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分担研究報告書

「個体レベルでの発がん物質評価系の検証」に関する研究

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研究要旨：Cre/loxP システムを用いたヒト活性型 $Hras^{G12V}$ トランスジェニックラット（Hras250）の肺に Cre リコンビナーゼ発現アデノウイルスを感染させることによって、活性型 $Hras^{G12V}$ 遺伝子の発現を誘導し肺腺がん、扁平上皮がんあるいはそれらの混合した腺扁平上皮がんを発生させることが可能である。肺がんを発生させたラットにおいて、血清 N-ERC 濃度が有意に上昇していたことから、N-ERC が肺がんモデルラットの血清診断マーカーとして有用であることが示唆された。本肺がんモデルラットにおいて N-ERC を血清診断マーカーとして用いることにより、肺がんの発生を簡便に評価可能なモデルになり得ると考えられた。

A．研究目的

肺癌は発生母地の違いとして小細胞がんと非小細胞がんに分類され、肺癌の約 80%は発生母地の不明な非小細胞癌（扁平上皮がん、腺がん、大細胞がん、その他のがん）とされる。疫学的には、扁平上皮癌および小細胞がんの発生には喫煙との関連が極めて強く、腺がんは非喫煙者にも発生する。肺がんにおける遺伝子変異は、Kras の変異は喫煙者に多く、非喫煙者では EGFR の変異の頻度が高い。

肺がんの動物モデルとして、ラットには 2,2-dihydroxydi-n-propylnitrosamine（DHPN）、N-nitrosobis(2-hydroxypropyl)amine（BHP）、4-(Methyl-nitrosamino)-1-(3-pyridyl)-1-butanone（NNK）等の発がん物質を投与するモデルがあり、マウスには NNK、urethane、4-Nitroquinoline-1-oxide（4-NQO）、2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline（MeIQx）等を投与し肺がんを誘発する化学発がんモデルがある。これら化学発がんモデルでは、主に腺がんが発生し、扁平上皮がんの発生は稀である。化学発がんモデルでは、通常、発がん物質の投与後 25ー50 週程度の期間が必要である。

我々は Cre リコンビナーゼにより活性型ヒト $Hras^{G12V}$ が発現誘導されるトランスジェニックラット（Hras250）を確立している（図1）。ラットはマウスに比べて体が大きく解析に充分な試料の採取が可能な点で優れている。先に記したように、化学発がんでは扁平上皮がんを発生させることは困難であり、試験期間も長期間となる。これを克服するために、Ras トランスジェニックラットを用いて、短期間に肺がんを発生させることが可能な新たな肺がんモデルを確立した。このモデルにおいては、肺腺がん、扁平上皮がんあるいは腺扁平上皮がんが発生する。本研究では、本肺がんモデルラットにおいて、肺がんの発生を簡便に評価可能な血清診断マーカーの確立を行った。

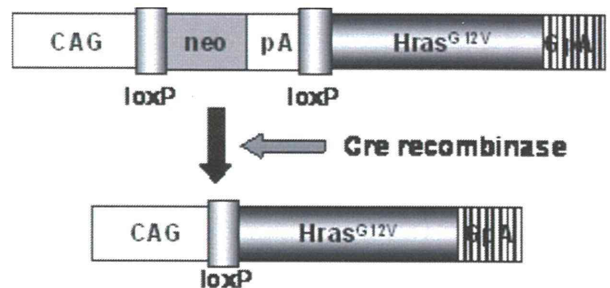

図１　Hras250 ラットの導入遺伝子

B．研究方法

（１）ラット肺がんの発生

Cre リコンビナーゼ発現アデノウイルスを HEK293 細胞に感染させて、Cre リコンビナ

ーゼ発現アデノウイルスを増幅し精製した。精製した Cre リコンビナーゼ発現アデノウイルスを、Hras250 トランスジェニックラットの気管内に噴霧し（$4x10^9$ ifu/ml, 500 μl）、肺胞に感染させることによって肺がんを発生させた。

（２）病理解析

Hras250 ラットの肺に Cre リコンビナーゼ発現アデノウイルスを感染させ、2-4 週後に肺組織を採取し、ホルマリンまたはパラホルムアルデヒドで固定した。パラフィン包埋後、薄切し H&E 染色を行った。

（３）Erc/Mesothelin の測定

組織における遺伝子発現量は RT-PCR 法により行った。血清に遊離した Erc/Mesothelin の N 末側タンパク（N-ERC）はラット ELISA システムを用いて測定した。

(倫理面への配慮)

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C．研究結果

気管内噴霧スプレーを用いて Cre リコンビナーゼ発現アデノウイルスを Hras250 ラットの肺内に投与した。Hras250 ラットでは 3ー6 週後に肺に多数の結節がみられた。これら結節性の病変は腺がん、または扁平上皮がんおよび腺扁平上皮がんであった。

我々はラット膵管がんの血清診断マーカーとして N-ERC を同定している。N-ERC は GPI アンカーをもつ膜結合蛋白である 71 kDa の Erc 蛋白がプロテアーゼにより切断され、遊離した 31 kDa の N 末側蛋白（N-ERC）である。Erc は腎がんモデルである Eker ラットの腎がん細胞で高発現する遺伝子（*Erc*; expressed in renal cell carcinoma）として同定されたものである。ヒトでは中皮腫・卵巣がんで Mesothelin、さらにヒト膵がん細胞株の培養上清中で MPF として同定されている。また、ヒト肺腺がんで高発現しているとの報告もある。そこで、N-ERC がラット肺がんの血清診断マーカーとして応用できるか検討した。

