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UV induced foot duplication in regenerating hydra is mediated by metalloproteinases and modulation of the Wnt pathway

LAKSHMI-SUREKHA KRISHNAPATI[1], ROHINI LONDHE[1], VAISHALI DEOLI[1], APURVA BARVE[#,1], SAROJ GHASKADBI[2] and SURENDRA GHASKADBI[*,1]

[1]*Developmental Biology Group, MACS-Agharkar Research Institute and*
[2]*Department of Zoology, Savitribai Phule Pune University, Pune, India*

ABSTRACT We have shown earlier that irradiation with UV induces duplication of foot in regenerating middle pieces of hydra. The present study was undertaken to elucidate the underlying mechanism(s) leading to this curious phenomenon. UV irradiation induced duplicated foot in about 30% of regenerating middle pieces. Metalloproteinases are important in foot formation, while Wnt pathway genes are important in head formation in hydra. The effect of UV irradiation on expression of these genes was studied by *in situ* hybridization and q-PCR. In whole polyps and middle pieces, UV irradiation led to up-regulation of *HMP2* and *HMMP*, the two metalloproteinases involved in foot formation in hydra. *HMP2* expression was significantly increased starting from 30 min post exposure to UV at 254 nm (500 J/m^2), while *HMMP* showed significant up-regulation 6 h post UV exposure onwards. In middle pieces, increased expression of both metalloproteinases was observed only at 48 h. In whole polyps as well as in middle pieces, expression of *Wnt3* and β-*catenin* was detected within 30 min of UV exposure and was accompanied by up-regulation of *GSK3β, DKK3* and *DKK1/2/4*, inhibitors of the Wnt pathway. These conditions likely lead to inactivation of Wnt signaling. We therefore conclude that duplication of foot due to UV irradiation in regenerating middle pieces of hydra is a combined effect of up-regulation of metalloproteinases and inactivation of the Wnt pathway. Our results suggest that UV irradiation can be employed as a tool to understand patterning mechanisms during foot formation in hydra.

KEY WORDS: *Hydra, metalloproteinase, pattern formation, UV-induced foot duplication, Wnt signaling*

Introduction

Hydra, a representative of phylum Cnidaria, has been a popular model to study various developmental processes owing to its structural simplicity and remarkable capacity for regeneration (Ghaskadbi *et al.*, 2005). Hydra shows a simple body plan with oral/aboral axis, the formation of which depends on the concentration of several morphogenetic gradients along the axis. Various exogenous agents such as UV, lithium chloride, alsterpaullone, etc. have been used as tools to understand the molecular mechanisms responsible for pattern formation in hydra. Previous studies from our laboratory have shown that UV irradiation induces duplication of foot in about one third of regenerating middle pieces of trisected hydra and increases budding in intact hydra (Ghaskadbi *et al.*, 2005). Based on this work, we hypothesized that UV-induced formation of duplicated foot in regenerating hydra could involve up-regulation of matrix metalloproteinases (MMPs) and/or inactivation of Wnt pathway genes.

Metalloproteinases are responsible for degradation of macromolecules such as collagen, gelatine and other glycoproteins (reviewed in Birkedal-Hansen *et al.*, 1993 and Page-McCaw *et al.*, 2007). Degradation of these components initiates ECM-cell interactions which are essential for various processes during

Abbreviations used in this paper: UV, ultra violet.

***Address correspondence to:** Surendra Ghaskadbi. Coordinator, Developmental Biology Group, MACS-Agharkar Research Institute, G.G. Agarkar Road, Pune-411 004, India. Tel: +91-20-2532-5063. Fax: +91-20-2565-1542. E-mail: ghaskadbi@gmail.com

[#]Present address: Indian Institute of Science Education and Research, Pune -411 008, India

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embryonic development and morphogenesis (Sarras *et al.*, 1993, Zhang and Sarras, 1994). Earlier studies have shown that ECM-cell interactions are crucial for certain developmental processes in hydra (Sarras *et al.*,1994; Zhang and Sarras, 1994). Three metalloproteinases, *HMP2,* an Astacin metalloproteinase (Yan *et al.*, 2000), *HMMP*, a matrix metalloproteinase (Leontovich *et al.*, 2000) and *MMP-A3* another matrix metalloproteinase (Münder *et al.*, 2010; Aufschnaiter *et al.*, 2011) have been characterized from hydra and are shown to be involved in foot morphogenesis, regeneration and in separation of buds, respectively. Further, UV irradiation has been shown to induce *MMP*s in human skin and canine cornea (Brenneisen *et al.*, 2002, Brennan *et al.*, 2003, Chandler *et al.*, 2008, Amano, 2009).

