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The Mediator complex protein Med31 is required for embryonic growth and cell proliferation during mammalian development

Michael D. Risley, Christopher Clowes, Ming Yu, Karen Mitchell, Kathryn E. Hentges *

University of Manchester, Faculty of Life Sciences, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK

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ABSTRACT

During development, the mammalian embryo must integrate signals to control growth and proliferation. A failure in the ability to respond to mitogenic stimuli can cause embryonic growth restriction. We have identified a mouse mutant, *l11Jus15*, from a mutagenesis screen that exhibits growth defects and late-gestation lethality. Here we demonstrate that this phenotype results from a mutation in the Mediator complex gene *Med31*, which causes degradation of Med31 protein. The *Med31* mutant phenotype is not similar to other Mediator complex mouse mutants, and target genes of other Mediator proteins are expressed normally in *Med31* mutants, suggesting that Med31 has distinct target genes required for mammalian development. *Med31* mutant embryos have fewer proliferating cells than controls, especially in regions that expand rapidly during development such as the forelimb buds. Likewise, embryonic fibroblast cells cultured from mutant embryos have a severe proliferation defect, as well as reduced levels of the cell cycle protein Cdc2. *Med31* mutants have normal limb bud patterning but defective or delayed chondrogenesis due to a lack of *Sox9* and *Col2a1* expression. As the Mediator complex is a transcriptional co-activator, our results suggest that Med31 functions to promote the transcription of genes required for embryonic growth and cell proliferation.

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Introduction

During development, many factors are required for the growth of the mammalian embryo. At a molecular level, embryonic cells must integrate mitogenic signals to promote cell proliferation. Many groups of genes are required for embryonic growth, ranging from those involved in the cell cycle to transcription factors that promote cell proliferation in specific organs or tissues (Brodsky and Christou, 2004). Despite the expectation that many diverse genes are required for embryonic growth, relatively few embryonic growth control genes have been identified in mammals (Efstratiadis, 1998; Han and Carter, 2001).

The mouse is an important experimental model for the study of embryonic growth and intrauterine growth restriction. Genetic manipulations in the mouse enable the deletion of genes from the embryo, placenta, or both, allowing embryonic and extra-embryonic requirements for selected genes to be evaluated. Additionally, mutagenesis screens in the mouse provide a phenotype driven approach to identify genes required for developmental processes in an unbiased manner. We have isolated a mutant, *l11Jus15*, from a mouse balancer chromosome mutagenesis screen that demonstrates cell proliferation and growth defects during development (Kile et al., 2003). Using a positional cloning strategy, we identified a mutation in the Mediator complex protein Med31 in the *l11Jus15* mutant mouse.

The Mediator complex is a general transcriptional co-activator required for transcription from RNA polymerase II promoters. The Mediator complex contains as many as 30 proteins (Malik and Roeder, 2005) and shows conservation from yeast through human (Blazek et al., 2005). Interactions between individual Mediator proteins and nuclear hormone receptors, as well as other transcription factors, have been identified (Beyer et al., 2007; Chen and Roeder, 2007; Ge et al., 2008; Gordon et al., 2006; Rau et al., 2006; Stumpf et al., 2006; Zhu et al., 1997). These findings suggest that the Mediator complex serves as a bridge between gene-specific activators and the RNA polymerase to initiate transcription from Pol II promoters (Blazek et al., 2005).

Med31 was first identified in yeast (called *Soh1*) as a suppressor of the hyperrecombination mutant *hpr1* (Fan et al., 1996; Fan and Klein, 1994) and is a component of the Mediator complex in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Linder and Gustafsson, 2004). Med31 is located in the middle region of the Mediator complex and binds Med10 and Med21/Srb7 (Guglielmi et al., 2004). Med31 is one of the most conserved proteins in the Mediator complex and, in yeast, acts as a positive regulator for transcription of target genes (Koschubs et al., 2009). No specific transcription factor interaction partners have been identified for the Med31 protein, distinguishing it

* Corresponding author. Fax: +44 161 275 5586.
E-mail address: Kathryn.hentges@manchester.ac.uk (K.E. Hentges).