肺組織における Erc/Mesothelin の遺伝子発現を RT-PCR 法により検討した。Erc/Mesothelin 遺伝子の発現は、正常肺に比べて肺結節部位で高発現していた。さらに、血清中の N-ERC 濃度は肺がんの発生したラットにおいて高値を示し、未処置のコントロールラットと比較して有意に高かった。

D．考察

本肺がんモデルでは、肺腺がんおよび化学発がんでは発生させることが困難な扁平上皮がんが発生すると考えられる。扁平上皮がんは、腺がんに次いで発生頻度が高く、男性の肺がんのうち約 40%、女性では約 15%、全体では 30%程度を扁平上皮がんが占めていることから、本モデルの有用性は高いと考えられる。また、肺がんの発生したラットにおいて、肺病変部位で Erc/Mesothelin 遺伝子が高発現していた。さらに、血清 N-ERC 値が有意に高値を示したことから、N-ERC がラット血清診断マーカーとなることが示唆された。N-ERC はヒト中皮腫、卵巣がん患者において血清濃度が高くなっていることが既に報告されている。中皮腫のみならず肺がん患者の腺がんにおいても Erc/Mesothelin が高発現しているという報告もある。ラットにおいても肺がんの発生した個体で血清中の N-ERC 濃度は高値を示したことから、肺がんの血清診断マーカーにもなり得ると考えられた。今後、腺がん、扁平上皮がんおよび腺扁平上皮がんにおける Erc/Mesothelin の発現を詳細に検討することにより肺がんの診断マーカーとしての意義を検討したい。

E．結論

Hras250 ラット肺がんモデルにおいて、特に化学発がんでは発生させることが困難な扁平上皮成分を含むがんを短期間に発生させることが可能である。Hras250 ラットでは、肺腺がんおよび扁平上皮成分を含む肺がんが短期間に発生し、さらに、血清 N-ERC が本肺がんモデルラットにおいて血清診断マーカーとなり得ることから、発がん評価において簡便なヒト肺がんモデルシステムとな

ることが示唆された

F．健康危険情報
特記すべき事無し。

G．研究発表
１．論文発表
該当無し

２．学会発表
（国内）
Katsumi Fukamachi, Yutaka Ohshima, Yuto Sakai, Mitsuru Futakuchi, Hiroyuki Tsuda, Masumi Suzui, A serum tumor marker for preclinical trials of rat lung cancer model、第 70 回癌学会学術総会、名古屋、2011 年 10 月

H．知的所有権の出願・登録状況
１．特許取得
なし
２．実用新案登録
なし
３．その他
なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Ishizaka Y, Okudaira N, Okamura T.	Regulation of retrotransposition of long interspersed element-1 by mitogen-activated protein kinases.	-	Protein kinases	InTech	クロアチア	in press	

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ishizaka Y, Okudaira N, Tamura M, Iijima K, Shimura M, Goto M, Okamura T.	Modes of retrotransposition of long interspersed element-1 by environmental factors.	Frontiers in Microbiology	in press		
Okudaira N, Goto M, Abe Y, Tamura M, An Y, Kano S, Ishizaka Y, Okamura T.	Involvement of retrotransposition of long interspersed nucleotide element-1 in DMBA/TPA-induced skin carcinogenesis.	Cancer Sci.	102	2000-2006	2011

Chapter Number

Regulation of Retrotransposition of Long Interspersed Element-1 by Mitogen-Activated Protein Kinases

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1. Introduction

Our genome contains a higher amount of endogenous retroelements (~42 %) than mouse (~37 %) or fruit fly (~3.6 %) (1-3). Long interspersed element-1 (L1) is the most abundant of transposable elements, comprising ~17% of the genome (1-4). L1 is an autonomous endogenous retroelement that has evolved in a single, unbroken lineage for the past 40 million years in primates (5). A single human cell has more than 5x10^{5} copies of L1 (2,4), and most of them are functionally defective (6). However, 80 to 100 copies of L1 are competent for retrotransposition (L1-RTP) (7), and approximately 10 % of these are highly active for "copy and paste" (7). L1 is actively expressed in embryonal stem cells (8) and L1-RTP is induced in oocytes or early embryonic development (9-11). L1-RTP occurring in germ lines would function an intrinsic factor responsible for allelic variants among individuals (12,13). However, aberrant L1-RTP alternates critical gene structures, leading to the development of inborn errors (14). At the moment, at least 17 genetic diseases have been reported as sporadic cases of inheritable disorders caused by aberrant insertion of L1 (14). On the other hand, recent observations suggest that L1-RTP occurs in somatic cells. Strikingly, it was shown that copy numbers of L1 is increased in human brain tissues (15,16). Aberrant L1 insertions have been detected in c-*myc* gene and the *APC* gene in breast carcinoma and colon carcinoma, respectively (17,18). Moreover recent analysis demonstrated that L1 is frequently mobilized in human lung cancers and pancreatic carcinomas (19,20). These observations indicate that it is important to understand the mode of L1-RTP, but little is known about the cellular factors for the induction of L1-RTP in somatic cells. We herein summarize our current understanding of L1-RTP induction, with an emphasis on mitogen-activated protein kinases (MAPKs), which are activated by environmental compounds, and we discuss their roles in genome shuffling.