Numerous developmental processes including cell proliferation, migration and differentiation, fate specification, dorso-ventral patterning and maintenance of cell polarity are mediated by Wnt family of glycoproteins (Komiya and Habas, 2008). The canonical Wnt pathway involves inactivation of *GSK3*β followed by β-*catenin-Tcf* coupled activation of transcription of genes that play critical role in developmental processes (Broun *et al.*, 2005, Eastman *et al.*, 1999, Molenaar *et al.*, 1999). In hydra, Wnt3 is involved in setting up the Organizer and the genes involved in Wnt pathway are strongly expressed in the hypostome region (Hobmayer *et al.,* 2000; Bode 2003, Broun *et al.*, 2005). In the present study, we show that exposure to UV up-regulates the metalloproteinases, *HMP2* and *HMMP* and inactivates overall Wnt pathway by activating Wnt inhibitors in regenerating middle pieces of hydra, demonstrating that both these mechanisms contribute to formation of a duplicated foot.

Results

Duplication of foot in UV irradiated regenerating middle pieces

Our previous work has shown that exposure to UVC induces formation of duplicated foot in 33% of regenerating middle pieces of hydra (Ghaskadbi *et al.*, 2005). In the present study, we have treated whole polyps and middle pieces of *Hydra vulgaris* Ind-Pune with UVC and allowed these to recover in hydra medium for 4-5 days. Irradiated whole polyps recovered in medium for 24 h showed reduced body length with shortened tentacles or no tentacles with swollen head/hypostome in 90% animals. Foot specific staining was performed on regenerated middle pieces (Fig. 1A) which showed duplication of foot in about 30% of irradiated regenerating middle pieces (Fig. 1 B,C). UV irradiated middle pieces recovered in medium did not show significant difference in head morphology (Fig. 1C g-h). These data confirm our earlier observation (Ghaskadbi *et al.*, 2005). It may be mentioned here that the species used in the earlier study, *Pelmatohydra oligactis* is identical to *Hydra vulgaris* used in the present study. The reason behind the difference in name is that the species was earlier misnamed as *Pelmatohydra oligactis* and has since been taxonomically restudied and identified recently by us as *Hydra vulgaris* Ind Pune (Reddy *et al.*, 2011).

Induction of metalloproteinases by UV irradiation

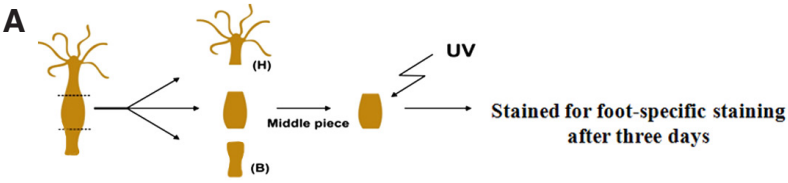
Whole mount *in situ* hybridization and q-PCRs were performed to compare the localization and expression levels of metalloproteinases in control and UV treated polyps. *In situ* hybridization showed up-regulation of metalloproteinases in UV treated polyps. Expression pattern of *HMP2* in control hydra was comparable to the reported pattern in *Hydra vulgaris* (Yan *et al.*, 2000), which showed localization in the basal region and extended along the lower body column (Fig. 2 Aa). UV exposure not only increased the expression of *HMP2* but also enhanced its domain of expression except in the hypostome region (Fig. 2 Ab).

In order to confirm the up-regulation of metalloproteinases by UV irradiation, q-PCRs were carried out for control- and UV-treated polyps and middle pieces. UV irradiation of whole polyps and middle pieces resulted in increased expression of both *HMP2* and *HMMP* (Fig. 4). Up-regulation of *HMP2* was observed as early as 30 min post-treatment and continued to increase up to 24 h as compared to control. Significant up-regulation of *HMMP* was seen after 6 and 24 h (Fig. 4A). Unlike in whole hydra, UV irradiation resulted in significant up-regulation of *HMP2* and *HMMP* at 48 h in middle pieces (Fig. 4B).

Effect of UV irradiation on Wnt signaling

Expression of *Wnt3*, β-*catenin, DKK3, DKK1/2/4* and *GSK3*β was studied by *in situ* hybridization and q-PCR. Whole polyps and middle pieces exposed to UVC at 500 J/m^2 and recovered in hydra

A



B

No. of UV treated middle pieces	No. of Hydra with ectopic foot	Percentage
187	57	30.48%

C

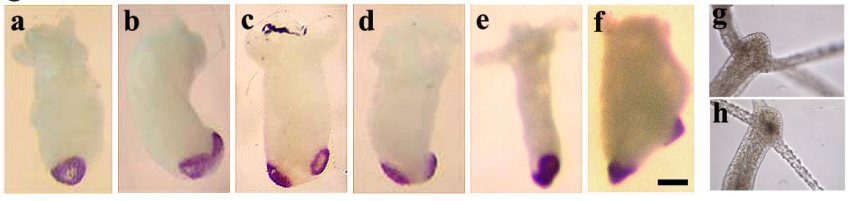


Fig. 1. UV irradiation-induced duplication of foot in regenerating middle pieces of hydra. *Middle pieces of hydra were irradiated with 500 J/m^2 UV at 254 nm and regeneration was monitored over a period of 4-5 days.* **(A)** *Schematic representation of trisection and irradiation of middle piece followed by foot-specific staining in the regenerates.* **(B)** *Quantification of UV irradiation-induced duplicated foot formation in the middle pieces.* **(C)** *UV irradiation-induced duplication of foot (b-f) as opposed to a single foot in unirradiated, control middle piece (a) was detected by foot specific staining. UV irradiated middle pieces recovered in medium (h) did not show significant difference in head morphology when compared to control (g). Scale bar, 200 μm.*

