Role of the scaffolding protein JLP in UVB-induced apoptosis

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Ultraviolet B (UVB) component of sunlight causes many adverse biological effects, including apoptosis, and eventually can lead to skin cancer. Growing evidence indicates that the UVB-induced signaling network is complex and involves diverse cellular processes. In this study the role of c-Jun NH_2-terminal kinase-associated leucine zipper protein (JLP), a scaffold protein for mitogen-activated protein kinase (MAPK) signaling cascades, was investigated in UVB-induced apoptosis. I found that UVB-induced skin epidermal apoptosis was reduced in *Jlp* knockout (KO) as well as in keratinocyte-specific *Jlp* KO mice compared to those of the controls. While exploring molecular mechanisms of the diminished apoptosis in *Jlp*-deficient mice, it was revealed that UVB-induced DNA repair system shows no evidence for the involvement of JLP in this process; however, UVB-stimulated p38 MAPK activation was impaired in both *Jlp* KO and keratinocyte-specific *Jlp* KO mice. Moreover, topical treatment of UVB-irradiated mouse skin with a p38 inhibitor significantly suppressed the epidermal apoptosis in wild-type mice, but not in *Jlp* KO mice. These findings suggest that JLP in skin basal keratinocytes plays an important role in UVB-induced apoptosis by modulating p38 MAPK signaling pathways. This is the first study to demonstrate a critical role for JLP in an *in vivo* response to environmental stimulation.

We are constantly exposed to environmental hazards, and our first protective barrier is the skin. UVB (280-320 nm) is mostly absorbed in the epidermis of the skin, and induces DNA photolesions, which if inefficiently repaired result in deleterious mutations. UVB irradiation also induces alterations in gene expression that are mediated by signaling molecules, including mitogen-activated protein kinases (MAPKs). The mammalian MAPK signaling system employs scaffold proteins, in part, to organize the MAPK signaling components into functional modules, thereby enabling the efficient activation of specific MAPK cascades. Growing evidence indicates that the UVB-induced signaling network is complex and involves diverse cellular processes, such as apoptosis and survival. To date, the mechanisms involved in regulating UVB-induced apoptosis pathways remain unclear. c-Jun NH_2-terminal kinase (JNK)-associated leucine zipper protein (JLP) functions as a scaffold protein for the JNK and p38 MAPK signalling modules. *In vivo* functions of scaffolding protein JLP remain largely unknown. To better understand the complex UVB response, in this study we investigated the function of JLP in UVB-induced apoptosis in the skin by analyzing *Jlp*-deficient mice. Our results suggest that JLP plays an important role in this apoptosis by modulating p38 MAPK signaling cascades.

Jlp$^{-/-}$ mice exhibited a lightened coat color and pale skin (**Fig. 1A**), as reported for homozygous dazzle mice by Krebs and Beutler, 2010. The dazzle mouse was generated by N-ethyl-N-nitrosourea mutagenesis on the C57BL/6 background and contains a missense mutation in the *Jlp* gene (Krebs and Beutler, 2010). These results indicate that the pigmentation defects can be attributed to the loss of normal JLP function. However, there were no obvious histological differences observed in the skin among control (*Jlp*$^{+/-}$), and *Jlp*$^{-/-}$ mice by H&E staining (**Fig. 1B,** bar, 100 µm) (E, epidermis; D, dermis; HF, hair follicle).

Next the effect of *Jlp* deficiency on UVB-induced apoptosis in mouse skin was examined. The backs of control and *Jlp*$^{-/-}$ adult mice were shaved and irradiated with 2.8 kJ/m^2 of UVB. After 24 hours, skin samples were obtained and fixed, and 20-µm-thick frozen sections were stained with an anti-active caspase-3 antibody and DAPI. As a result of immunohistochemical analysis the number of active caspase-3-positive cells, which were detected mostly in the epidermis (**Fig. 2A,** bar, 100 µm) was significantly decreased in *Jlp*$^{-/-}$ compared with control mice (**Fig. 2B,** ***P < 0.001).

These results suggested that JLP is a positive regulator of UVB-induced apoptotic pathways.