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2. Biology of L1-RTP

L1, a non-long terminal repeat (non-LTR)-type endogenous retroelement, encodes two proteins: open reading frames 1 and 2 (ORF1 and 2) (3). ORF1 is a cytoplasmic 40 kDa protein that is present within ribonucleoprotein complexes (21-23). ORF-1 associates in *cis* with L1-mRNA (24) and functions as a chaperone of L1-mRNA (25). ORF2 is a protein of about 150 kDa with dual activities as reverse transcriptase (RT) (26) and an endonuclease (27). ORF2 recognizes the 5'-TTAAAA hexanucleotide in the genome and induces a nick between 3'-AA and TTTT in the complementary strand (28,29). It has been proposed that the first-strand DNA is synthesized by target site-primed reverse transcription (3,29). ORF1 and 2 complete the entire process of L1-RTP and are competent for the induction of retrotransposition of *Alu*, a non-autonomous retroelements (30, 31).

3. Reported triggers of L1-RTP

As to the environmental factors that induce L1-RTP in somatic cells, Farkash *et al.* reported that gamma irradiation at 4.5 Gy induced L1-RTP (32). Independently, Deiniger's group reported that heavy metals of such as mercury, cadmium and nickel also induced L1-RTP (33,34). They also reported that nickel-induced L1-RTP is induced by a post-transcriptional mechanism (34). As to an environmental carcinogen, Stribinskis and Ramos found that benzo[*a*]pyrene (B[*a*]P) induced L1-RTP (35). An extensive analysis revealed that aryl hydrocarbon receptor (AhR), which serves as a receptor for such environmental pollutants as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (36), was required for the B[*a*]P-induced L1-RTP (35). Because TCDD, a non-genotoxic hydrocarbon carcinogen, did not induce L1-RTP, it was proposed that as one of the its mechanisms an AhR-dependent cellular response converts B[*a*]P into an active genotoxic compound, which in turn induces L1-RTP (35). Although the exact modes of L1-RTP are unclear, these studies inspired us to investigate the possibility that various environmental compounds can induce L1-RTP.

4. Induction of L1-RTP by an environmental compound and identification of p38 as a pivotal cellular factor

First, we found that 6-formylindolo[3,2-*b*]carbazole (FICZ), a tryptophan photoproduct, induced L1-RTP (37). FICZ is highly active, and even picomolar comncentratiom of the compound induced L1-RTP. In mammalian cells, six groups of MAPKs, namely extracellular signal-regulated protein kinase (ERK)1/2, ERK5, JNK, p38, ERK3/4 and ERK7/8, are identified, and are activated by intracellular and extracellular stimuli (38). Among these, cellular signal cascades of ERK1/2, p38 and JNK have been well characterized, because of the availability of inhibitors, including PD98,059, SB202190 and SP600125, respectively. Using these MAPK inhibitors, we found that FICZ-induced L1-RTP was dependent on p38 (37). Interestingly, the compound induced phosphorylation of cyclic-AMP responsive element binding protein (CREB), and the down-regulation of endogenous CREB by short interference RNA (siRNA) attenuated the induction of L1-RTP by FICZ. Moreover, a transfection-back experiment of cDNA that encoded a siRNA-resistant CREB restored the induction of L1-RTP. These data indicate that the induction of L1-RTP by FICZ depended on p38-CREB-dependent signaling. Intriguingly, L1-RTP by FICZ was not dependent on AhR, although FICZ is a candidate physiological ligand of AhR (39). In contrast, L1-RTP by FICZ was dependent on AhR nuclear translocator 1 (ARNT1), a binding partner of AhR (40).

AhR and ARNT1 are members of the basic helix-loop-helix/per-arnt-sim (bHLH/PAS) family, which are transcription factors involved in a variety of biological functions (41). Recently, it was shown that the bHLH/PAS family is functionally linked with environmental adaptation of living organisms (42). When AhR binds environmental compounds, it forms a heterodimer with ARNT1, which is recruited from the cytoplasm to chromatin and recognizes a xenobiotic responsive element (XRE) (36). It has been shown that the chromatin recruitment of ligand-bound AhR depens on the nuclear localization signal of ARNT1 (43), but there are no reports showing that ARNT1 functions as a receptor for environmental compounds. A cellular factor that cooperates with ARNT1 in FICZ-induced L1-RTP has yet to be identified.