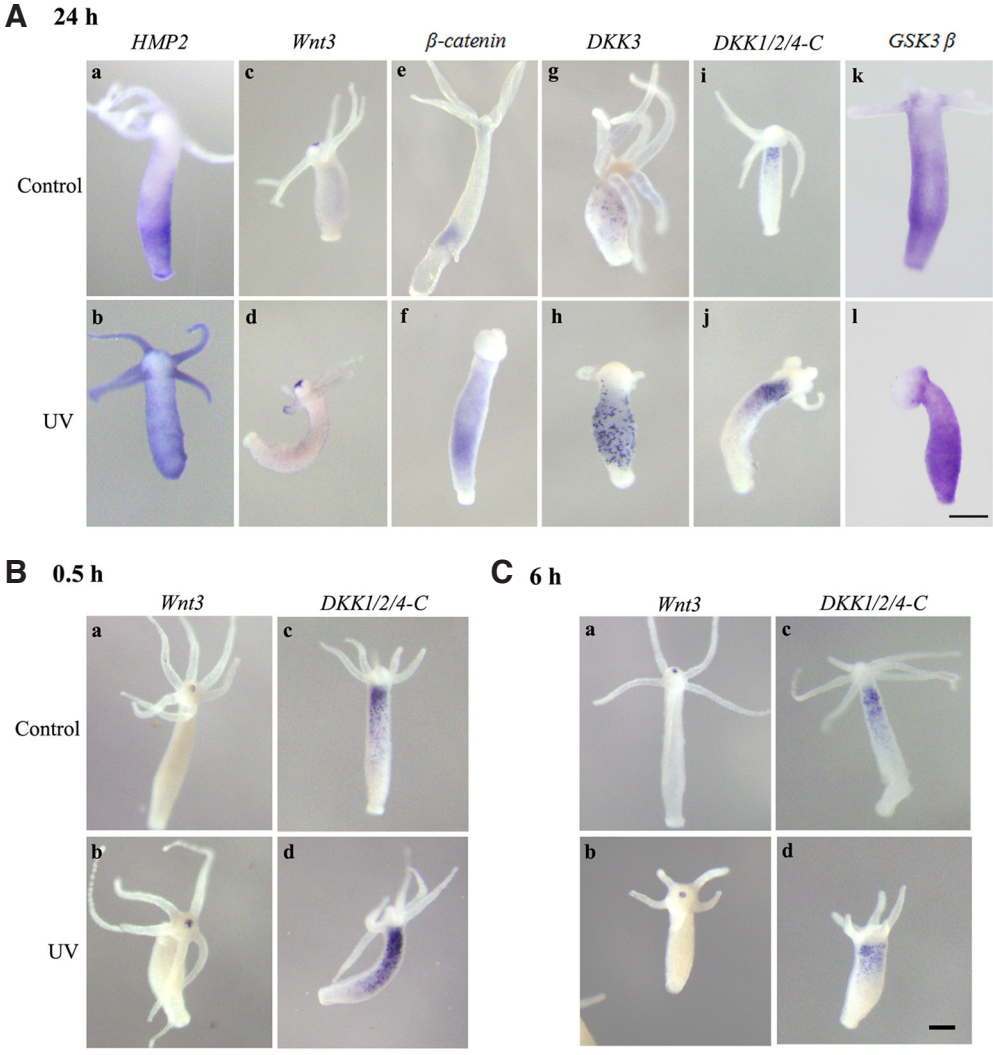


Fig. 2. Localization of *HMP2, Wnt3, β-catenin, DKK3, DKK1/2/4-C* and *GSK3 β* by whole mount *in situ* hybridization DIG labeled antisense probes were used for the detection of transcripts. (A) *Enhanced expression of* HMP2 **(b)**, Wnt3 **(d)**, β-catenin **(f)**, DKK3 **(h),** DKK1/2/4-C **(j)** *and* GSK3 β **(l)** *was observed in UV treated whole hydra post 24 h recovery in hydra medium as compared to controls* **(a**,**c**,**e**,**g**,**i**, **k)**, *respectively. Expression of* Wnt3 *and* DKK1/2/4-C *in irradiated polyps after 0.5* **(B)** *and 6 h* **(C)** *recovery. Strong expression of* Wnt3 *and* DKK1/2/4-C *was seen in irradiated polyps after 0.5 h (B, b, d) and 6 h (C, b, d) recovery. Scale bar, 200 μm.*