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from the other Mediator complex proteins that have been studied in mouse mutants (Ito et al., 2002, 2000).

Surprisingly, Med31 is not essential in *S. cerevisiae* (Fan and Klein, 1994) although it is required for septation in the fission yeast *S. pombe* (Grallert et al., 1999). In *Drosophila*, maternal *Med31* is required for the establishment of cell fate and anterior–posterior axis formation during embryogenesis (Bosveld et al., 2008). Here we show that *Med31* mutant mouse embryos have defects in cell proliferation and are smaller than control littermates from mid-gestation. This mutant phenotype is different to the phenotypes observed from targeted deletions of Med1 and Med24 (Ito et al., 2002, 2000). An analysis of the target genes of other Mediator complex proteins demonstrates that *Med31* mutants express these target genes at levels comparable to wild-type littermates, allowing us to conclude that *Med31* target genes are distinct from those of other Mediator complex proteins. Fibroblasts isolated from mutants also exhibit a proliferation defect and lack the cell cycle protein cdc2. *Med31* mutant embryos also have reduced expression of *cyclin B* and *mTOR*, genes involved in cell proliferation. *Sox9* and *Col2a1*, genes required for limb bud proliferation, are not expressed in *Med31* mutant limb buds. These experiments therefore identify *cdc2*, *cyclin B*, *mTOR*, *Sox9*, and *Col2a1* as putative *Med31* target genes. These results demonstrate that *Med31* is required for mammalian development and regulates genes that promote proliferation during embryogenesis.

Materials and methods

Embryo dissection and analysis

l11Jus15 mutation carriers were set up for timed matings. The day of the vaginal plug was considered as E0.5. At the desired time point, embryos were dissected out of their decidua and placed on ice for 30 min. Prior to fixation, embryos were imaged while submerged in PBS using a Leica MZ6 microscope and DFC420 camera.

Genotyping, mutation mapping, and Med31 sequencing

Genomic DNA obtained from ear punches of adult mice or yolk sacs of embryos was subjected to PCR to differentiate between C57BL/6 (mutant) and 129S5 (wild-type) strains of mice. For mutation mapping, and some experimental crosses, mice were bred to wild-type 129S5 mice to remove the balancer chromosome. Mice without the balancer chromosome were mated and their offspring genotyped to determine if they were C57BL/6, 129S5, or heterozygous in the 'balancer region'. Adult mice with a chromosome that was partially of C57BL/6 and partially of 129S5 background in the chromosome 11 balancer region were designated as 'recombinant'. Out of a total of 458 genotyped mice, 82 were found to be recombinant (17.9%). Recombinants were mated with *l11Jus15* mice and offspring genotyped at 4 weeks of age. The presence of homozygous C57BL/6 offspring in a litter demonstrated the absence of the mutation causing the *l11Jus15* phenotype. The absence of any homozygous C57BL/6 pups indicated that the mutation was present. Genotyping primer sequences and *Med31* sequencing primers are listed in Supplementary Table 1. PCR products were purified by precipitation and sequenced using Big Dye 1.1 (ABI).