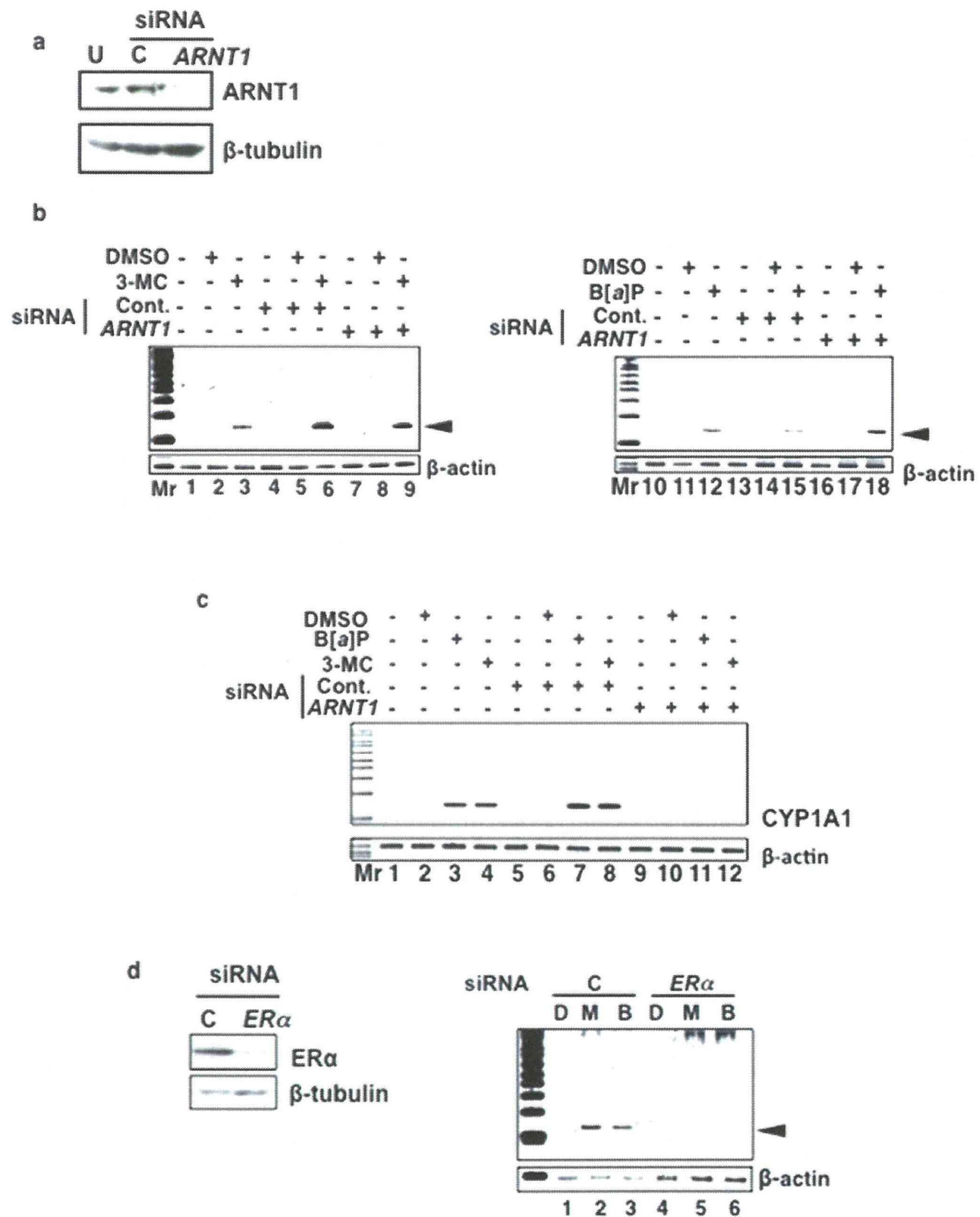
5. MAPKs required for L1-RTP by FICZ

To explore the involvement of MAPKs in L1-RTP, we extended our experiments to explore whether environmental carcinogens induce L1-RTP. In two-stage chemical carcinogenesis, it has been shown that skin tumors develop by treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA) plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (44). DMBA functions as an initiator and activates *H-ras* gene, whereas TPA functions as a tumor promoter thorough non-genotoxic effects (45). However, how TPA induces tumor progression remains to be clarified. We first analyzed whether L1-RTP is involved under skin carcinogenesis. When transgenic mice harboring human L1 as a transgene (hL1-EGFP mouse) were subjected to DMBA/TPA-induced skin carcinogenesis, L1-RTP was frequently observed in the DMBA/TPA-induced skin tumors (46). Interestingly, *in vitro* experiments revealed that both DMBA and TPA were active for the induction of L1-RTP. On the other hand, *in vivo* experiments, in which hL1-EGFP mice were transiently treated with DMBA or TPA suggested that L1-RTP in the skin tumors was attributable to the effects of the repeated treatment with TPA. Notably, we observed that the mode of L1-RTP by DMBA and TPA was different. DMBA-induced L1-RTP was dependent on both AhR and ARNT1, whereas TPA-induced L1-RTP required neither protein. Instead, it depended on ERK1/2 and epidermal growth factor receptor (EGFR). Since Balmain *et al* (44) originally reported on DMBA/TPA-induced two-stage carcinogenesis, a major issue of cancer research is to clarify the mechanism of the TPA-induced tumor promotion. Using genetically-engineered mice, it has been proven that TPA-induced tumor promotion depends on ERK1/2 and EGFR (47,48). Interestingly, TPA-induced L1-RTP was shown to be dependent on these molecules, suggesting that the genome shuffling by L1-RTP is linked with the mode of TPA-dependent tumor promotion.