medium for 0.5, 6, 24 and 48 h were used for *in situ* hybridization. *In situ* hybridization in UV irradiated whole polyps recovered after 24 h showed up-regulation of *Wnt3, β-catenin, DKK3, DKK1/2/4* and *GSK3*β (Fig. 2A). Enhanced expression of *Wnt3* and *β-catenin* post UV irradiation suggests possible activation of Wnt pathway. Though the expression of *Wnt3* was restricted to the hypostome, its expression was more intense in UV treated polyps (Fig 2A, d) as compared to controls (Fig 2A, c). Similarly, *β-catenin* expression was enhanced in the body column with strongest signal in the budding region in UV treated polyps as compared to controls (Fig 2A e,f). Simultaneously, expression of inhibitors of Wnt pathway, *DKK3, DKK1/2/4-C* and *GSK3*β was also enhanced as a result of UV irradiation. In control hydra, expression of *DKK3* was seen in the differentiating nematocytes of gastric region (Fig. 2A, g) and *DKK1/2/4-C* in gland cells in a graded expression pattern with high expression from sub-tentacle region (Fig. 2A, i) as previously reported (Fedders *et al.*, 2004; Augustin *et al.*, 2006). Upon irradiating with UV, expression of *DKK3* (Fig. 2A, h) and *DKK1/2/4-C* (Fig. 2A, j) was increased as compared to the control polyps (Fig. 2A g,i). Similarly, in control hydra, expression of *GSK3β* was seen all over the body and at the tentacle boundaries (Fig. 2A k) whereas in UV treated hydra (Fig. 2A, l) *GSK3*β expression was more intense than control hydra (Fig.2A, k).

In situ hybridization in whole polyps recovered after 0.5 and 6 h also showed up-regulation of *Wnt3* and *DKK1/2/4-C* (Fig. 2B, C). In middle pieces, expression of *Wnt3* was seen in regenerating heads in controls (Fig. 3A, a; 3B, a) while its expression was enhanced in UV treated middle pieces after 0.5 (Fig. 3A, b) and 6 h (Fig. 3B, b). However, expression of *Wnt3* after 24 and 48 h recovery did not show specific pattern in UV treated middle pieces (Fig. 3C, b and 3D, b), while *DKK1/2/4-C* expression was increased in regenerating heads from early recovery and continued till 48 h (Fig. 3C, d; 3D, d).

Analysis by q-PCR also showed up-regulation of *Wnt3* immediately after UV irradiation till 24 h, with a maximum expression at 6 h (Fig. 5A). Up-regulation of *β-catenin, DKK3* and *GSK3*β was also observed in UV irradiated hydra, confirming the *in situ* hybridization results (Fig. 5A). Comparison of expression patterns of *Wnt3*, *DKK3* and *GSK3* β clearly shows that *DKK3* and *GSK3* β are up-regulated when *Wnt3* becomes active and down-regulated when Wnt signaling decreases (Fig. 5B), which points towards overall inactivation of Wnt pathway. Middle pieces also showed

up-regulation of *DKK3* and *GSK3* β along with *Wnt* and β-*catenin* (Fig. 6) confirming the overall down-regulation of Wnt pathway.

Discussion

The genesis of this study is in the curious observation that UV irradiation induces duplication of foot in regenerating middle pieces of hydra (Ghaskadbi *et al.*, 2005). UV irradiation at the dose of 500 J/m^2 was found to reprogram patterning processes in about a third of the middle pieces. When a hydra is cut into pieces, each piece, except for the extreme oral/aboral tissues, regenerates into small adult polyp maintaining the head-base polarity (Bode, 2003). This maintenance of axial polarity is due to the presence of different morphogen gradients (Bode, 2009). We hypothesized that UV irradiation could affect these morphogen gradients that specify the oral/aboral axis, leading to duplication of foot in regenerates. One of the signaling pathways critical for head specification and formation in hydra is the Wnt pathway (Bode, 2009). Therefore we examined the effect of UV irradiation on Wnt pathway genes in intact and regenerating pieces of hydra. The other possibility we considered and experimentally tested was the possible effects of UV radiation on the expression of metalloproteinase genes. As mentioned earlier, two metalloproteinases in hydra, HMP2, an Astacin metalloproteinase and HMMP, a matrix metalloproteinase participate in foot formation (Leontovich *et al.*, 2000, Yan *et al.*, 2000). It is known from previous studies that UV radiation leads to up-regulation of MMPs in human skin (Brenneisen *et al.*, 2002, Brennan *et al.*, 2003; Amano, 2009). Also, it has been demonstrated that Snail family transcription factors induced by UV via mitogen-activated protein kinase (MAPK) pathway can modulate *MMP* induction (Chandler *et al.*, 2008), while in keratinocytes UV irradiation produces a variety of cytokines that can induce metalloproteinases in human skin (Dong *et al.*, 2008).

