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Fat facets deubiquitylation of Medea/Smad4 modulates interpretation of a Dpp morphogen gradient

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SUMMARY

The ability of secreted Transforming Growth Factor β (TGFβ) proteins to act as morphogens dictates that their influence be strictly regulated. Here, we report that maternally contributed *fat facets* (*faf*; a homolog of USP9X/FAM) is essential for proper interpretation of the zygotic Decapentaplegic (Dpp) morphogen gradient that patterns the embryonic dorsal-ventral axis. The data suggest that the loss of *faf* reduces the activity of Medea (a homolog of Smad4) below the minimum necessary for adequate Dpp signaling and that this is likely due to excessive ubiquitylation on a specific lysine. This study supports the hypothesis that the control of cellular responsiveness to TGFβ signals at the level of Smad4 ubiquitylation is a conserved mechanism required for proper implementation of a morphogen gradient.

KEY WORDS: USP9X/FAM, Smad4/Medea, TGFβ/BMP, Deubiquitylation, Dorsal-ventral axis, *Drosophila*

INTRODUCTION

TGFβ signals are pleiotropic regulators of animal development. In *Drosophila*, the TGFβ family member Dpp, a homolog of vertebrate BMP2/4, initiates signal transduction with a complex of receptor kinases. One of the receptors then phosphorylates Mad, a member of the Smad family of TGFβ signal transducers. Once phosphorylated, Mad translocates to the nucleus, forms a complex with its sister Smad protein Medea (Med) and regulates gene expression in cooperation with tissue-specific co-factors (Derynck and Miyazono, 2008).

During early embryogenesis, Dpp plays a key role in orchestrating dorsal-ventral (DV) axis formation. Prior to *dpp* zygotic transcription, maternally contributed Mad and Med are present in every cell. At cellular blastoderm stage, a dorsal to ventral extracellular gradient of Dpp protein is generated via a system of extracellular regulation that is translated quantitatively by each cell into intracellular levels of phosphorylated Mad (pMad). At specific thresholds of Mad phosphorylation, cells implement distinct developmental programs. Perturbation of the Dpp gradient (*dpp* mutations) or its interpretation (*Mad* or *Med* mutations) results in aberrant cell fate decisions and abnormal development (Shimmi et al., 2005).

Recent evidence indicates that Med (a homolog of vertebrate Smad4) is also a tunable determinant of cellular responses to TGFβ signals. In vertebrate embryos, Smad4 monoubiquitylation opposes the formation of Smad complexes, providing a mechanism by which nuclei monitor the presence of extracellular ligands and activate gene expression appropriately. In *Xenopus*, knockdown of the Smad4 ubiquitin ligase Ectodermin (Ecto/TRIM33/Tif1-γ) causes expansion of mesoderm markers, whereas knockdown of the Smad4 deubiquitylase USP9X/FAM causes defective mesoderm induction (Dupont et al., 2005; Dupont et al., 2009). In mouse embryos, epiblast-specific *Ecto* (*Trim33* – Mouse Genome Informatics) knockout leads to upregulation of Nodal-dependent mesoderm-induction (Morsut et al., 2010). At present it is unclear whether this mechanism for Smad4 regulation is truly general or vertebrate specific.

The Smad4 deubiquitylase USP9X/FAM is homologous to *Drosophila fat-facets* (*faf*), suggesting conservation of Smad4/Med regulation by deubiquitylation (supplementary material Fig. S1) (Chen et al., 2000). This hypothesis is supported by studies in *Drosophila* where expression of *Xenopus* Ecto led to phenotypes similar to mutations in *Med* that were rescued by co-expression of *Drosophila* Faf (Dupont et al., 2009). In addition, sequence analysis (Konikoff et al., 2008) found that a conserved lysine (Lys^{519} in human Smad4; Lys^{738} in Med) is the residue through which Ecto and USP9X regulate Smad4 activity (Dupont et al., 2009). To date, no developmental roles for Faf in TGFβ signaling have been identified via mutational analyses in any species.

Here, we report the maternal and zygotic requirement for *faf* in Dpp signaling during DV patterning. We analyzed *faf* transheterozygous genotypes that generate embryos capable of surviving beyond the *faf*-null phenotype of blastoderm arrest. We found that a subset of these are dominant maternal enhancers of *dpp* mutations that engender defects in DV axis formation. We were able to rescue *faf* enhancement of *dpp* with a non-ubiquitylatable form of Med. Taken together, the data suggest an important developmental role for Faf as a Med deubiquitylase during Dpp DV signaling. Overall, our study reveals that deubiquitylation is a highly conserved mechanism employed by cells to fine-tune their interpretation of TGFβ signals.

MATERIALS AND METHODS

Fly stocks

Mutant strains are: faf^{BP}, faf^{F08}, faf^{B3}, faf^{BX3}, faf^{B4}, faf^{B5}, faf^{B6} (Fischer-Vize et al., 1992), faf^{EP381} (Berger et al., 2001), dpp^{hr4}, dpp^{hr27}, dpp^{Hin61} (St Johnston et al., 1990), sog^{y506} (Ferguson and Anderson, 1992), Med^{8} (Wisotzkey et al., 1998), Med^{15}, Med^{17} (Hudson et al., 1998) and Mad^{12} (e.g. Sekelsky et al., 1995). Transgenic strains are: nos.Gal4:VP16-MVD1 (van Doren et al., 1998), UASp.GFP-αTub84B (Grieder et al., 2000) and act-lacZ-CB1 (Bourgouin et al., 1992).

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Genetics

Assays of dominant maternal enhancement, zygotic lethality, maternal effect lethality, synthetic lethality and transgenic rescue of *faf* enhancement were conducted using standard methods (Sekelsky et al., 1995). Stage of lethality tests were as described (Takaesu et al., 2006). Alignments and phylogenetic trees were as described previously (Konikoff et al., 2010).

Cuticles and embryos

Cuticle scoring followed Wharton et al. (Wharton et al., 1993). Non-fluorescent antibody labeling followed Johnson et al. (Johnson et al., 2007). Double labeling employed anti-lacZ (Organon Teknika) and anti-Hnt (DSHB- 1G9) detected with biotinylated goat anti-mouse or anti-rabbit and Vecta Stain Elite (Vector Labs). RNA in situ double labeling of embryos with *rho* or *dpp* or *sog* and lacZ cDNAs or antibody double labeling with anti-pMad and anti-lacZ were as described previously (Takaesu et al., 2002; Shimmi et al., 2005). Fluorescence double labeling of embryos followed methods of Quijano et al. (Quijano et al., 2010) using anti-Hnt and anti-lacZ. Additional antibodies employed in unfertilized eggs and embryos were anti-Flag (Sigma) and anti-Bonus (Beckstead et al., 2001). Secondary antibodies were Alexa Fluor goat anti-rabbit, anti-mouse and anti-guinea pig (Molecular Probes).

Transgenes

A Med-K738R cDNA was generated by site directed mutagenesis from Med-wt in pAcpA (McCabe et al., 2004). These were subcloned as *Mlu*I-*Nhe*I fragments into a modified pUASP vector (Rørth, 1998) with a novel multiple cloning site. Human Smad4-K519R and Smad4-wt cDNAs in pRK5 (Dupont et al., 2009) were excised with *Eco*RI-*Sal*I and subcloned as *Eco*RI-*Pme*I fragments into the modified pUASP vector. All UASP plasmids were verified by sequencing prior to generating multiple transgenic lines by standard methods.