To gain insight into the mechanisms underlying the attenuated UVB-induced apoptosis in *Jlp*-deficient mice, the DNA repair capability of keratinocytes lacking JLP was investigated. Primary keratinocytes derived from control ($Jlp^{+/-}$) and $Jlp^{-/-}$ P0 mice were irradiated with 160 J/m^2 dose of UVB. The cells were incubated for 0 hr, 1 hr and 2 hrs and processed for the detection of 6-4PP. As a result, the 6-4PP lesions were removed with similar kinetics in the control and $Jlp^{-/-}$ keratinocytes (**Fig. 3**), suggesting that JLP is not involved in the DNA excision repair of UVB-induced damage, and furthermore that the inhibition of apoptosis observed in $Jlp^{-/-}$ mice is not due to a decreased accumulation of DNA damage.

Next question was whether JLP ablation perturbs the normal MAPK activation response to UVB irradiation. The backs of control and $Jlp^{-/-}$ adult mice were shaved and treated with (UVB (+)) or without (UVB (-)) 2.8 kJ/m^2 dose of UVB. Thirty minutes after irradiation, cell lysates were prepared from the skin samples, and analyzed by Western blotting (50 µg lysate/lane) for activated JNK (p-JNK), p38 (p-p38), and ERK (p-ERK). While the p-ERK levels were similar between the control and $Jlp^{-/-}$ mice, modest and substantial decreases in the levels of p-JNK and p-p38, respectively, were observed in the skin samples of $Jlp^{-/-}$ mice (**Fig. 4A**, compare lanes 3 and 4). The UVB-induced p38 activation was further analyzed by immunohistochemistry. Control and $Jlp^{-/-}$ adult mice were shaved and treated with or without UVB as in Fig. 4A. Thirty minutes after irradiation, skin samples were obtained and fixed, and 20-µm-thick frozen sections were stained with anti-p-p38 antibody and DAPI. As shown in **Fig. 4B** (bar, 50 µm) and **C** (n.s., not significant; **P < 0.01), the p-p38 immunopositive signals in the epidermis were significantly lower in $Jlp^{-/-}$ mice compared to control mice.

To examine whether JLP expressed in skin basal keratinocytes is responsible for UVB-induced apoptosis, keratinocyte-specific *Jlp* conditional KO (cKO) mice were generated by crossing mice carrying *Jlp* loxP-flanked (floxed) alleles with Keratin5-Cre (K5-Cre) transgenic mice. The region-specific expression of Cre recombinase in the K5-Cre mice was confirmed using the *Rosa26-lacZ* reporter (*R26R*). Frozen sections (20-µm-thick) from the back skin of *R26R* and *R26R*;K5-Cre P5 mice

were stained in X-gal solution for 24 hours at 37˚C (**Fig. 5 B,** bar, 30 μm). The loss of JLP expression in keratinocytes was assessed by Western blotting of cell lysates prepared from primary keratinocytes isolated from control (*Jlp*$^{flox/+}$*;K5-Cre*) and *Jlp* cKO (*Jlp*$^{flox/flox}$*;K5-Cre*) mice (**Fig. 5A**). Results indicated that the *Jlp* gene was successfully disrupted in keratinocytes by K5-Cre-mediated recombination. The UVB-induced apoptosis and p38 MAPK activation in the control and *Jlp* cKO mice were then analyzed. Control and *Jlp* cKO adult mice were shaved and irradiated with UVB, and skin specimens were subjected to immunohistochemistry as in Fig. 2A. Consistent with the findings in *Jlp*$^{-/-}$ mice (Fig. 2A, B), the *Jlp* cKO mice exhibited significantly decreased apoptosis in the epidermis in response to UVB irradiation, compared with control mice (**Fig. 5C**, bar, 100 μm; **D**, *P < 0.05). Moreover, reduced levels of p-p38 were observed in the skin samples of *Jlp* cKO mice by Western blotting (**Fig. 5E**) as in Fig. 4A. The UVB-induced p38 activation was also analyzed by immunohistochemistry, and as a result the p38 immunosignals in the epidermis were found significantly lower in the *Jlp* cKO mice compared with control mice (**Fig. 5F,** bar, 50 μm**; G,** n.s., not significant; *P < 0.05).

