may act in a similar pathway. Similar to *p38IP* mutant embryos, p38 is not activated in the primitive streak (between arrows) or the mesoderm that accumulates in the streak of *NIK* mutant embryos (arrowhead, Figure 6O). These data indicate that NIK may act upstream of p38 in a pathway to promote EMT and that this pathway is independent of Fgf signaling. This possibility is also supported by the similarities in expression of mesoderm markers in $p38IP^{RRK/RRK}$ and *NIK* mutant embryos as compared to *Fgf8* or *Fgfr1* mutant embryos (Deng et al., 1994; Sun et al., 1999; Xue et al., 2001; Yamaguchi et al., 1994).

DISCUSSION

Here we identify a p38-interacting protein (p38IP) that is required to bind and activate p38 during mouse development. Loss of p38IP function causes severe embryonic defects. A splicing mutation in *p38IP* results in a variety of incompletely penetrant phenotypes including neural tube, eye, and gastrulation defects. A stronger *p38IP* allele results in completely penetrant gastrulation defects in which mesoderm migration away from the primitive streak is impaired, although mesoderm induction, patterning, and migratory behavior are not affected. These defects arise due to disruption of p38 activation leading to a decreased ability of specified mesoderm cells to downregulate the E-cadherin protein and complete EMT.

Outlined in our model (Figure 7), our data reveal an additional mechanism of E-cadherin regulation in the gastrulating mouse embryo that is independent of the Fgf8/Snail pathway. We show that NIK/p38/p38IP do not affect *Snail* expression or the normal repression of *E-cadherin* transcription. Instead, the NIK/p38/p38IP pathway is required to properly downregulate E-cadherin protein expression. Our results suggest that two pathways—Fgf/Snail and NIK/p38/p38IP—act independently and converge on E-cadherin expression: one at the transcriptional level and one at the protein level to insure the rapid downregulation of E-cadherin and a robust EMT; and disruption of either pathway leads to defective gastrulation.

p38IP Regulates p38 Activity In Vivo

We demonstrate that p38IP specifically binds p38 in both yeast two-hybrid and coimmunoprecipitation assays. We mapped the binding domain to the C-terminal region of p38IP. Significantly, the mutations in both the $p38IP^{RRK}$ and $p38IP^{drey}$ alleles result in deletion of the C-terminal domain of p38IP, and these mutant proteins cannot bind to p38. Furthermore, we demonstrate that p38 activation in vivo is compromised in both $p38IP^{RRK}$ and $p38IP^{drey}$ mutants. Future experiments are required to elucidate the biochemical mechanism(s) by which p38IP functions to regulate p38.

We found that p38IP binds to only p38α in a yeast two-hybrid assay, yet previous studies of *p38*α mutant embryos demonstrated that *p38*α is essential for development of the placenta but not for gastrulation (Adams et al., 2000; Mudgett et al., 2000). This raises the possibility that p38IP may be required for activation of other p38 isoforms in vivo. To begin to investigate this possibility, we determined the expression pattern of *p38*β, *p38*γ, and *p38*δ to determine if the other p38 isoforms are expressed in the primitive streak. *p38*γ and *p38*δ are expressed in the extraembryonic regions of the gastrula while *p38*β is expressed in the epiblast (Figure S1). This data combined with studies in explanted mesoderm in which an inhibitor of p38α and p38β prevents EMT (Figure 6) suggest that p38IP may be required for activation of both p38α and p38β in vivo. An alternate explanation is that p38α may normally mediate EMT in the primitive streak but in the absence of p38α, p38β may be able to compensate either by altering its normal expression pattern or activity. In support of these ideas, we examined activation of p38 in *p38*α mutant gastrula stage embryos and found that p38 is phosphorylated normally throughout the epiblast and streak (I.E.Z. and L.N., unpublished data). In addition, we cannot exclude the possibility that p38IP is also required for the function of other, yet to be discovered, factors.

p38 Is Downstream of NIK

Our data provide an intriguing link between p38 activation and the NIK pathway. This relationship to NIK is surprising as NIK activates JNK but not p38 in cultured cells (Su et al., 1997). Similarly, Misshapen (Msn), the *Drosophila* NIK homolog, activates JNK to regulate dorsal closure (Su et al.,

1998), and Msn couples Frizzled and Disheveled to JNK activation to regulate planar cell polarity in the *Drosophila* wing and eye (Paricio et al., 1999). Interestingly in this context, JNK acts redundantly with Dp38a and Dp38b, suggesting that Msn is capable of regulating p38 activity in vivo. During mouse embryogenesis, a role for NIK in eye and neural tube morphogenesis has not been determined as *NIK* mutant embryos die around E9.5 (Xue et al., 2001). However, *JNK* mutant embryos (either single or double mutants) do not exhibit the severe gastrulation defects observed in *NIK* mutant embryos (Kuan et al., 1999; Sabapathy et al., 1999), suggesting that NIK may activate JNK-independent pathways in vivo. Indeed, our studies indicate that p38 activity is specifically disrupted in *NIK* mutant embryos, and mutations in either *NIK* or *p38IP* result in similar gastrulation defects, providing additional in vivo support for a link between NIK and p38 activation.