Reports from earlier studies have shown that DNA damaging agents such as aluminium trigger morphological and behavioural changes, including DNA damage, in green and brown hydra (Kovačević *et al.*, 2007). Previous results from our laboratory have shown that *XPF,* a UV responsive gene, is expressed in higher amounts in the ectoderm than in endoderm. Further, it is expressed in significantly high levels in the interstitial stem cells that are truly multipotent (Barve *et al.*, 2013). Efforts are currently on to find out if XPF indeed participates in the nucleotide excision repair in hydra. In the present work, we have confirmed, using a much larger sample size, that irradiation of middle pieces with UV leads to formation of duplicated foot in about one third of the cases. Our quantitative PCR data show that UV irradiation leads to up-regulation of *HMMP* and *HMP2*, which may have been primarily responsible for the formation of duplicated foot. Wnt is responsible for setting up the head organizer in hydra (Hobmeyer *et al.*, 2000), and inhibits the formation of a foot in the near vicinity. In the present study, we believe that up-regulation of Wnt inhibitors, *DKK3, DKK1/2/4-C* and *GSK3*β simultaneously with *Wnt* may have contributed to the formation of duplicated foot. Up-regulation of *DKK1,* a homolog of *DKK3,* by UV irradiation has been reported (Grotewold and Rüther, 2002; Shou *et al.*, 2002). Further, reversal of Wnt induced morphological alterations in fibroblasts due to simultaneous expression of *DKK1* and *Wnt-2* has been demonstrated (Fedi *et al.*, 1999). Our results point towards a similar mechanism in which simultaneous up-regulation of *Wnt* and its inhibitors, *DKK3* and *DKK1/2/4-C* may have resulted in overall inactivation of Wnt pathway. Thus, formation of duplicated foot by UV irradiation is probably due to induction of *HMP2* and *HMMP* in the body column along with overall inhibition of Wnt pathway. Another possibility for duplicated foot formation could be change in head- and foot-activating gradients by activated Wnt in such a way that it inhibits foot inhibition resulting in the formation of duplicated foot. At present, we do not have any data suggesting a connection between DNA damaging ability of UV radiation and its effects on patterning during regeneration in hydra.

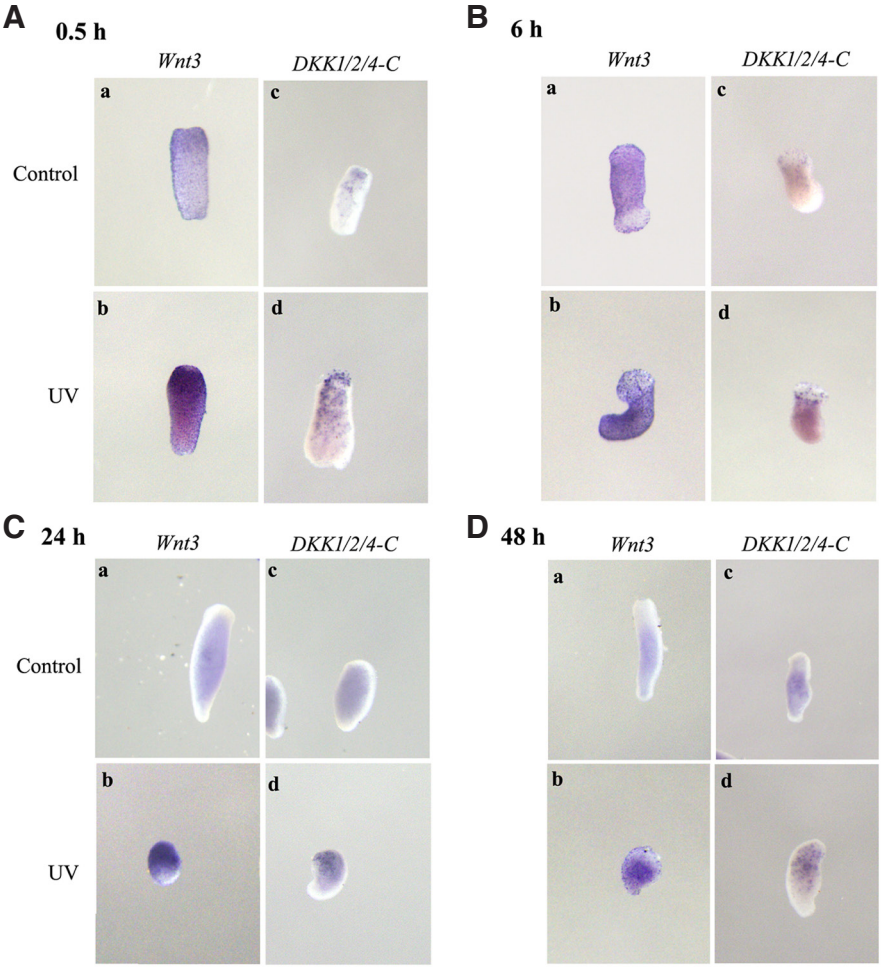
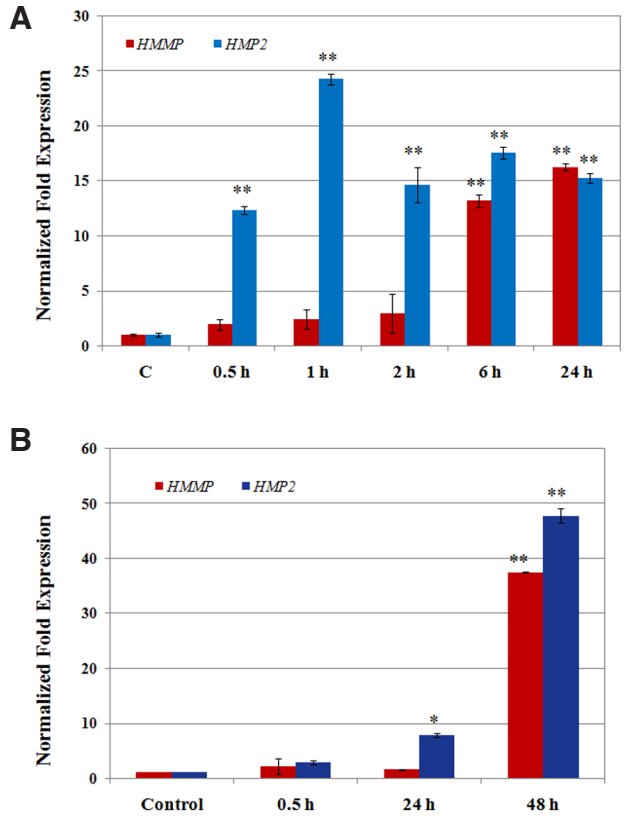