Final investigation was done to find out whether p38 signaling is required for UVB-induced apoptosis in mouse skin by using SB203580, a small molecule inhibitor of p38. The backs of adult mice were shaved and topically treated with 40 μL of either vehicle or SB203580 (0.5 μmol) 1 hour before and just after UVB irradiation at a dose of 2.8 kJ/m^2. After 24 hours, skin specimens were subjected to immunohistochemistry as in Fig. 2A. Topical treatment of UVB-irradiated mouse skin with the p38 inhibitor significantly reduced the number of active caspase-3-positive cells in the epidermis of wild-type mice (**Fig. 6 A**, bar, 100 μm; **B**, ***P < 0.001), but not *Jlp* KO mice (**Fig. 6 C,** bar, 100 μm; **D,** n.s., not significant), indicating that p38 signaling plays a pro-apoptotic role in response to UVB exposure.

In this study, the role of JLP in UVB-induced apoptosis in skin epidermal tissues was examined, and found that *Jlp*-deficient mice exhibit a substantially reduced apoptotic response. This is the first demonstration of a critical role for JLP in an *in vivo* response to environmental stimulation. It is also observed that conventional *Jlp* KO mice and keratinocyte-specific *Jlp* cKO mice, in which *Jlp* is disrupted in K5-expressing basal cells, exhibit almost identical effects on UVB-induced apoptosis (Figs 2, 4, 5). Thus, the lack of JLP expression in basal

keratinocytes is most likely responsible for the decreased susceptibility of the *Jlp* mutant mice to UVB-induced stress. The UVB-induced activation of p38 MAPK was significantly attenuated in the epidermis of *Jlp* KO and *Jlp* cKO mice (Figs. 4C, 5G). In addition, topical application of a p38 inhibitor to the skin significantly suppressed the UVB-induced apoptosis in wild-type mice, but not in *Jlp* KO mice (Fig. 6). It is therefore likely that JLP functions as a scaffolding factor for pro-apoptotic p38 pathways following UVB stimulation in basal keratinocytes. However, it is possible that JLP and/or p38 expressed in cells or tissues other than keratinocytes also affect the regulation of the UVB-induced apoptosis independently or cooperatively. At present, the detailed mechanisms underlying UVB-induced JLP-p38 signaling remain unclear. However, considering evidence that UVB exposure stimulates the generation of reactive oxygen species (ROS), and that ROS regulate p38 activation, UVB-induced ROS may activate JLP-mediated p38 signaling pathways. JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JIP3 or Sunday Driver), which is highly homologous with JLP in its sequence and domain structure, has been identified as a scaffold protein for JNK signaling pathways. Recently, Ongusaha *et al*. (2008) analyzed JSAP1/JIP3 knockdown cultured cells, and reported that Rho-associated kinase 1 plays an essential role in UVB-induced apoptosis by regulating JSAP1/JIP3-JNK pathways. Thus, upon UVB stimulation, the scaffold proteins JSAP1 and JLP may be responsible for the efficient activation of JNK and p38 signaling pathways, respectively, leading to apoptosis. In addition, it is also possible that JSAP1 and JLP scaffolds are partially redundant in the regulation of JNK and/or p38 signaling pathways in response to UVB irradiation, although to date, no functional redundancy between JSAP1 and JLP has been reported. Future studies, including the analysis of keratinocyte-specific *Jsap1* cKO and *Jsap1* and *Jlp* double cKO mice, will be required to clarify this issue. The current study identified the scaffold protein JLP as a novel positive regulator of UVB-induced apoptosis. It will be interesting to determine whether *Jlp*-deficient mice exhibit an increased susceptibility to skin cancers, especially basal cell carcinoma, in response to UVB irradiation.

Figure 1.

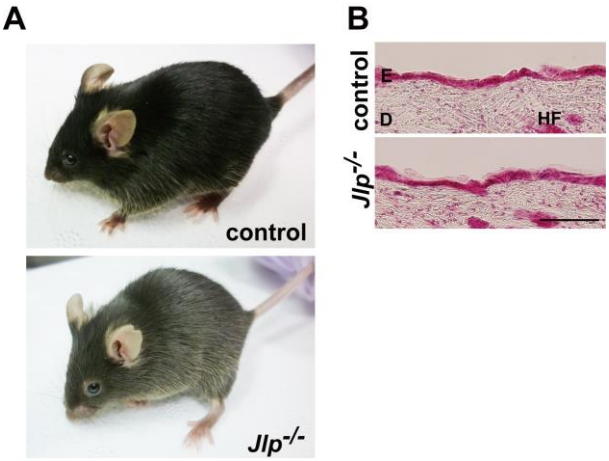


Figure 2.