RESULTS

Given that USP9X deubiquitylates Smad4, we tested the hypothesis that *faf*, the fly USP9X homolog, plays a role in Dpp signal transduction, a process in which Med is a major component. Two events that depend on Dpp signaling are adult wing vein formation and DV patterning in the early embryo. A study of wings from adults generated by a set of inter se crosses between eight *faf* alleles did not identify any defects. We did note that transheterozygous *faf* mutant females are sterile, as reported by Fischer-Vize et al. (Fischer-Vize et al., 1992). This prompted us to examine the possibility that *faf* plays a role in Dpp signaling during embryonic DV patterning.

faf mutations are dominant maternal enhancers of *dpp* mutations

We began with the classic assay for dominant maternal enhancement of *dpp* recessive lethal mutations that led to the discovery of *Mad* and *Med* (Sekelsky et al., 1995; Raftery et al., 1995). Missense mutations in the Dpp pro-domain (dpp^{hr4}; G402E) (Wharton et al., 1996) impact ligand cleavage, dimerization or stability and are almost completely recessive; they survive at near wild-type levels when heterozygous but are absolutely lethal when homozygous. In this assay, a female parent with a heterozygous mutation at a second locus reduces the survival of the *dpp* recessive allele as a heterozygote from near wild type to near absolute lethality. For *Mad* and *Med*, the explanation is that the reduction in the dose of functional maternal RNA for either of these signal transducers in combination with a reduction in the dose of fully functional Dpp leads to a diminished Dpp morphogen gradient, ventralization of the embryo and death.

Employing females heterozygous for seven of our eight *faf* alleles (faf^{EP381} is a P insertion in the first intron creating a viable allele that was tested as a homozygote), we found that faf^{EP381} displayed maternal enhancement of dpp^{hr4} and that faf^{F08} (missense mutation in the catalytic histidine) displayed dominant maternal enhancement. Mating to faf^{EP381} homozygous females reduced the survival of dpp^{hr4} progeny to 26% of expected. For comparison, mating to Med^{15} and Med^{17} (missense mutations) homozygous females reduced the survival of dpp^{hr4} to less than 5% of expected. Mating to faf^{F08} heterozygous females reduced dpp^{hr4} survival to 10% of expected. For comparison, mating to Med^{8} (nonsense mutation) heterozygous females reduced dpp^{hr4} survival to 5% of expected (Fig. 1E; supplementary material Table S1A). Stage of lethality assays revealed that faf^{F08} enhancement of dpp^{hr4} led these individuals to die as embryos and that there was no effect on dpp^{hr4} survival in the reciprocal cross between faf^{EP381} males and dpp^{hr4} females (supplementary material Table S2). These results are consistent with data for both *Mad* and *Med* (Sekelsky et al., 1995; Raftery et al., 1995).

We then analyzed cuticles from the two *faf* maternal enhancement crosses and compared them with dpp^{hr4} homozygous and *Med* enhanced dpp^{hr4} cuticles. The analysis revealed that *faf* enhanced dpp^{hr4} progeny displayed defects in DV patterning similar to those of ventralized dpp^{hr4} homozygous and *Med* enhanced dpp^{hr4} progeny (Fig. 1A-D; supplementary material Table S3A). Shared defects include a herniated head skeleton at the anterior, misshapen and/or internalized Filzkorper at the posterior, dorsal extension of the ventral denticle belts and a partially U-shaped body. These similarity of *faf* enhanced dpp^{hr4} embryos to dpp^{hr4} homozygotes and *Med* enhanced dpp^{hr4} embryos suggest that maternal Faf deubiquitylation is important for Dpp signaling during DV patterning.

faf zygotic mutants display DV defects

The maternal enhancement experiments showed that *faf* mutations can sensitize embryonic development to defective Dpp signaling. Therefore, we sought to determine whether *faf* mutations alone can cause defects comparable with mutations in the Dpp pathway. In these studies, we analyzed both transheterozygous zygotic and maternal mutants by assessing survival and cuticle phenotypes. We employed an inter se strategy with eight *faf* mutant alleles to generate *faf* transheterozygous zygotic mutant progeny (e.g. faf^{F08}/faf^{B3}). We found that many *faf* zygotic mutant combinations are fully viable but others generate adults at less than 50% of the expected ratio. In three genotypes, only 25% of the expected *faf* mutants survived to adulthood (Fig. 1J; supplementary material Table S1B). A reduction in *faf* zygotic mutant survival had not been noted before.

In cuticle studies of genotypes with reduced survival, we observed that *faf* transheterozygous zygotic mutants displayed a range of defects in DV patterning (Fig. 1F-I; supplementary material Table S3B). The most severely affected *faf* zygotic mutants (faf^{F08}/faf^{B3}, 27% of expected) displayed DV defects that resembled *dpp* null genotypes. Other *faf* zygotic mutants generated cuticles similar to dpp^{hr4} homozygous and dpp^{hr4} enhanced cuticles. This data suggests that, in addition to the maternal requirement identified in enhancement assays, there is also a zygotic requirement for *faf* in Dpp signaling during DV patterning.

faf maternal mutants form an allelic series that generates a gradient of DV defects

We continued the inter se experiment for a second generation to confirm a maternal requirement for *faf* in Dpp DV signaling. We employed transheterozygous females bearing all combinations of the eight *faf* mutants in crosses to wild-type males. The progeny will be heterozygous for a *faf* mutant allele derived from their