Fig. 3. Localization of *Wnt3 and DKK1/2/4-C* in middle pieces by whole mount *in situ* hybridization. Wnt3 **(Ab, Bb)** *and* DKK1/2/4-C **(Ad, Bd)** *expression is up-regulated after early recovery time points, 0.5 and 6 h post UV treatment.* DKK1/2/4-C *expression is continued in regenerating pieces after 24* **(Cd)** *and 48 h recovery* **(Dd)** *while no specific pattern was observed for* Wnt3 *expression.*



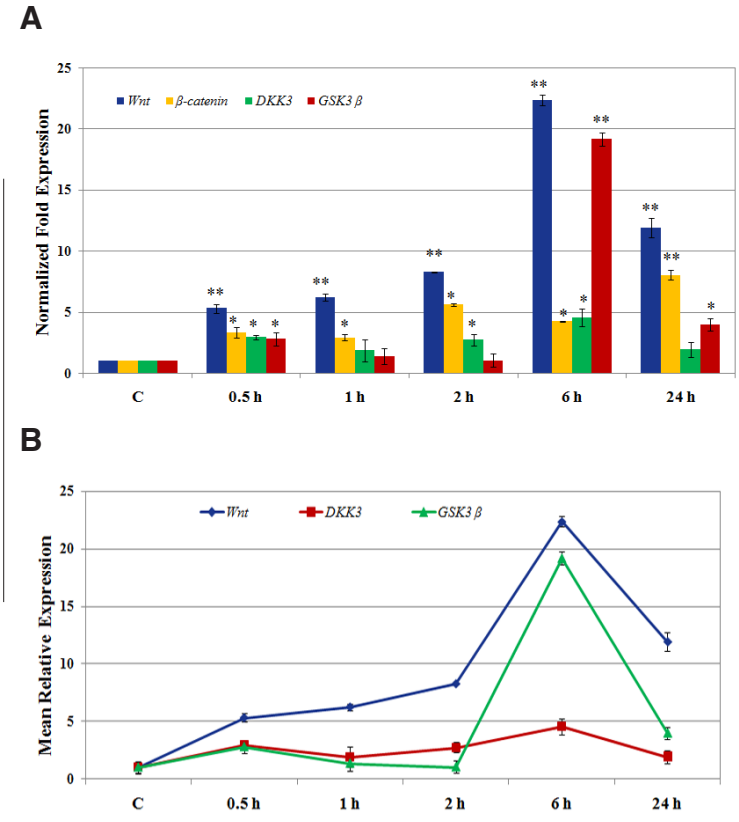


Fig. 4 (Left). Up-regulation of metalloproteinases with UV irradiation in whole polyps and middle pieces as assessed by q-PCR. (A) *Significant up-regulation of* HMMP *and* HMP2 *was observed in whole polyps and middle pieces with UV treatment. In whole polyps, treatment with UV at 500 J/m² resulted in up-regulation of* HMP2 *from 0.5 h and continued till 24 h, whereas* HMMP *showed significant up-regulation from 6 h post UV treatment.* **(B)** *Middle pieces when irradiated with UV and allowed to recover in hydra medium for 0.5, 24 and 48 h showed significant up-regulation of* HMP2 *and* HMMP *at 48 h. Data are represented as mean of three biological replicates with SD. X axis shows different time points and Y axis shows fold change obtained after normalizing C_T values against* Hyactin. **Statistically significant ($p < 0.01$); **statistically significant ($p < 0.001$).*

Fig. 5 (Right). Effect of UV irradiation on Wnt signaling in whole polyps as assessed by q-PCR. (A) *Significant up-regulation of* Wnt *and* β-catenin *along with Wnt pathway inhibitors,* DKK3 *and* GSK3 β *was observed.* **(B)** *Data from* **(A)** *was used to show the trend for* Wnt3, DKK3 *and* GSK3 β. *Data are represented as mean of three biological replicates with SD. X axis shows different time points and Y axis shows fold change obtained after normalizing C_T values against* Hyactin. *** statistically significant ($p < 0.001$); * statistically significant ($p < 0.01$).*