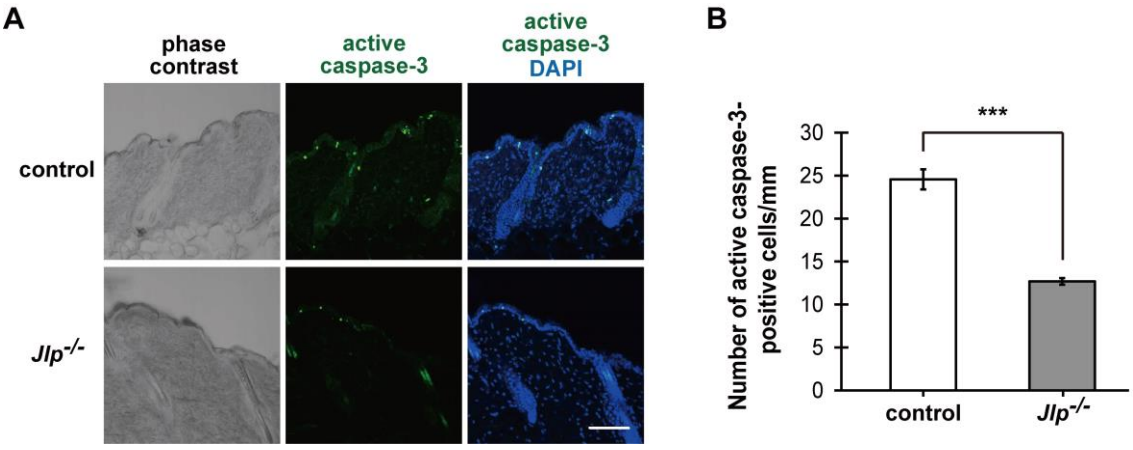


Figure 3.

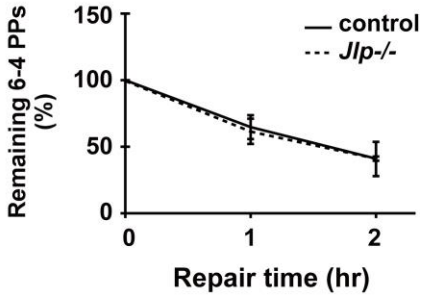
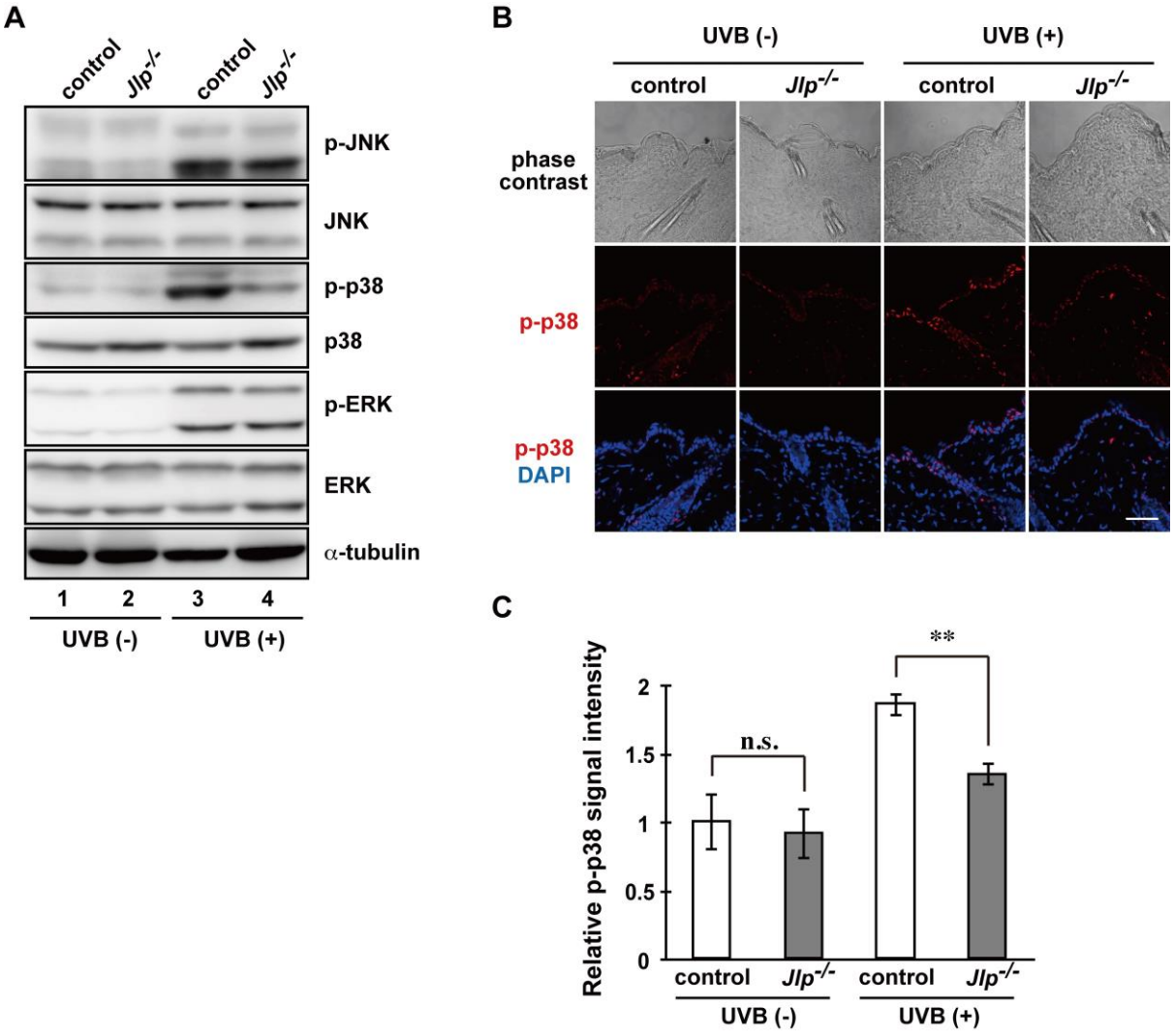