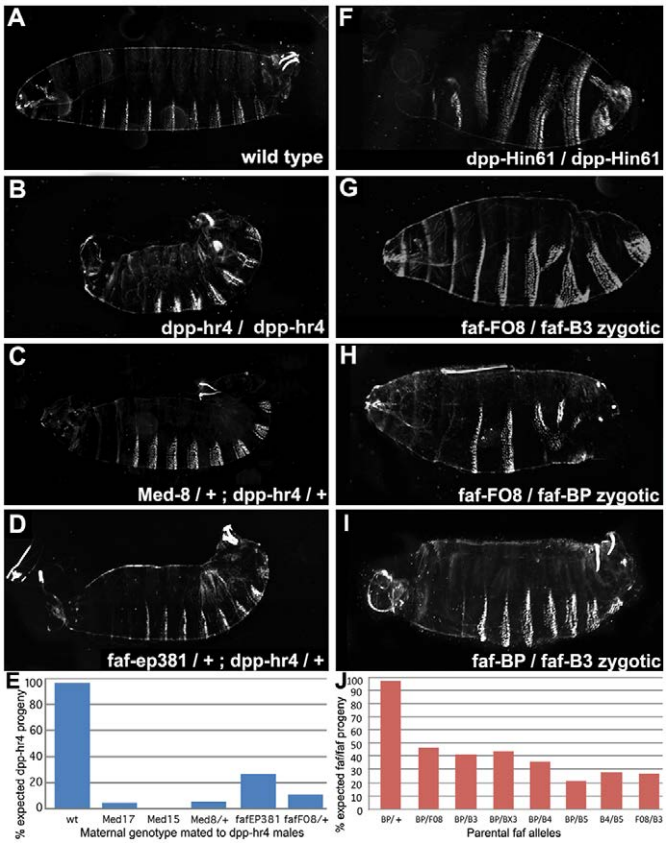

Fig. 1. *faf* enhancement of *dpp* and *faf* zygotic lethality is associated with DV defects. Cuticles in lateral view with anterior leftwards and dorsal upwards. The maternally contributed allele is listed first. (**A**) Wild type. Broad white ventral denticle belts (bottom), narrow white Filzkorper (upper right corner) and internal head skeleton (left side) are visible. (**B**) Homozygous dpp^{hr4} ventralized cuticle with a short curved body, dorsally extended denticles, herniated head and defective Filzkorper. (**C**) Dominant maternal enhancement of dpp^{hr4} by Med^{8} is visible in a ventralized Med^{8} and dpp^{hr4} double heterozygous cuticle similar to dpp^{hr4}. (**D**) Maternal enhancement of dpp^{hr4} by faf^{EP381} is visible in a ventralized faf^{EP381} and dpp^{hr4} double heterozygous cuticle similar to the dpp^{hr4} and the Med^{8} and dpp^{hr4} double heterozygous cuticle. (**E**) Maternal enhancement of dpp^{hr4} by *Med* and *faf* females. The percentage of expected dpp^{hr4} progeny from crosses to *Med* or *faf* heterozygous females is compared with mating with wild-type females (first bar). Numerical data are in supplementary material Table S1A. (**F**) dpp^{Hin61} homozygous null cuticle is fully ventralized with a herniated head, ectopic denticle belt replacing the Filzkorper and ventral denticle belts encircling the body. (**G**) faf^{F08}/faf^{B3} ventralized cuticle with dorsally extended denticles and an ectopic denticle belt replacing the Filzkorper. (**H**) faf^{F08}/faf^{BP} ventralized cuticle with dorsally extended denticles and poorly developed Filzkorper. (**I**) faf^{BP}/faf^{B3} ventralized cuticle with herniated head, dorsally extended denticles and misplaced Filzkorper. (**J**) Zygotic lethality of *faf* transheterozygous genotypes. The percentage of expected *faf* progeny from representative crosses is compared with those generated by a *faf* null allele mated to wild type (first bar). Numerical data are in supplementary material Table S1B.

mother and a wild-type *faf* allele from their father. The paternal wild-type allele allows normal development and defects are attributed to the *faf* mutation in the maternal parent.

Embryos derived from *faf* homozygous mutant females primarily die prior to the onset of embryonic *dpp* expression (Fischer-Vize et al., 1992). However, our second-generation crosses revealed that embryos from a subset of *faf* transheterozygous mutant females survive past this point and that their cuticles display DV defects similar to ventralized *dpp* mutants. Importantly, cuticles derived from embryos generated by these *faf* mutant females could be ordered into an allelic series. As opposed to the zygotic requirement for *faf* in DV patterning, which was revealed by combinations of strong hypomorphic alleles, the graded maternal requirement for *faf* was identified in combinations of weak hypomorphic alleles (Fig. 2A-G).

The least affected cuticles were derived from faf^{B4}/faf^{B5} females. Next in severity are faf^{B4}/faf^{B6}, faf^{F08}/faf^{B4} and faf^{B4}/faf^{BX3} cuticles. The most severe DV defects are seen in faf^{B3}/faf^{B5} cuticles. Embryos derived from stronger transheterozygous combinations, such faf^{B6}/faf^{BP}, do not generate any cuticle, owing to early embryonic lethality associated with the *faf*-null phenotype of blastoderm arrest. The *faf* maternal mutant allelic series creates a gradient of DV defects that parallels the phenotypes of an allelic series constructed for *dpp* (Wharton et al., 1993).

faf mutations enhance *Med* mutations and can partially suppress *sog* mutations

If the mechanism by which USP9X deubiquitylates Smad4 is operating in Dpp DV signaling, then one prediction is that reducing *faf* and *Med* dose together in double heterozygous embryos will reduce the amount of functional Med to the point of interfering with Dpp signaling. A corollary is that if the effect of the double heterozygote is non-reciprocal, then the more crucial component is the one that must be compromised in the mother to obtain an effect.

In our analysis, we observed that a subset of *faf* and *Med* double heterozygous genotypes displayed the synthetic lethality predicted by the USP9X-Smad4 mechanism. In these cases, fewer than half the expected number of double heterozygous progeny survived (Fig. 2J; supplementary material Table S1C). In all combinations with synthetic lethality, the female parent was heterozygous for Med^{8} while the male parent contributed a *faf* mutant allele, indicating that maternal reduction in *Med* is more damaging to Dpp DV signaling than maternal reduction in *faf*. Cuticle studies of double heterozygous genotypes with reduced survival revealed that the lethality was due to defects in DV patterning (Fig. 2H,J; supplementary material Table S3C). Data prior to this point revealed that *faf* plays a role in Dpp DV signaling and now *faf-Med* synthetic lethality suggests a mechanism: *faf* impacts Dpp DV signaling via interactions with *Med*. This suggestion is supported by results from crosses of *faf* mutants and Mad^{12} (null allele for Dpp signaling) (Sekelsky et al., 1995). All *Mad* and *faf* double heterozygous genotypes displayed normal survival and wild-type cuticles.

We then examined the ability of *faf* to suppress dorsalization defects caused by to mutations in *sog*. If *dpp* mutations are able to partially suppress *sog* mutant phenotypes (Francois et al., 1994), then mutations affecting Dpp DV signaling pathway components will have the same effect. We validated the prediction with a mutation in *Med* and found that strong *faf* mutant alleles have the same effect (Fig. 2K-L; supplementary material Table S3D). Taken together, our survival assays and cuticle data suggest that maternal and zygotic *faf* activity plays a positive role in Dpp DV signaling via interactions with *Med*.

Loss of hindsight and *rhomboid* in *faf* mutants phenocopy Dpp signaling mutants

To confirm the role of *faf* in Dpp signaling, we next visualized established Dpp target genes (Hindsight and *rhomboid*) in genetic combinations displaying cuticular DV defects. Hin is expressed in