Induction of duplicated foot by UV is observed only in a third of the irradiated middle pieces. It is worth considering the possible reasons behind this, especially in view of increased expression of metalloproteinases in whole hydra (present study), where no ectopic foot is induced. Two possibilities may be considered. One concerns localization of the organizing centres in hydra. The two organizing centres are localized at the two extremities of the hydra polyp and one would expect that their molecular influences would be significantly reduced in the middle pieces (that lack them), making reprogramming of patterning possible. The other possibility is that UV irradiation affects fate of some of the multipotent stem cells, the interstitial cells of hydra, which are mostly localized in the body column and are absent from the extremities.

The mechanisms of foot formation in hydra are poorly understood. While a large number of studies on head formation have led to a better understanding of the process, not much is known about specification and formation of foot. A better understanding of the same would lead to crucial information on evolution of axial polarity in metazoans. The present study provides an experimental framework to probe the mechanisms of foot formation in hydra further.

Materials and Methods

Maintenance of culture

Clonal culture of *Hydra vulgaris* Ind-Pune (Reddy *et al.*, 2011) was maintained in hydra medium (Sugiyama and Fujisava, 1977) at 18°C with 12 hour day and night cycle. Hydra polyps were fed on alternate days with *Artemia salina* nauplii and the medium was changed four to five hours after feeding.

Exposure of polyps and middle pieces to UV radiation and foot specific staining

Hydra were trisected and the middle pieces were irradiated with 500 J/m² UV at 254 nm. These were allowed to regenerate for 4-5 days. Head regeneration was assessed by presence of hypostome and tentacle buds. Visual criteria are not sufficient to detect foot regeneration. Therefore, foot specific staining was performed in control and UV irradiated middle pieces

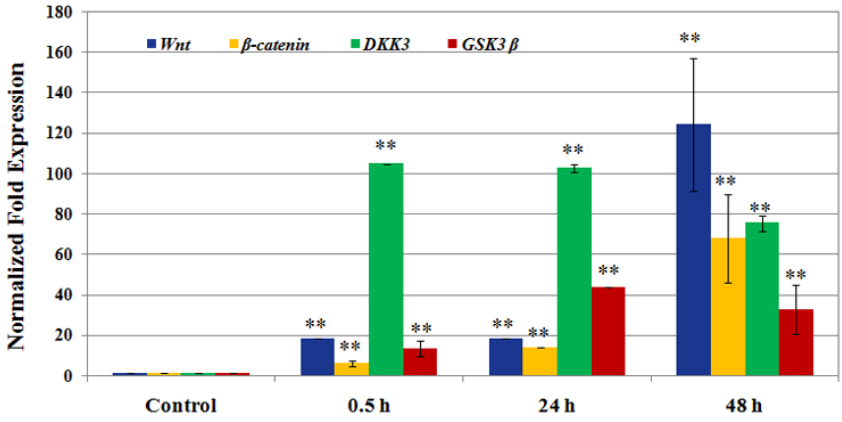


Fig. 6. Modulation of Wnt signaling by UV irradiation in middle pieces of hydra. *Up-regulation of* DKK3 *and* GSK3 β *along with* Wnt3 *and* β-catenin *in UV irradiated middle pieces suggests overall inactivation of Wnt pathway. Data are represented as mean of three biological replicates with SD. Y axis shows fold change obtained by normalizing* C_T *values against* Hyactin *and X axis shows different time intervals. **Statistically significant (p < 0.001).*

as described earlier (Hoffmeister and Schaller, 1985) with few modifications. Briefly, regenerating middle pieces were incubated in 3 ml of freshly prepared staining solution containing citric acid (65.5 mM), trisodium citrate (34.5 mM), 2,2-azino-di (3-ethyl-benzthiazoline-sulfonic acid-6) ammonium salt (ABTS, Sigma, U.S.A) (0.1%) and hydrogen peroxide (0.003%) for 5-10 min at room temperature. The reaction was terminated by transferring the tissue to 1X PBS followed by imaging using SZX16 Stereo microscope (Olympus U.S.A).

For studies on gene expression, 48 h starved whole hydra polyps or middle pieces of trisected hydra were irradiated with UVC at 254 nm at 500 J/m^2. After UV irradiation, whole polyps were allowed to grow in hydra medium for various durations, such as, 0.5, 1, 2, 6 and 24 h and the middle pieces were harvested after 0.5, 6, 24 and 48 h post UV irradiation. 45 to 50 hydra polyps or about 60 middle pieces of trisected hydra were used for a given time point for RNA extraction, while 10-15 hydra polyps or middle pieces of trisected hydra were used for *in situ* hybridization for a given time point.