Figure 4.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control
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JNK
p-p38
p38
p-ERK
ERK
α-tubulin
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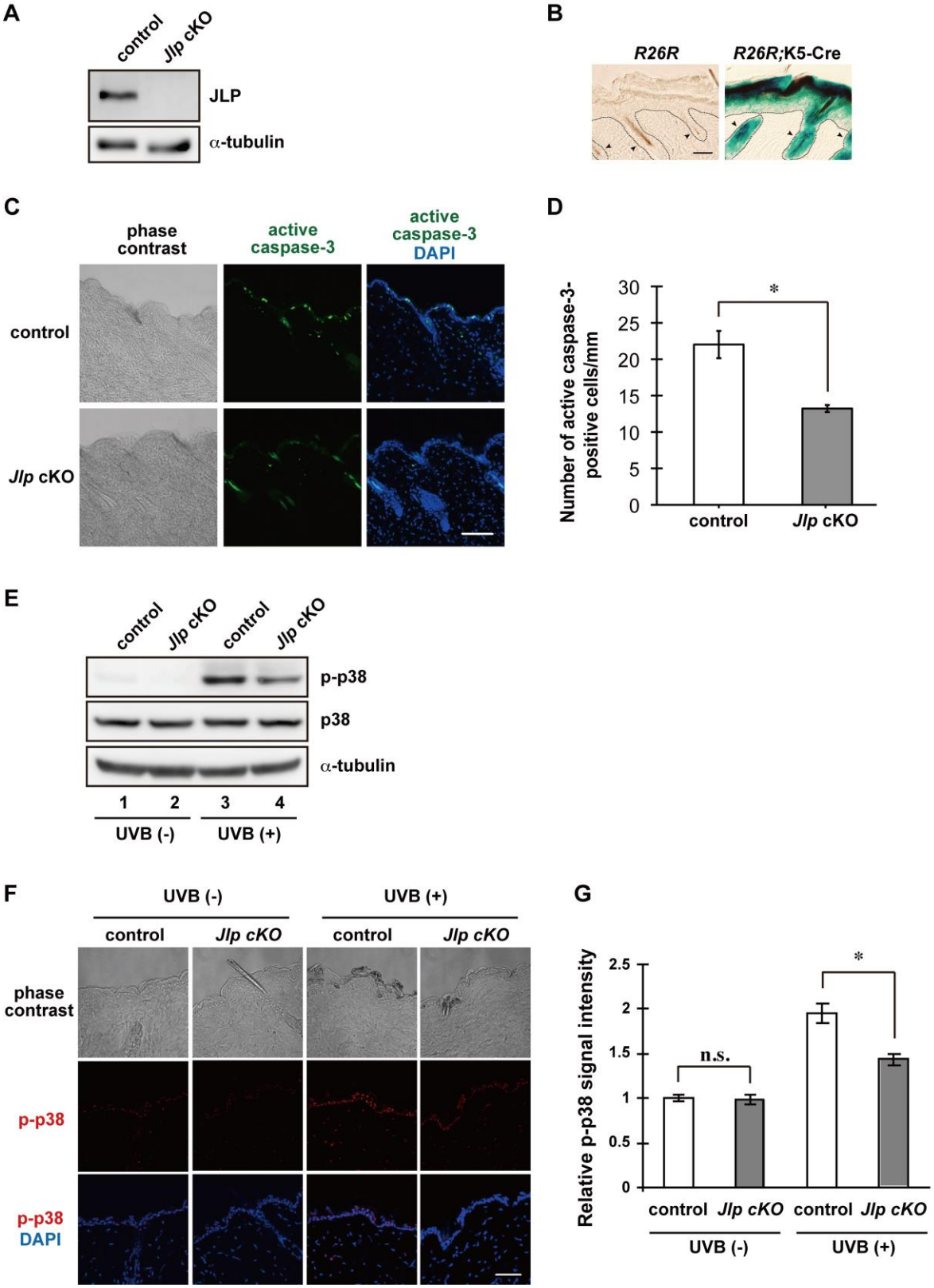
2
3
4
UVB (-)
UVB (+)
B
UVB (-)
UVB (+)
control
Jlp-/-
control
Jlp-/-
phase contrast
p-p38
p-p38
DAPI
C
Relative p-p38 signal intensity
2
1.5
1
0.5
0
n.s.
**
control
Jlp-/-
control
Jlp-/-
UVB (-)
UVB (+)