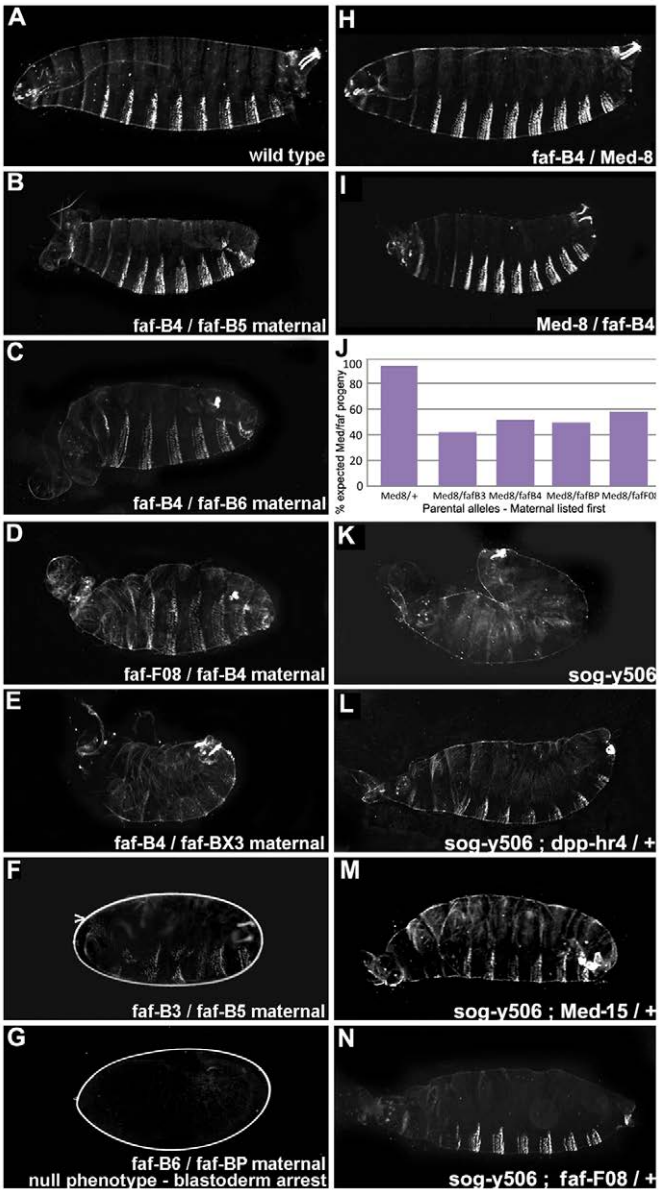

Fig. 2. *faf* maternal mutants generate a gradient of DV defects, whereas *faf* zygotic heterozygosity partially enhances *Med* and suppresses *sog* mutants. Cuticles in lateral view derived from mating of the indicated *faf* transheterozygous female to a wild-type male. (**A**) Wild type. (**B**) faf^{B4}/faf^{B5} cuticle displays modest ventralization with a herniated head, poorly developed Filzkorper and slightly extended ventral cuticles similar to a haploinsufficient dpp^{Hin46} heterozygous cuticle (Wharton et al., 1993). faf^{B4} is an in-frame six-residue insertion at position 279 and faf^{B5} is a frame-shift after amino acid 2150. These widely spaced alternations each generate a weak hypomorphic allele (Fisher-Vize et al., 1992; Chen and Fisher, 2000). (**C**) faf^{B4}/faf^{B6} cuticle displays increased ventralization with a herniated head, defective Filzkorper and denticle belts that extend throughout the ventral half of the cuticle similar to a dpp^{hr56} homozygous cuticle. faf^{B6} is a nonsense mutation at position 459 that functions as an near protein-null allele. (**D**) faf^{F08}/faf^{B4} cuticle displays significant ventralization with a herniated head, defective Filzkorper, malformed dorsal cuticle and denticle belts that extend well into the dorsal half of the embryo similar to a dpp^{hr92} homozygous cuticle and to cuticles from *Med* mutant germline clones with partial paternal rescue (Hudson et al., 1998). faf^{F08} is a missense mutation in a catalytic histidine (H1986Y) that generates an allele impacting Dpp signaling, as assayed by dpp^{hr4} enhancement. (**E**) faf^{B4}/faf^{BX3} cuticle also displays significant ventralization with a herniated head, defective Filzkorper, malformed dorsal cuticle and extended denticle belts similar to a dpp^{Hin46}/dpp^{e87} cuticle. faf^{BX3} is an in-frame deletion of 15 bp shortly after the catalytic domain that generates a strong hypomorphic allele. (**F**) faf^{B3}/faf^{B5} cuticle appears almost fully ventralized within the vitelline membrane. Its rudimentary cuticle contains one anterior denticle belt that fully encircles the embryo (indicated by the white arrowhead on the left outside the vitelline membrane) similar to a dpp^{Hin94}/dpp^{Hin95} cuticle. faf^{B3} is a nonsense mutation at position 71 that generates a protein null allele. (**G**) faf^{B6}/faf^{BP} embryo remains intact inside the vitelline membrane but contains no cuticle due to early embryonic lethality associated with the *faf*-null phenotype of blastoderm arrest. (**H**) *faf* mutation contributed by a heterozygous mother yields a faf^{B4} and Med^{8} double heterozygous cuticle that appears wild type. Note that *Med* and *faf* are both on chromosome 3, so we do not employ '+' to represent the homolog, even though *Med* on the faf^{B4} chromosome is normal, as is *faf* on the Med^{8} chromosome. (**I**) *Med* mutation contributed by a heterozygous mother yields a Med^{8} and faf^{B4} double heterozygous cuticle that is similar to dpp^{hr4}. (**J**) Maternal enhancement of *faf* mutants by Med^{8} heterozygous females. The percentage of expected Med^{8} and *faf* mutant double heterozygous progeny is compared with those generated by mating of Med^{8} heterozygous females with wild-type males (first bar). Numerical data are in supplementary material Table S1C. (**K**) sog^{y506} hemizygous dorsalized cuticle containing extremely truncated denticles, completely U-shaped body, herniated head and Filzkorper defects. (**L**) sog^{y506} hemizygous and dpp^{hr4} heterozygous cuticle with partially restored denticles and curved body. (**M**) sog^{y506} hemizygous and Med^{15} heterozygous cuticle with partially restored denticles, normal body shape and misplaced, misshapen Filzkorper. (**N**) sog^{y506} hemizygous and faf^{F08} heterozygous cuticle with partially restored denticles, normal body shape and poorly developed Filzkorper.

the amnioserosa, the dorsal-most embryonic tissue and the one that requires the highest level of Dpp signaling (e.g. Raftery et al., 1995). Each of the Hin experiments was consistent with cuticle data derived from the same cross (Fig. 3A-G).

The dominant maternal enhancement of dpp^{hr4} by faf^{F08} led to a loss of Hin-expressing cells, though less severe in this example than Med^{17} enhancement of dpp^{hr4} and homozygosity for dpp^{hr4}. Zygotic transheterozygous combinations of strong *faf* mutant alleles also displayed reduced Hin expression. Embryos derived from *faf* maternal transheterozygous mutants for which a subset survive past the null phenotype exhibit essentially no Hin expression. This closely resembles dpp^{hr4} homozygous embryos and *Med* mutant germline clones (Hudson et al., 1998). *Med* maternal and *faf* paternal double heterozygous embryos display reduced Hin expression whereas embryos from the reverse cross (*Med* paternal and *faf* maternal) are wild type.

We extended the analysis to *rhomboid* (*rho*) expression in the dorsal ectoderm of cellular blastoderm (stage 5) embryos: *rho* is the earliest known target of Dpp signaling in embryonic DV patterning (Yu et al., 2000) and assay of *rho* transcription is also a standard means of evaluating Dpp activity (e.g. Ross et al., 2001). If *faf* mutations influence dorsal ectoderm *rho* expression, this would further support a role in Dpp DV signaling (Fig. 3H-N).

The dominant maternal enhancement of dpp^{hr4} by faf^{F08} led to loss of *rho* expression in the central region of the dorsal ectoderm. This phenotype mimicked Med^{17} enhancement of dpp^{hr4} and