6. MAPKs are involved in the induction of L1-RTP by carcinogens

Given that environmental compounds seemed to induce L1-RTP by involving different cellular proteins, we investigated other carcinogens such as B[*a*]P and 3-methylcholanthrene (3-MC). Consistent with a previous report (35), B[*a*]P induced L1-RTP in an AhR-dependent manner (46). Additionally, 3-MC also induced L1-RTP in an AhR-dependent manner (46). However, we found that the L1-RTP was induced even when siRNA against *ARNT1* was transfected into the cell (Fig. 1b, lanes 9 and 18). The siRNA clearly suppressed the mRNA expression of *CYP1A1* (Fig. 1c, lanes 11 and 12), indicating that the siRNA effectively abrogated the function of endogenous ARNT1 protein. These data support the idea that ARNT1 is dispensable for the induction of L1-RTP by these compounds. Because it has been

1



2

3 Fig. 1. B[*a*]P and 3-MC induced L1-RTP depending on AhR and ERα, but not on ARNT1. An
4 L1-RTP assay was performed according to the procedures described (37,46). Briefly, Huh-7
5 cells from a human hepatoma cell line were transfected with pEF06R on day 0, then treated
6 with 0.5 μg/mL puromycin for two days (days 1-3). The cells were then trypsinized and

replated for treatment with the compounds. Two days after the addition of 3 μM B[*a*]P or 1 μM 3-MC, the cells were harvested and their DNA extracted. No cytotoxicity was caused by 3 μM B[*a*]P or 1 μM 3-MC (data not shown). For the PCR-based assay, a spliced form of *EGFP* cDNA (140 bp in length) was amplified by PCR with primers specific for the separated exons of *EGFP* cDNA. The amplified DNA was then loaded onto an agarose gel and detected after staining with Vistra Green. As an internal control, the same samples were used as templates for the amplification of β-actin. **a**. Effects of *ARNT1* siRNA on the down-regulation of endogenous ARNT1. Western blot analysis was performed on day 2 after the transfection of *ARNT1* siRNA. U, untreated; C, control siRNA; *ARNT1*, *ARNT1* siRNA. **b**. L1-RTP caused by B[*a*]P and 3-MC was independent of ARNT1. The PCR-based assay of the effects of *ARNT1* siRNA is shown. Huh-7 cells were transfected with pEF06R on day 0 and then selected from days 1-3. On day 3, the cells were trypsinized, replated, and further transfected with control or *ARNT1* siRNA. On day 4, the cells were again divided into three groups and treated with DMSO, 3-MC (left panel), or B[*a*]P (right panel). After two days, DNA was extracted and subjected to a PCR-based assay. The arrowhead indicates the PCR-amplified band corresponding to the induction of L1-RTP. **c**. *ARNT1* siRNA effectively blocked the mRNA expression of *CYP1A1*, which was induced by the compounds. Huh-7 cells were first transfected with control or *ARNT1* siRNAs. On day 2 after transfection, the cells were trypsinized, replated, and treated with B[*a*]P or 3-MC. RT-PCR analysis was performed on day 2 after the addition of the compounds. **d**. ERα is required for the induction of L1-RTP by B[*a*]P or 3-MC. In this experiment, MCF-7 cells from a human breast carcinoma cell line were used. Using a similar experimental protocol, the effect of *ERα* siRNA on the induction of L1-RTP was examined. As an internal control, β-actin was amplified.

Cellular factors		Inducers				
		FICZ	B[*a*]P	3-MC	DMBA	TPA
AhR		-	○	○	○	-
ARNT1		○	-	-	○	-
ERα		N.T.	○	○	-	-
MAPK	SB202190	○	○	○	-	-
	SP600125	○	○	○	-	-
	PD98,059	N.T.	N.T.	N.T.	-	○

○, dependent; -, independent; N.T., not tested.
The induction of L1-RTP was examined by a PCR-based assay (see legend for Fig. 1).

Table 1. Summary of cellular factors required for L1-RTP by environmental compounds

shown that AhR forms a complex with estrogen receptor α (ERα) (49), we further tested the involvement of ERα in the induction of L1-RTP. Interestingly, the transfection of *ERα* siRNA attenuated L1-RTP induced by these compounds (Fig. 1d, lanes 5 and 6). In addition, we found that CREB was definitely phorphorylated (Fig. 2a, lane 4), and checked the effects of MAPK inhibitors on the induction of L1-RTP by 3-MC. As shown in Fig. 2b, SB202190 attenuated the induction of L1-RTP (lane 8), whereas SP600125 did not (lane 10). To further