RNA extraction and cDNA synthesis

Control and UV treated hydra polyps were homogenised in 1 ml of Tri Reagent for total RNA extraction (Chomczynski, 1993). After homogenization, chloroform (200 µl) was added to the homogenate, mixed well and centrifuged at 13000 rpm for 15 min at 4°C. RNA was precipitated from the aqueous phase using 500 µl of isopropanol at -20°C for 30 min. RNA pellet recovered after centrifugation was washed with 70% ethanol, air-dried, and dissolved in nuclease-free water. RNA was quantified using Nanodrop spectrophotometer and the integrity was checked on 1% formaldehyde agarose gel. Genomic DNA contamination was eliminated using RQ1 RNase free DNase as per manufacturer's instructions (Promega) followed by cDNA synthesis (Verso cDNA synthesis kit) and q-PCR.

TABLE 1

PRIMER SEQUENCES USED FOR QUANTITATIVE REAL-TIME PCR

Gene name	Primer sequence
ActinFW	CAATTGAACACGGAATTGTA
ActinREV	AGTAAGAAGGACAGGGTGTTC
HMMPFW	GGTTGCAGTTCACGAGATAG
HMMPREV	CATCAGACAGCTGAATATTTCTTAC
HMP2FW	ACTGATTTGTGTGGGTTTACT
HMP2REV	CAGTTCCTCTAGAAGGTGTTTC
Wnt3FW	AACAGCCAGCAGAGAAAG
Wnt3REV	CAACGACAGTGGACAGATT
BetacateninFW	TCAGGCTTCTATTATGGTCAAC
BetacateninREV	GCAGTCACACCAACCAAT
DKK3FW	GTGTAAGAGAACCAGCCATAAA
DKK3REV	ACTTGAGCTCCAACGTAAAC
GSK3FW	TTGTTACTAGGCCAACCTATTT
GSK3REV	TCATTTCACGAATCTGTTCTCT

Quantitative real-time PCR (q-PCR)

Analysis of expression of Wnt pathway genes and metalloproteinases was carried out by q-PCR. Primers were designed using IDT RealTime PCR Design Tool (Table 1). Initially, semi-quantitative PCRs were performed using q-PCR primers to obtain a single amplicon. Absence of genomic DNA contamination in the RNA was confirmed by performing PCR using DNase treated RNA as a template. This was followed by q-PCRs for adult as well as middle pieces of hydra. Comparable un-irradiated samples served as controls. PCR was performed in a StepOnePlus™ System (Applied Biosystems, U.S.A.) using Power SYBR Green Master Mix (Invitrogen, U.S.A.) and primers. The PCR conditions were as follows. Initial denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 10 s, denaturation at 95°C for 15 s, extension at 60°C for 1 min and final denaturation at 95°C for 15 sec. Crossover points (C_T) were recorded, and quantification was carried out using the standard $2^{-\Delta\Delta CT}$ method. All C_T values were corrected by normalizing against the housekeeping gene *Hyactin*. Data are presented as mean fold change in gene expression relative to untreated control samples. Three biological replicates were carried out and the data are presented as mean ± SD (standard deviation), and statistical significance was calculated by Student's t test.

Localization of transcripts by in situ *hybridization*

Whole mount *in situ* hybridization using digoxygenin (DIG) labeled riboprobes was carried out as described by Martinez *et al.,* (1997) with a few modifications. Sense and antisense riboprobes were prepared using T7 or SP6 RNA polymerases (Roche). Briefly, hydra polyps and middle pieces were relaxed in 2% urethane, followed by overnight fixation in 4% paraformaldehyde at 4°C. Polyps were permeabilized with proteinase K and refixed overnight in 4% paraformaldehyde at 4°C. Prehybridization was carried out in prehybridization buffer (50% formamide, 5X SSC, 1X Denhardt's solution, 200 mg/ml tRNA, 0.1% Tween 20, 0.1% CHAPS, 100 µg/ml heparin) to block non-specific hybridization sites. Hybridization with DIG labeled probes was carried out for ~60 h followed by washes with hybridization solution and SSC. Polyps were washed in maleic acid buffer with Tween 20 (100 mM maleic acid, 150 mM NaCl and 0.1% Tween 20). This was followed by incubation in anti DIG-alkaline phosphatase conjugate antibody (1:3000) overnight at 4°C. Color reaction was detected by alkaline phosphatase staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt (BCIP) system.

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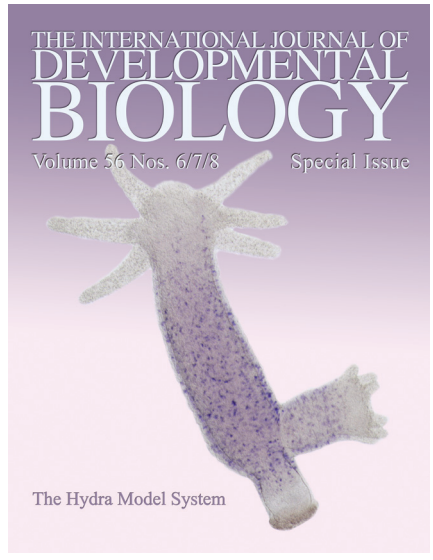

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