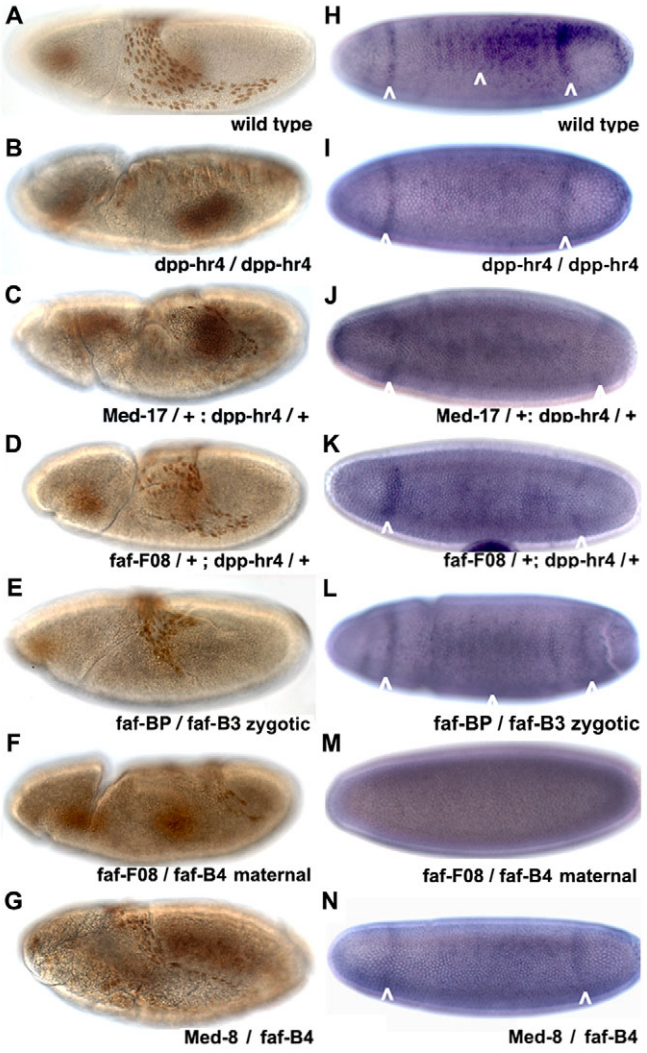

Fig. 3. Loss of Hnt and *rho* in *faf* mutant genotypes suggests reduced Med function in Dpp DV signaling. (A-G) Stage 10/11 embryos in lateral view depicting Dpp-dependent Hnt expression in the large cells of the amnioserosa. Dpp-independent Hnt expression in the foregut and hindgut (below the plane of focus) act as an internal control for staining. (**A**) Wild-type embryo. (**B**) dpp^{hr4} homozygous ventralized embryo with a few scattered amnioserosa cells. (**C**) Med^{17} and dpp^{hr4} maternally enhanced embryo appears similar to dpp^{hr4}. (**D**) faf^{F08} and dpp^{hr4} maternally enhanced embryo is similar to the ventralized Med^{17} and dpp^{hr4} embryo. (**E**) faf^{BP}/faf^{B3} embryo appears weakly ventralized with a reduced number of amnioserosa cells. (**F**) Embryo from a faf^{F08}/faf^{B4} female mated to a wild-type male is similar to dpp^{hr4}. (**G**) Med^{8} maternal and faf^{B4} paternal double heterozygous embryo is similar to the weakly ventralized faf^{BP}/faf^{B3} zygotic embryo but with fewer amnioserosa cells. (H-N) Stage 5 embryos in dorsal/lateral views revealing Dpp-dependent *rho* transcription in the dorsal ectoderm. (**H**) Wild-type embryo with a central dorsal stripe of *rho* expression bounded by wider domains (arrowheads). (**I**) dpp^{hr4} homozygous embryo with no central stripe of *rho*. (**J**) Med^{17} and dpp^{hr4} maternally enhanced embryo is similar to dpp^{hr4}. (**K**) faf^{F08} and dpp^{hr4} maternally enhanced embryo is similar to dpp^{hr4}. (**L**) faf^{BP}/faf^{B3} embryo appears weakly ventralized with faint *rho* expression in the dorsal ectoderm. (**M**) Embryo from faf^{F08}/faf^{B4} female mated to a wild-type male has passed syncytial blastoderm arrest but displays no *rho* expression. (**N**) Med^{8} maternal and faf^{B4} paternal double heterozygous embryo is similar to the weakly ventralized faf^{BP}/faf^{B3} zygotic embryo but with further reduced *rho* expression.

homozygosity for dpp^{hr4}. Zygotic transheterozygous combinations of strong *faf* mutant alleles also displayed reduced *rho* central region expression though less severe in this example than Med^{17} enhancement of dpp^{hr4} and homozygosity for dpp^{hr4}. Embryos derived from *faf* maternal transheterozygous mutants for which a subset survive past the null phenotype exhibit no *rho* expression in any region of the dorsal ectoderm, a phenotype also seen in embryos derived from homozygous Med^{15} females. *Med* maternal and *faf* paternal double heterozygous embryos display reduced *rho* expression in the central region. Each of the *rho* experiments was consistent with the cuticle and Hin data derived from the same cross.

We replicated all of these results with a second missense mutation in the prodomain-dpp^{hr27} (E316K) (Wharton et al., 1996). For example, Med^{8} dominant maternal enhancement of dpp^{hr27} led to the survival of 3% of dpp^{hr27} individuals, whereas faf^{F08} enhancement led to survival of 4%. One hypothesis that explains all of the data is that loss of the Faf deubiquitylase reduces Med activity below the level needed for gene expression in tissues requiring the highest amount of Dpp. This hypothesis for the relationship between Faf and Med in Dpp signaling is analogous to that between USP9X and Smad4 in vertebrate TGFβ signaling (Dupont et al., 2009).

faf mutations do not affect *dpp* or *sog* transcription or pMad activation/dorsal localization

We then analyzed an alternative hypothesis for the role of *faf* in Dpp DV signaling. During DV patterning the roles of opposing morphogen gradients composed of Dpp and Sog are well known. *dpp* is transcribed in the dorsal half of the embryo and then post-translational mechanisms, such as extracellular interactions with Sog transcribed in the ventral-lateral region of the embryo, create a gradient of Dpp activity that induces five distinct cell fates along the DV axis (Shimmi et al., 2005). Assays of cuticles or Hin or *rho* expression in *faf* mutants cannot formally exclude the possibility that *faf* modulates DV patterning by regulating *dpp* and/or *sog* transcription. We conducted *dpp* and *sog* situ hybridization studies using *faf* maternal mutants for which a subset of embryos survive past the null phenotype and zygotic mutants that display DV defects. Both faf^{B4}/faf^{B5} maternal and faf^{F08}/faf^{BP} zygotic mutant embryos contain wild-type *dpp* RNA (Fig. 4A-C) as well as wild-type *sog* RNA (Fig. 4D-F) expression. This suggests that the alternative hypothesis of transcriptional activation is false.

We also analyzed pMad expression in *faf* maternal and zygotic mutants. This assay can provide evidence that will allow us to further eliminate the possibility that *faf* influences Dpp DV signaling via interactions with *Mad*, rather than *Med*. Both maternal and zygotic *faf* mutant combinations display normal pMad activation and localization to the dorsal-most region at stage 5 (Fig. 4G-I). These pMad results are reproducible in multiple maternal and zygotic *faf* mutants (supplementary material Fig. S2) and are similar to pMad expression in dpp^{hr4} heterozygous embryos that have wild-type DV patterning (supplementary material Fig. S3). These data confirm our hypothesis that *faf* blocks Dpp DV signaling downstream of Mad. We noted that *faf* maternal mutants show a slightly broader pMad stripe and believe this is due to reduced Med activity in a Dpp feedback loop (Wang and Ferguson, 2005).