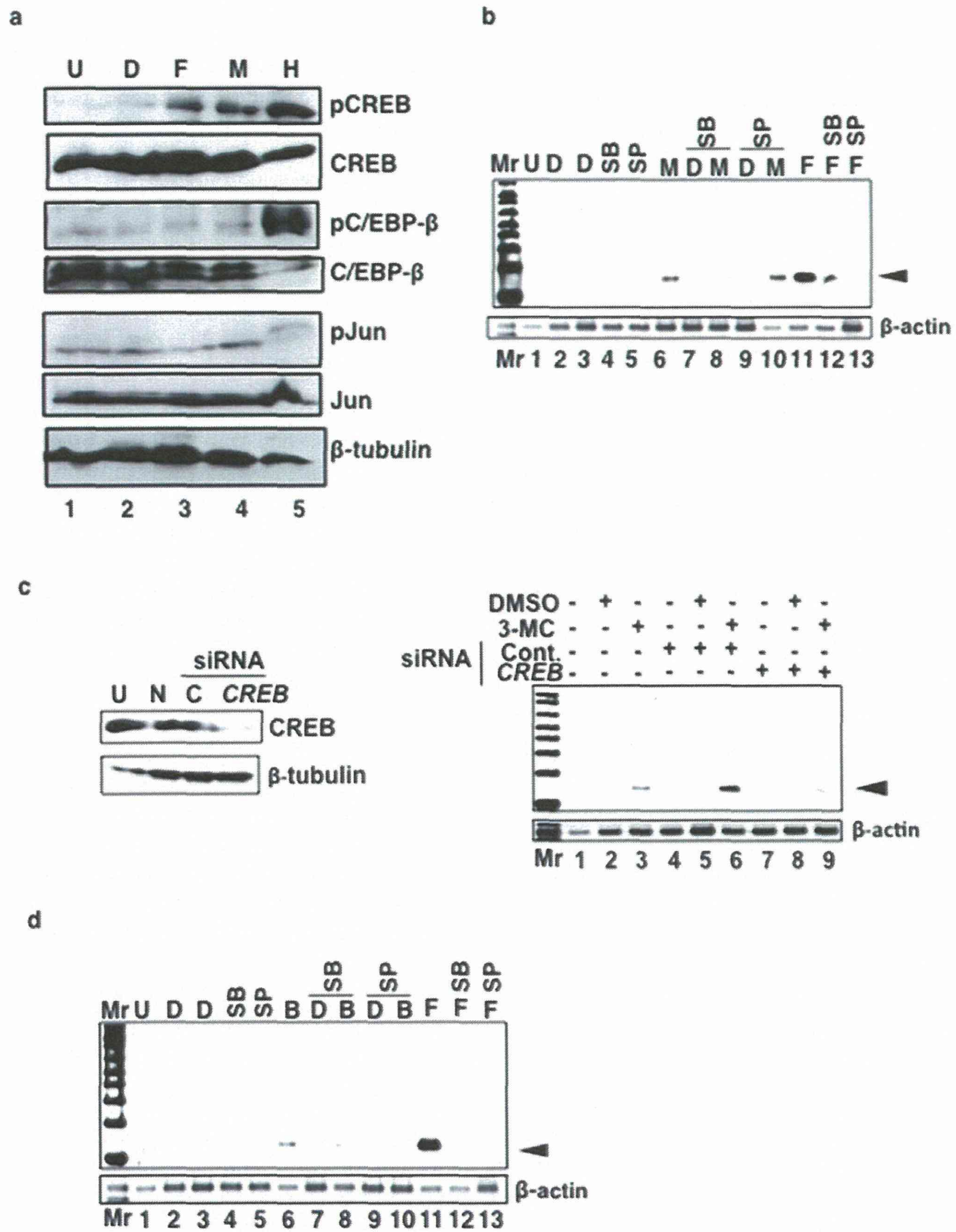
identify a candidate substrate of p38, we examined the effects of *CREB* siRNA. The transfection of *CREB* siRNA abrogated the induction of L1-RTP by 3-MC (Fig. 2c). These data suggest that L1-RTP by 3-MC is induced by the cooperative function of AhR and ERα depending on a signal cascade involving the p38-CREB pathway. Our data also indicate that the induction of L1-RTP by B[*a*]P is dependent on p38 and JNK (Fig. 2d, lanes 8 and 10).

Although further study is required, our current understanding is that various environmental compounds induce L1-RTP by combinations of the bHLH/PAS family and MAPKs (Table 1). L1-RTP was differentially induced by FICZ, DMBA, B[*a*]P, 3-MC and TPA. Most of the compounds examined, with the exception of DMBA, depended on MAPKs. Moreover, the L1-RTP by carcinogens depended on AhR, whereas FICZ did not. It is important to collect more information about chemical compounds active in the induction of L1-RTP and to elucidate the involvement of MAPKs.

It has been proposed that L1-RTP is controlled at the transcriptional and post-transcriptional levels. *In vitro* experiments revealed that that the expression of L1 is tightly regulated by methytlation of CpG in the region of 5′-LTR. In normal somatic cells, the 5′-LTR of L1 is methylated at CpG (50,51), but it is hypomethylated in transformed cells (52). It has been consistently reported that treatment with B[*a*]P induced the hypomethylation of CpG in HeLa cells (53). Moreover, it was reported that L1-5′UTR has a ubiquitously active antisense promoter that encodes small interfering RNAs, which effectively suppressed the retrotransposition of L1 (54). These observations indicate that epigenetic alternation of the 5′-LTR was proposed as the activation mode of L1-RTP by the compound. However, the following *in vitro* experiments suggested the presence of another regulatory system of L1-RTP. A reporter construct was transfected into cultured cells, and treatment with the compounds increased the frequency of L1-RTP. Because the reporter construct (*e.g.*, pEF06R, which carries *EGFP* cDNA as a reporter gene) contained a potent CMV promoter (32,34), L1-mRNA was strongly expressed when it was transfected into cultured cells. Even under such conditions, remarkable effects on the induction of L1-RTP were detectable by additing inducers such as FICZ, B[*a*]P, and 3-MC (37,46). Data indicate the presence of an additional regulatory system in which cellular proteins regulate the induction of L1-RTP. One possible mode of regulation is the chromatin recruitment of ORF1.