Figure 5.


A
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Jlp cKO
JLP
α-tubulin
B
R26R
R26R;K5-Cre
C
phase contrast
active caspase-3
active caspase-3 DAPI
control
Jlp cKO
D
Number of active caspase-3-positive cells/mm
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25
20
15
10
5
0
*
control
Jlp cKO
E
control
Jlp cKO
control
Jlp cKO
p-p38
p38
α-tubulin
1
2
3
4
UVB (-)
UVB (+)
F
UVB (-)
UVB (+)
control
Jlp cKO
control
Jlp cKO
phase contrast
p-p38
p-p38 DAPI
G
Relative p-p38 signal intensity
2.5
2
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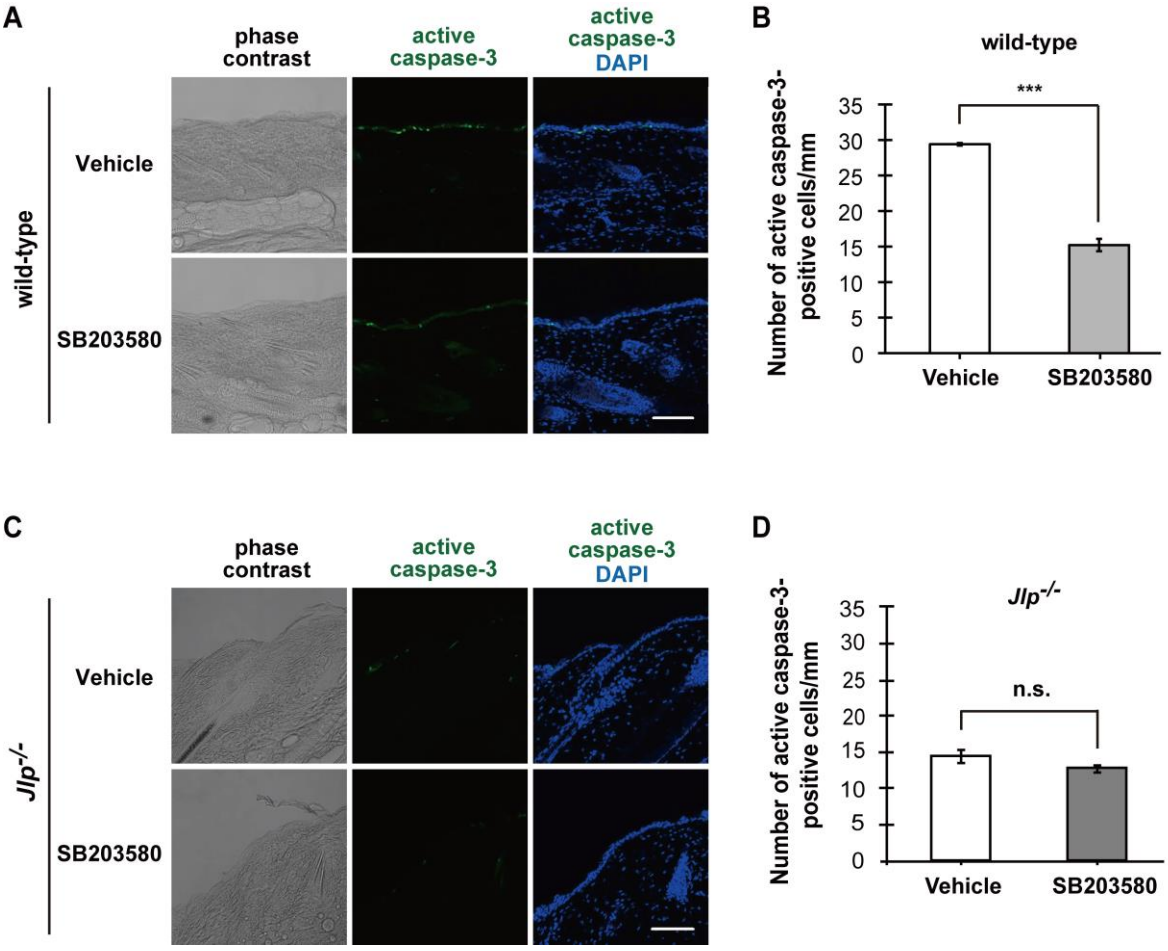
0
n.s.
*
control
Jlp cKO
control
Jlp cKO
UVB (-)
UVB (+)