Taken together, all of the data are consistent with our hypothesis for the role of *faf* in Dpp signaling: loss of maternal or zygotic *faf* leads to a reduction in Med activity, insufficient Dpp signal transduction and DV defects. Thus, Faf deubiquitylation of Med during Dpp DV signaling is the first genetically defined

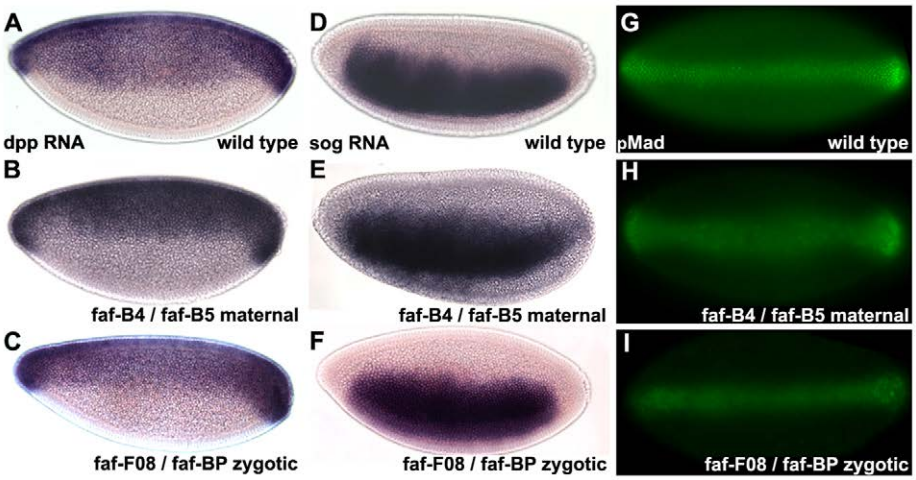

Fig. 4. *faf* zygotic and maternal mutant embryos display wild-type *dpp* and *sog* transcription and pMad activation. (A-F) In situ hybridization to stage 5 embryos in lateral view. (**A**) Wild-type *dpp* is visible in the dorsal half of the embryo with slight ventral extensions in terminal regions. (**B**) faf^{B4}/faf^{Bt5} maternal embryo from a female whose progeny survive beyond the *faf*-null phenotype (see Fig. 2B) displays wild-type *dpp*. (**C**) faf^{F08}/faf^{BP} zygotic embryo, from a cross generating cuticles with DV defects (see Fig. 1H), displays wild-type *dpp*. (**D**) Wild-type *sog* is visible in the ventral half of the embryo but absent in terminal regions and the ventral-most 5-10%. (**E**) faf^{B4}/faf^{B5} maternal mutant embryo displays largely wild-type *sog*. (**F**) faf^{F08}/faf^{BP} zygotic mutant embryo displays wild-type *sog*. (G-I) Antibody labeling of stage 5 embryos shown in dorsal view. (**G**) Wild-type pMad activation and localization is visible in a narrow stripe atop the dorsal-most region of the embryo with slightly wider expression at the termini. (**H**) faf^{B4}/faf^{B5} maternal mutant embryo displays normal pMad activation and a slightly broader pMad stripe. (**I**) faf^{F08}/faf^{BP} zygotic mutant embryo exhibits wild-type pMad activation and localization.

developmental event employing the conserved USP9X-Smad4 regulatory mechanism. We then wondered whether the mechanism is conserved at the level of the deubiquitylated lysine in Med.

Medea Lys^{738} is deubiquitylated by maternal Faf during Dpp DV signaling

We examined whether the USP9X-Smad4 regulatory mechanism is conserved at the level of the deubiquitylated lysine in Med using transgenic rescue experiments that were evaluated with survival, cuticle and Hnt data. We compared the ability of a wild-type Med transgene (Med-wt) to rescue *faf* and *Med* dominant maternal enhancement of dpp^{hr4} with the rescuing ability of a ubiquitin-resistant Med transgene. Our phylogenetic analysis showed that Lys^{519} in human Smad4 (targeted by Ecto and USP9X) is conserved as Lys^{738} in Med (Konikoff et al., 2008). For the non-ubiquitylatable Med transgene, we assumed the homologous lysine was the Faf target and created Med-K738R. We used arginine as the replacement to avoid disturbing Med protein structure with a dissimilar (non-negatively charged) amino acid. The USP9X-Smad4 model predicts that the non-ubiquitylatable transgene (Med-K738R) will be hyperactive in Dpp signaling and thus rescue dpp^{hr4} embryos from *faf* or *Med* maternal enhancement better than Med-wt.

We conducted the rescue experiment with two different mutants (faf^{F08} and Med^{8}), at two discrete levels of transgene expression and with two distinct sets of transgenes. Basal levels of expression are generated by the P transposase minimal promoter plus first intron and the K10 3′UTR that are present in the UASP vector (Rørth, 1998). Overexpression is driven by nos.Gal4 (Gal4:VP16-nos.3′UTR, line MVD1) (van Doren et al., 1998) from a chromosome also containing UASP.eGFP to monitor nos.Gal4 expression. Figure S4 (supplementary material) reveals that relative levels of transgene expression in these two genotypes are essentially identical for two different transgene insertions (Med-wt and Med-K728R) in unfertilized eggs and stage 5 embryos, strongly suggesting that position effects will not interfere with this assay. Last, all balancer chromosomes were marked with transgenic lacZ to allow positive identification of experimental embryos during the Hnt analysis. Results from all rescue experiments were compared with the original, non-transgenic dpp^{hr4} enhancement cross.

Rescue assays with faf^{F08} were the most informative. The logic is that loss of the deubiquitylase will be rendered less consequential in the presence of a non-ubiquitylatable and thus hyperactive Med-K738R transgene, only if Lys^{738} is the ubiquitylated lysine. If any other lysine were ubiquitylated, then the loss of *faf* would be compensated for by Med-K738R at the same level as Med-wt. The most telling result was with the basal promoter (Fig. 5G; supplementary material Table S4A). Here, expression of Med-wt increased the survival of dpp^{hr4} progeny 1.6-fold. By contrast, expression of Med-K738R increased the survival of dpp^{hr4} progeny 4.2-fold. Thus, Med-K738R performed 2.65-fold better in the rescue of dpp^{hr4} with basal expression. Once the transgenes were driven at ectopic levels with nos.Gal4, then Med-wt was equal to Med-K738R as both reached a rescue ceiling in this assay at roughly 80% of expected.

The survival results are supported by cuticle assays (Fig. 5A-F, left column; supplementary material Table S5A). A high percentage of cuticles show DV defects in the faf^{F08} enhancement control and in the basal rescue experiment with Med-wt. The basal experiment with Med-K738R shows an intermediate level and the ectopic rescue experiments show a low percentage of cuticles with DV defects. The cuticle data for faf^{F08} rescue is perfectly mirrored by Hnt data with the added advantage of explicitly identifying dpp^{hr4} heterozygous embryos (Fig. 5A-F, middle and right columns). The data validates Lys^{738} as the ubiquitylation site associated with Faf modulation of Med activity.