Quantitative real-time PCR

RNA was prepared from individual tissues from CD1 E15.5 embryos, or E10.5 wild-type or *l11Jus15* mutant whole embryos, using TriReagent (Sigma). Five micrograms of RNA from each tissue was treated with RNase-free DNaseI (Promega) and then reverse transcribed with random primers (Promega) and BioScript reverse transcriptase (BioLine). cDNA was added to quantitative real-time PCR reactions using a qPCR core kit (RT-QP73-05, Eurogentec) and the validated *Med31* TaqMan assay (Mm00651937_g1, Applera UK), or SYBR green reagent. Each reaction was performed on three separate embryos for biological replicates. For *Med1*, *Med28*, and *Med31* target gene *qPCR*, E14.5 fetal organ RNA was isolated from at least three embryos of each genotype. GATA-1 target gene primer sequences were provided by Tilman Borggrefe (personal communication). Published qPCR primer sequences for Med28 targets (Beyer et al., 2007), *cyclin B* (Tschop and Engeland, 2007), and *mustn1*(Gersch and Hadjiargyrou, 2009) were used as described. mTOR primer sequences were forward: TACGTCACCATGGAGCTTCGA and reverse: CAAATCTGCCAATTCTGGTGG. All reactions were normalized to GAPDH levels using primer sequences forward: AACTCGGCCCCCAACACT and reverse: TCTAGGCCCCTCCTGTTATTATG. Each qPCR reaction was performed in triplicate for technical replicates.

Whole mount immunohistochemistry and cell counting

Dissected embryos (Supplemental Information) were fixed in 4% paraformaldehyde (PFA) in PBS for 1 h and stored in 100% methanol. Rehydrated embryos were stained with primary antibody: anti-CD31 at 1:100 dilution (BD Pharmingen; cat 550274); anti-phosphohistone H3 at 1:100 dilution (Upstate Biotechnology; cat 06-570) for 1 h at room temperature. Secondary antibody was used at 1:250 dilution, and staining was developed using the VECTASTAIN Elite ABC Kit (Vector Laboratories). Proliferating cells were counted using Image Pro Plus 6.2 software (Media Cybernetics) and analyzed statistically using SPSS version 15 (SPSS Inc.).

MEF cell cultures and growth curve assays

Fibroblast isolation and culture conditions are listed in Supplemental Information. For growth curve experiments, P5 cells were plated in 6-well plates at 2.5×10^4 cells per well and counted using a hemocytometer after 1, 3, 5, and 7 days. Mice without the balancer chromosome were used in these crosses, so that wild-type 129S5 embryos were recovered along with heterozygotes and *l11Jus15* mutants.

Generation of FLAG-Med31 constructs

Mouse Med31 cDNA was obtained from Gene Services (Cambridge, UK) in the pCMV-SPORT6 vector (IMAGE Clone 5709831). PCR products of full-length Med31 cDNA were generated using primer sequences Med31-NotI-GCGGCCGCGATGGCTGCGGCCGT and Med31-SalI-AGAGTCGACCCTAAGAGCCTGACCTGG for cloning into pFLAG-CMV2 (Sigma). Primers L15-01-GCAGATTCTGCACTGGTAGCACTACTCTCG and L15-02-CCGAGAGTAGTGGAACCAGTGCAGAATCTG were used for site-directed mutagenesis to create the mutant version of *Med31*. *Pfu* Ultra polymerase and buffer (Stratagene) were used for PCR mix according to manufacturer's instructions. Cycling conditions for site directed mutagenesis were as follows: 95 °C 30 s, 12 cycles of 95 °C 30 s, 55 °C 1 min, 68 °C 5 min. *DpnI* was then added to the PCR reaction for 2 h at 37 °C to digest plasmid DNA, but not the PCR product. Following digestion, the DNA was transformed, and minipreps performed on all colonies. The presence of the *Med31* ENU mutation was confirmed by sequencing.

HEK293 cell culture and transfections

HEK293 cells were cultured in T75 flasks and passaged at confluency. Culture media was as follows: DMEM (BioWhittaker), 10% FCS (BioWhittaker), 1xPen/Strep (BioWhittaker), 1× L-glutamine (BioWhittaker). For transfections, cells were passaged into 6-well plates at 2×10^6 cells per well in antibiotic free media. The next day, at 80–90% confluency, cells were transfected using Lipofectamine 2000 (Invitrogen). To block proteasome activity 10 µm Lactacystin

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