7. The chromatin recruitment of ORF1 is MAPK-dependent

Because it has been postulated that ORF1 is present in the cytoplasm (21-23) and carcinogen-induced L1-RTP was dependent on AhR, it is plausible that ORF1 is functionally associated with the bHLH/PAS family. To prove this, we evaluated the association of ORF1 and AhR by an immunoprecipitation followed by Western blot analysis with a polyclonal antibody to human ORF1. Intriguingly, ORF1 and AhR were associated even under normal conditions (Okudaira N, submitted). More importantly, we detected that recruitment of ORF1 into the chromatin-rich fraction was coupled with L1-RTP. As reported, chromatin recruitment of ORF1 was induced by FICZ in a MAPK-dependent manner. It is interesting that the chromatin recruitment of ORF1 was induced by FICZ, although FICZ-induced L1-RTP was not dependent on AhR (37). Interestingly, ARNT1 was associated with ORF1 when FICZ was added to the culture medium (37). Although the precise role of the MAPK is unclear, these data suggest that the chromatin recruitment of ORF1 is the important regulatory step in L1-RTP, where at least p38 is involved as a crucial cellular factor.



1

2 Fig. 2. MAPK is required for the induction of L1-RTP by B[*a*]P or 3-MC. **a**. Phosphorylation
3 of MAPK substrates induced by 3-MC. Huh-7 cells were analyzed on day 2 after the
4 addition of the compound. U, untreated; D, dimethylsulfoxide (DMSO); F, FICZ; M, 3-MC;
5 H, H_2O_2. **b**. Effects of MAPK inhibitors on L1-RTP induced by 3-MC. SB and SP are

SB202190 and SP600125, respectively. U, untreated; D, DMSO; F, FICZ; M, 3-MC; H, H_2O_2. Of note, L1-RTP caused by 3-MC was attenuated by SB (lane 8), but not by SP (lane 10). The arrowhead indicates the induction of L1-RTP. **c.** CREB is required for L1-RTP induced by 3-MC. Left panel: Western blot analysis detected efficient down-regulation of the endogenous protein by *CREB* siRNA. U, untreated; N, non-transfected; C, control siRNA; *CREB*, *CREB* siRNA. Right panel: PCR-based assay after the transfection of *CREB* siRNA. *CREB* siRNA attenuated L1-RTP induced by 3-MC (lane 9). **d.** Effects of MAPK inhibitors on L1-RTP induced by B[*a*]P. Reagents similar to those described in Fig. 2b were used. L1-RTP by B[*a*]P was attenuated by both SB (lane 8) and SP (lane 10).

8. Roles of MAPK on L1-RTP

It has been supposed that the increase of transposable elements coupled with evolution (7). Even in *Candida albicans*, an L1-like structure is present as a functional gene (55). On the other hand, the bHLH/PAS family, which has a variety of biological functions including the metabolism of xenobiotics, maintenance of the circadian rhythm, cellular responses to hypoxia, and neuronal differentiation (41,42), is also well conserved from lower species to mammals (56). Interestingly, *AhR* homologs are also present in the genomes of *Drosophila melanogaster* and *Caenorhabditis elegans* (56). Although no direct evidence on the functional relationship between these two biological phenomena has been claimed, our observation is the first to demonstrate the functional link of these biological events. Moreover, data suggest that MAPKs are involved in the bHLH/PAS-dependent L1-RTP. MAPKs are involved in cellular response to intracellular and extracellular stress (38,57), and it is plausible that MAPKs mediate various stresses in the induction of L1-RTP, resulting in genome shuffling. Random mutagenesis by L1-RTP may give emerging novel organisms to survive in altered environments.

It is important to clarify the roles of MAPKs in the induction of L1-RTP. At present, at least two functions of MAPKs can be postulated. As explained, environmental compounds activate MAPKs, by which the chromatin recruitment of ORF1 is induced as a necessary step in L1-RTP. ORF1 functions in *cis* with L1- mRNA and functions as a chaperon of L1-mRNA (24,25). Using MAPK inhibitors, we observed that L1-RTP was abrogated concomitantly with the reduced chromatin recruitment of ORF1. These observations suggest that MAPK activation drives the mobilization of ORF1 to chromatin, by which retroelements are translocated to chromtin.