To firmly establish the conservation of Med deubiquitylation at the level of the target lysine, we repeated the faf^{F08} rescue experiment with two human Smad4 UASP transgenes (Smad4-wt

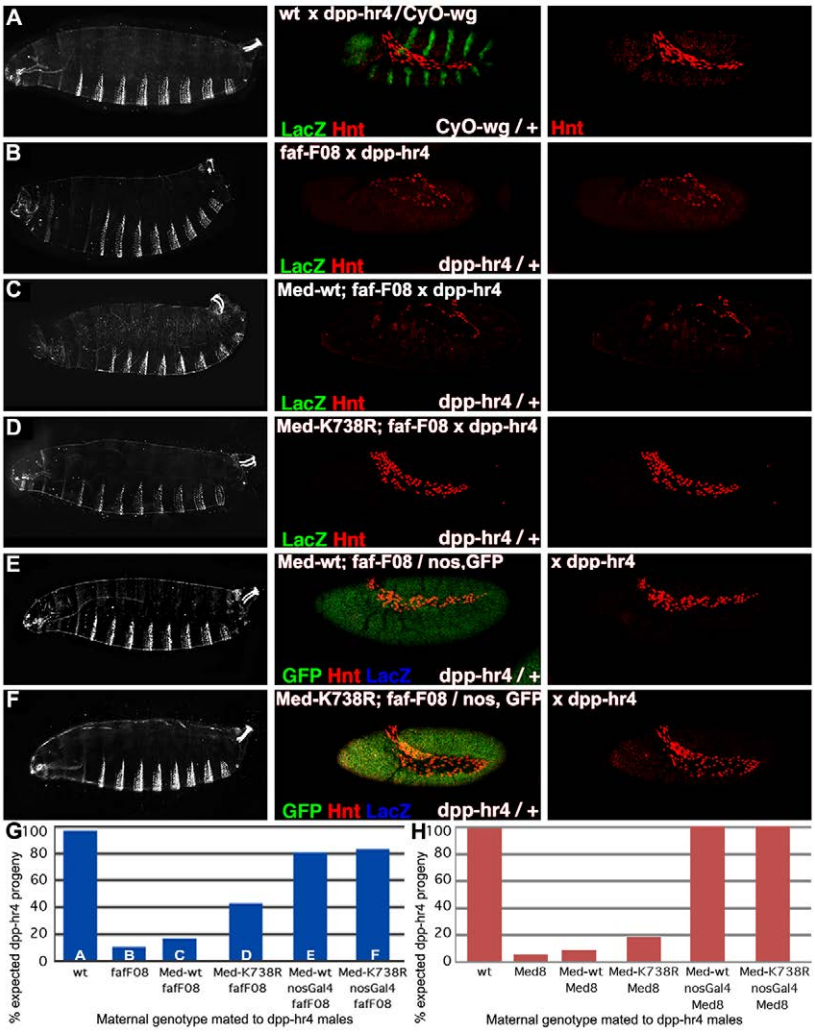

Fig. 5. Med-K738R rescues *faf*^{F08}- and *Med*^{8} enhancement of *dpp*^{hr4} better than Med-wt. (A-F) Cuticles (left). Stage 10/11 embryos (middle) showing endogenous Hnt (red), transgenic *lacZ* from the balancer opposite *dpp*^{hr4} (green, A-D; blue, E,F) and transgenic eGFP (green, E,F). Red channel only (Hnt, right). Absence of *lacZ* indicates the *dpp*^{hr4} chromosome. (**A**) Wild-type cuticle and embryo inheriting balancer chromosomes from crosses between wild-type females and *dpp*^{hr4} heterozygous males. (**B**) Ventralized cuticle and embryo with significant reduction in Hnt generated by *faf*^{F08} dominant maternal enhancement of *dpp*^{hr4}. (**C**) Ventralized cuticle and embryo with little Hnt indicate that basal maternal expression of a Med-wt transgene does not effectively rescue *faf*^{F08} enhancement of *dpp*^{hr4}. (**D**) Wild-type cuticle and normal Hnt indicate that basal maternal expression of a Med-K738R transgene rescues *faf*^{F08} enhancement of *dpp*^{hr4} better than Med-wt. (**E**) Wild-type cuticle and normal Hnt indicate that nos.Gal4 maternal expression of Med-wt effectively rescues *faf*^{F08} enhancement of *dpp*^{hr4}. (**F**) Wild-type cuticle and normal Hnt indicate that nos.Gal4 maternal expression of Med-K738R also effectively rescues *faf*^{F08} enhancement of *dpp*^{hr4}. (**G**) Bar graph depicting transgenic rescue of *faf*^{F08} dominant maternal enhancement of *dpp*^{hr4} by Med-wt and Med-K738R with basal and nos.Gal4 expression. The percent of expected *dpp*^{hr4} progeny obtained in crosses to wild-type and *faf*^{F08} heterozygous females (first and second bars) is compared with matings of *faf*^{F08} heterozygous females bearing four different transgene/promoter combinations. Letters in each bar indicate data corresponding to the panels above. There is 2.65-fold better rescue with basal expression of Med-K738R versus Med-wt (column D versus C). Numerical data are in supplementary material Table S4A. (**H**) Transgenic rescue of *Med*^{8} dominant maternal enhancement of *dpp*^{hr4} by Med-wt and Med-K738R with basal and nos.Gal4 expression. There is 2.25-fold better rescue with basal expression of Med-K738R versus Med-wt. Numerical data are in supplementary material Table S4C.

and Smad4-K519R). The relative performance of the two Smad4 transgenes (2.14-fold better rescue with Smad4-K519R) was nearly identical to that of Med-wt versus Med-K738R at basal levels (supplementary material Fig. S5; Table S4B, Table S5B).

A rescue assay with the *Med*^{8} null allele then controlled for the possibility that Med-K738R is neomorphic. If Med acquired an unusual activity via the K738R substitution that allowed it to rescue *faf*^{F08} enhancement but this was not due to hyperactivity of normal functions, then Med-K738R should not rescue a *Med* loss-of-function allele. If K738R leads to hyperactivity owing to ubiquitin resistance, then it should rescue *dpp*^{hr4} embryos from *Med*^{8} enhancement better than Med-wt.

Again, the most telling result was with the basal promoter (Fig. 5H; supplementary material Table S4C). Here Med-wt expression increased the survival of *dpp*^{hr4} progeny 1.6-fold. By contrast, Med-K738R expression increased the survival of *dpp*^{hr4} progeny 3.6-fold. Thus, Med-K738R performed 2.25-fold better in the rescue of *dpp*^{hr4} with basal expression. Once the transgenes were driven at ectopic levels with nos.Gal4, Med-wt again closed the gap in performance. These survival results are strongly supported by cuticle assays and Hnt data (supplementary material Fig. S6, Table S5C). We repeated the experiment with the Smad4 transgenes. The relative performance of the two transgenes (1.75-fold better rescue with Smad4-K519R) was only slightly lower than for Med-wt versus Med-K738R at basal levels (supplementary material Fig. S7; Table S4D, Table S5D).

Overall, the rescue data indicate that Lys^{738} is the key residue by which Med is regulated by the Faf deubiquitylase. These results strongly suggest that regulation of Med/Smad4 deubiquitylation by Faf/USP9X is a conserved molecular and developmental mechanism regulating TGFβ responsiveness.

DISCUSSION

Faf deubiquitylase regulates Dpp signaling and embryonic DV patterning

The existence of a ‘Smad4 ubiquitylation cycle’ has been recently proposed in vertebrate model systems that requires the ubiquitin ligase Ecto and the deubiquitylase USP9X. These studies suggested that Ecto can monoubiquitylate Smad4 at Lys^{519} and that this interferes with binding to R-Smads because Lys^{519} falls within a crucial interaction surface. Subsequently, USP9X deubiquitylation of Smad4 at Lys^{519} restores Smad4 function. The function of Ecto as an inhibitor of TGFβ signaling was validated by mouse knockout studies demonstrating that Ecto restrains early Nodal/Smad4 signaling (Dupont et al., 2012). However, USP9X activity has not yet been validated through any genetic test.