p38IP and Neural Tube Defects

Neural tube defects (NTDs) occur when the neural tube fails to close completely during embryogenesis leading to exencephaly/anencephaly and spina bifida. NTDs are one of the most common birth defects observed in humans and represent a complex genetic disease (reviewed in Copp et al., 2003). The mouse has provided over 100 potential candidate genes for NTDs, and in some instances mutations in these genes have been identified in human patients (reviewed in Copp et al., 2003; Zohn et al., 2005). Furthermore, identification of hypomorphic mutations associated with NTDs indicates that these types of mutations will reveal many more potential candidate genes. We show that a severe allele of *p38IP* results in gastrulation defects whereas the milder *drey* allele results in exencephaly and spina bifida. Future experiments will determine if the morphogenic defects in *drey* mutant mice such as the failure to close the neural tube and eye defects are due to specific defects in p38 activation.

EMT and Metastasis during Cancer

The EMT that occurs in the primitive streak of the gastrulating mouse embryo and during invasion and metastasis of tumor cells involve regulation of *E-cadherin* expression by Snail (reviewed in Barrallo-Gimeno and Nieto, 2005). Furthermore, the loss of E-cadherin expression is a central event in the transition of tumors from noninvasive to invasive carcinomas (Batlle et al., 2000; Cano et al., 2000). Here we present evidence of a Snail-independent pathway in which p38 is required in the primitive streak to downregulate E-cadherin expression at the posttranscriptional level. Whether this Snail-independent, p38-dependent pathway also functions in EMT during tumor invasion remains to be determined.

EXPERIMENTAL PROCEDURES

Mouse Strains and Genotyping

drey was identified in a screen for recessive ENU-induced mutations that cause morphological abnormalities at E12.5 (Garcia-Garcia et al., 2005; Kasarskis et al., 1998; Zohn et al., 2005). The *drey* mutation was generated on a C57BL/6J genetic background and backcrossed to C3H. In a mapping cross of 480 opportunities for recombination, *drey* was mapped between Massachusetts Institute of Technology (MIT) simple sequence length polymorphism (SSLP) markers D3mit6 and D3mit137. For high-resolution mapping, additional polymorphic DNA markers were generated based on nucleotide repeat sequences (see http://mouse.ski.mskcc.org/ for primer sequences). A mouse embryonic stem (ES) cell line carrying an insertion in the *p38IP* gene (RRK304) was obtained from BayGenomics http://baygenomics.ucsf.edu/ database. Other mutant mouse lines used were Fgf8 and NIK (Meyers et al., 1998; Xue et al., 2001).

Molecular Identification of *drey* Mutation

cDNAs of the p38IP gene were amplified by RT-PCR (Superscript One-Step RT-PCR, Invitrogen) using RNA from E10.5 *drey*/*drey* and C57BL/6 control embryos. The splice site junctions around exon 14 were sequenced and a T to C transition discovered in the splice donor consensus sequence. Sequencing was confirmed using ten additional *drey*/*drey* mutant embryos.

Analysis of Mutant Phenotype

Whole-mount and section RNA in situs were performed as described (Holmes and Niswander, 2001; Liu et al., 1998). p38IP expression pattern was determined using an antisense RNA probe synthesized from IMAGE clone: 2598858 or by expression of the lacZ transcript in $p38IP^{RRK/+}$ embryos. Immunofluorescence experiments were performed as described (Timmer et al., 2002) using anti-E-cadherin antibody (1:100 dilution of anti-uvomorulin antibody; Sigma F3648), Hoechst (10 μg/ml; clone DECMA-1, Sigma), anti-Phospho-p38 MAP Kinase (Thr180/Tyr182) antibody (1:100 dilution; Cell Signaling #9211), anti-Phospho-ATF2 antibody (1:100 dilution; Cell Signaling #9221), anti-Phospho-CREB antibody (1:100 dilution; Cell Signaling #9191), anti-Integrin-α5 (1:100; BD-Pharmingen #553319), anti-fibronectin (1:100; Sigma F3648), anti-β-catenin (1:1000; Sigma C7082).

Culture of Primitive Streak Explants

Primitive streak explants were dissected from E7.5 wild-type and mutant embryos and grown on fibronectin-coated plates for 3 days as described (Ciruna and Rossant, 2001). Media was supplemented with 20 μM SB203580 (Calbiochem) in DMSO and/or anti-E-cadherin (1:100 dilution of anti-uvomorulin antibody; Sigma).

Interaction of p38α with p38IP

To identify p38-interacting proteins, we used a kinase-inactive mutant of p38α fused with the GAL4 DNA binding domain (pGBT9-p38(AF)) as bait and performed a yeast two-hybrid screen of a Hela cell cDNA library constructed in the activation domain plasmid pACT as described by the manufacturer (BD Clontech). Sequencing showed that the clone encodes amino acids 381–733 of p38IP (1–733). PCR and Southern blots were used to clone full-length human p38IP from a phage library. The specificity of the interaction was tested by immunoprecipitation as described (Ge et al., 2002).

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/cgi/content/full/125/5/957/DC1/.

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Accession Numbers

The NCBI accession numbers for the human p38IP and mouse p38IP sequences reported in this paper are AF093250 and AF139179, respectively.