Figure6.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phase contrast
active caspase-3
active caspase-3 DAPI
wild-type
Vehicle
SB203580
B
wild-type
***
Number of active caspase-3-positive cells/mm
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30
25
20
15
10
5
0
Vehicle
SB203580
C
phase contrast
active caspase-3
active caspase-3 DAPI
Jlp-/-
Vehicle
SB203580
D
Jlp-/-
n.s.
Number of active caspase-3-positive cells/mm
35
30
25
20
15
10
5
0
Vehicle
SB203580

学位論文審査報告書（甲）

1．学位論文題目（外国語の場合は和訳を付けること。）

Role of the scaffolding protein JLP in UVB-induced apoptosis

（紫外線誘導性アポトーシスにおける足場タンパク質 JLP の役割）

2．論文提出者 (1) 所　属　生命科学　専攻　講座

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3．審査結果の要旨（600～650 字）

紫外線が皮膚がんの危険因子であることはよく知られている。紫外線応答に関する研究は精力的に行われているが，紫外線応答は複雑であり，分子レベルでの十分な理解には至っていない。また，MAP キナーゼ(MAPK)経路の足場タンパク質は，シグナル伝達の特異性規定因子である。しかし足場タンパク質の *in vivo* における役割については，ほとんど解析されていないのが現状である。

本研究では，JLP 単純ノックアウト（KO）マウス，および基底細胞特異的 JLP KO［JLP cKO(K5-Cre)］マウスの作出・解析を行い，紫外線 B（UVB）誘導性アポトーシスがこれら JLP 欠失マウスにおいて有意に抑制されることを見出した。6-4 型ダイマー（6-4PP）を指標に DNA 損傷の修復能を調べたところ，野生型細胞と JLP 欠失細胞の間で有意な差異は認められなかった。一方，UVB 照射による皮膚表皮での p38 MAPK の活性化は，JLP KO 及び JLO cKO(K5-Cre)マウスにおいて，有意に抑制された。さらに，p38 MAPK インヒビターを用いた塗布実験を行い，野生型マウスでは UVB 誘導性アポトーシスが顕著に抑制されるが，JLP KO マウスの場合には、ほとんど影響がないことを確認した。以上のことから，JLP-p38 シグナル経路は UVB 誘導性アポトーシスを仲介する重要な細胞内情報伝達経路であることが強く示唆された。

本論文は、環境刺激に対する足場タンパク質 JLP の *in vivo* での役割を初めて明らかにした労作であり、学位に値すると評価された。

4．審査結果　(1) 判　定（いずれかに○印）　(合　格)　・　不合格

(2) 授与学位　博　士（　理学　）