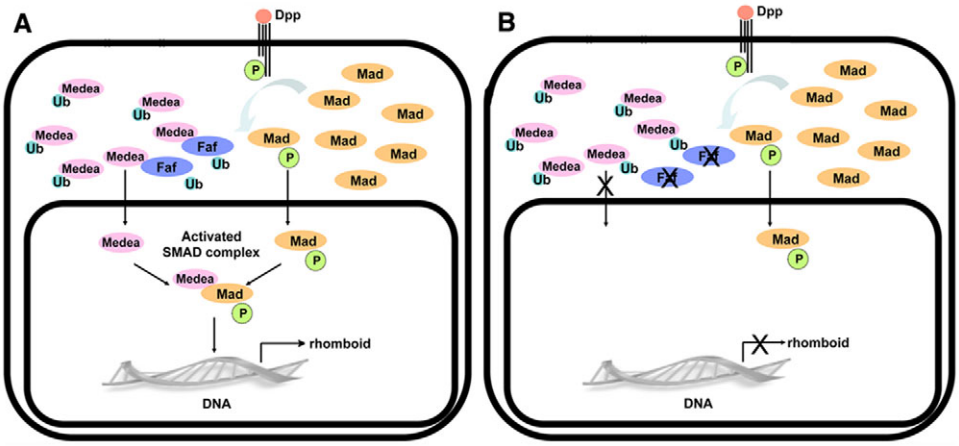

Fig. 6. Model for *fat facets* activity in Dpp DV signal transduction. Events downstream of a Dpp ligand in a dorsal cell from a blastoderm stage embryo are depicted. Arrows represent the movement of information via phosphorylation (P) or changes in protein subcellular localization. Cells contain pools of maternally contributed Mad (yellow), monoubiquitylated Med (purple with green ubiquitin attached) and Faf (blue). (**A**) Wild type. Information flows from Dpp to transmembrane receptors to Mad via phosphorylation on serine (blue arrow). pMad then forms an activated Smad complex with deubiquitylated Med in the nucleus where they drive transcription of *rho*. This results in normal DV axis formation. (**B**) *fat facets* mutant. Information flows normally from Dpp to receptors to pMad but in the absence of Faf there is no deubiquitylated Med to form activated Smad complexes. This prevents the activation of *rho* and leads to ventralization of the embryo.

Here, we provide genetic evidence that reversible ubiquitylation of Med can limit Dpp responsiveness. In *faf* mutants, defective deubiquitylation renders embryonic cells unable to respond appropriately to Dpp and results in defective DV patterning. This conclusion is supported by multiple observations. (1) *faf* mutants act as dominant maternal enhancers of *dpp* mutations leading to defective DV axis formation in a manner comparable with *Mad* and *Med* mutants. In addition, by reducing the levels of Dpp signaling in *sog* mutants, *faf* mutants can partially rescue DV defects caused by loss of *sog*, as shown for *dpp* mutants. (2) *faf* maternal mutant genotypes generate a gradient of DV defects similar to that seen in a *dpp* alleleic series. (3) *faf* mutants interact with *Med* mutants in a non-reciprocal manner, strongly suggesting that *faf* acts in the Dpp pathway by modifying *Med* activity. (4) Mutation of Med-K738 and human Smad4-K519 render Med and Smad4 more active then their wild-type counterparts and thus less susceptible to *faf* mutation – most likely as a result of resistance to the activity of a ubiquitin ligase that operates unopposed in the absence of Faf. We summarize these findings in a model depicting the role of Faf in Dpp DV signaling in Fig. 6.

An intriguing feature of our genetic analyses is that females heterozygous for faf^{BP}, a complete deletion of the *faf* locus, do not enhance dpp^{hr4}, but females heterozygous for faf^{F08} and females homozygous for faf^{EP381} do. Our interpretation is that *faf* maternal activity must be reduced below one-half dose in the presence of one-half dose of *dpp* zygotic activity to generate maternal enhancement. Only homozygosity for the faf^{EP381} insertion and heterozygosity for the faf^{F08} missense mutation in the catalytic histidine reduce *faf* activity to that extent. Thus, faf^{F08} fits the criterion (phenotypic effect is more severe than a deletion) for a dominant-negative allele for *faf* functions in Dpp DV signaling. From this perspective, the requirement for *faf* is less stringent than the requirement for *Mad* and *Med*. For *Mad* and *Med*, reduction to one-half dose is sufficient to engender dominant maternal enhancement. The less stringent requirement for *faf* is consistent with the fact that maternally contributed *Med* mutations, but not *faf* mutations, generate synthetic lethality in double heterozygous mutant individuals.

A ubiquitylation cycle required for morphogen interpretation

The importance of a conserved zygotic extracellular system that regulates embryonic DV differentiation via the generation of a robust Dpp/BMP morphogen gradient is well known (e.g. Piccolo et al., 1996). An equally relevant intracellular system employing ubiquitin is now being recognized that acts in parallel to control Dpp signal transduction (Xia et al., 2010). Our data extend this recognition by showing that maternal intracellular regulation of Med activity via ubiquitylation and deubiquitylation is a fundamental feature of Dpp DV signaling.

Our study using *faf* mutants showed that a maternally programmed intracellular balance of Med regulative ubiquitylation and deubiquitylation is required for the zygotic extracellular system to operate and can even compensate for an excess of Dpp, as shown by the partial rescue of DV defects in *sog* and *faf* double mutants. Furthermore, *faf* mutations do not affect pMad activation or dorsal localization (except as a consequence of interfering with Med activity in a Dpp feedback loop). This suggests that the level of available Med (non-ubiquitylated) is a key quantitative variable, in parallel with pMad that cells employ to interpret the Dpp gradient.

These data closely mirror results obtained in mouse embryo knockouts for the Smad4 ubiquitin ligase Ecto. In this case, the absence of a Smad4-inhibitory mechanism, and thus unrestrained Smad4 activity, caused cells to respond to levels of extracellular Nodal/intracellular phospho-Smad2 that would normally be too small to activate gene expression. Thus, a global picture emerges whereby cells keep Smad4 constantly in check, and that this is essential for their ability to sense quantitative differences in R-Smad activity.

However, one wonders why do cells need such a cycle, instead of simply fine-tuning Smad4 expression levels? One interesting possibility is that Smad4 monoubiquitylation, which is promoted by TGFβ signals in mammalian cells, might serve to continuously clear active Smad complexes from promoters. This hypothesis is supported by recent findings indicating that Ecto is recruited to TGFβ target promoters in a Smad4-dependent fashion and that the enzymatic activity of Ecto on Smad4 is promoted when Ecto is bound to chromatin (Agricola et al., 2011). In this respect, Smad4

ubiquitylation favors a dynamic state for R-Smads, keeping them exposed to fluctuations in extracellular concentrations of TGFβ ligands (Schmierer and Hill, 2007).

The Smad4 ubiquitin cycling model implies the existence of a ubiquitin ligase for Med. One candidate is Bonus, the *Drosophila* protein most closely related to the three vertebrate Tif1 proteins (Beckstead et al., 2001). A second is Highwire, a ligase for Med at the neuromuscular junction (McCabe et al., 2004). dSmurf, a ligase shown to affect Mad but not Med in Dpp DV signaling, is not a candidate (Liang et al., 2003). We tested *bonus* and *highwire* mutants for DV phenotypes and found they are inconsistent with those predicted for a Med ubiquitin ligase. Additional candidates are currently under investigation.

In summary, our study reveals that Med deubiquitylation by Faf is a conserved mechanism required for proper interpretation of the Dpp morphogen gradient and embryonic DV axis formation.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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