Another possible role for MAPKs is related to the activity of the APOBEC family. It has been proposed that APOBEC family functions as innate restriction factors that suppress the activity of endogenous retroelements (58). Originally, it was postulated that the APOBEC family inhibits HIV-1 infection by editing C to T via deaminase activity (58). Vif, a gene product of HIV-1, degrades APOBEC proteins, causing infected cells to become permissive for HIV-1 infection (59). We previously showed that all members of the APOBEC family exhibit inhibitory activity toward L1-RTP (60). However, it was recently postulated that the APOBEC has dual activity (61) and inhibits the activity of RT (62). In *in vitro* experiments in which APOBEC3G were added to the reaction of RT in the synthesis of viral DNA, APOBEC interfered with elongation of the viral DNA (62). Interestingly, it has been shown that C/EBP-β bound APOBEC3G and attenuate the inhibitory activity of APOBEC3G (64). Moreover, it was demonstrated that the mutation of serine at 228 (S228), the phosphorylation of which is correlated with the cytoplasmic localization of the molecule (63), abolished both

binding and inhibitory activity on APOBEC3G (64). Given that C/EBP-β is a substrate of p38 (38), a plausible model is that p38 augments the blocking activity of C/EBP-β on APOBEC3G via phosphorylation.

9. Further implications

Ataxia telangiectasis mutated (ATM), a phosphoinositide 3-kinase, has a functional link with L1-RTP (16). In an intriguing recent observation, the copy number of L1 increased in the brain tissues of patients with ATM (16). L1-RTP is consistently increased in the brain tissue of *ATM*-knock out mouse. Although these observations suggest that ATM functions as a negative regulator of L1-RTP, Gasior *et al.* originally reported that ATM was required for the induction of L1-RTP (65). Because of controversial observations regarding the role of ATM in L1-RTP, we focused on MAPKs in the current study.

Recent observations revealed that genome shuffling by L1-RTP in human somatic cells is a source of interindividual genomic heterogeneity (12,13). In addition, independent research groups reported that L1-RTP is frequently induced in tumors (19,20), suggesting the involvement of L1-RTP in the development of carcinogenesis. Importantly, L1 proteins are active on the retrotransposition of *Alu* (30,31), a non-autonomous retoroelement. On the other hand, it has been shown that *Alu* induces genomic instability via non-allelic homologous recombination (66). Thus, it is important to understand the activation mechanisms of L1. Our current observations support the idea that the chromatin recruitment of ORF1, which is controlled by cooperative regulation by members of the bHLH/PAS family and MAPKs, is a critical step in the regulation of L1-RTP. If this is the case, L1-RTP induction in the genome is selectively determined by cellular factors. Because AhR is a transcription factor that recognizes specific nucleotide element (36), carcinogens possibly induce L1-RTP in the genomes in the vicinity of the *cis*-element.

As observed in the analysis of L1-RTP by B[*a*]P and 3-MC, L1-RTP was not induced via the classical pathway controlled by both AhR and ARNT1. Our data suggest that L1-RTP is not necessarily induced by genotoxic activities of these compounds, further implying that L1-RTP is a novel type of genomic instability by which cellular cascades activated by environmental compounds lead to genome shuffling and generate stable phenotypes of the affected cells. The suppression of L1-RTP in somatic cells by targeting MAPK activity may be a novel strategy to protect the development of intractable diseases that include carcinogenesis.

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All authors declare that they have no conflict of interest for the current work.

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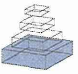

Modes of retrotransposition of long interspersed element-1 by environmental factors

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Approximately 42% of the human genome is composed of endogenous retroelements, and the major retroelement component, long interspersed element-1 (L1), comprises ~17% of the total genome. A single human cell has more than 5 × 10^5 copies of L1, 80~100 copies of which are competent for retrotransposition (RTP). Notably, L1 can induce RTP of other retroelements, such as *Alu* and *SVA*, and is believed to function as a driving force of evolution. Although L1-RTP during early embryogenesis has been highlighted in the literature, recent observations revealed that L1-RTP also occurs in somatic cells. However, little is known about how environmental factors induce L1-RTP. Here, we summarize our current understanding of the mechanism of L1-RTP in somatic cells. We have focused on the mode of L1-RTP that is dependent on the basic helix–loop–helix/per–arnt–sim (bHLH/PAS) family of transcription factors. Along with the proposed function of bHLH/PAS proteins in environmental adaptation, we discuss the functional linking of L1-RTP and bHLH/PAS proteins for environmental adaptation and evolution.

Keywords: LINE-1, environmental factors, retrotransposition, bHLH/PAS family

INTRODUCTION

Approximately half of the human genome (~45%) is composed of transposable elements, most of which are endogenous retroelements (~42%; Lander et al., 2001; Bannert and Kurth, 2004). Notably, humans have more endogenous retroelements than do mice (~37%) or fruit flies (~3.6%), implying a possible role for endogenous retroelements in evolution (Kazazian, 2004). In humans, ~8% of the genome comprises human endogenous retroviruses (HERV) that have gene structures similar to the long-terminal repeats (LTRs) of lentiviruses (Bannert and Kurth, 2004). By contrast, ~34% of retroelements are of non-LTR types, half of which are long interspersed element-1 (L1). A single human cell contains more than 5 × 10^5 copies of L1, 80~100 copies of which are competent for genome shuffling by retrotransposition (RTP; Brouha et al., 2003). Interestingly, ~10% of such RTP-competent L1 sequences are "hot" and account for more than 80% of the total RTP activity (Brouha et al., 2003).

L1 is actively expressed in embryonic stem cells (Georgiou et al., 2009), and L1-RTP occurs in oocytes and during early embryonic development (van den Hurk et al., 2007; Kano et al., 2009). L1-RTP is also coupled with neuronal cell differentiation (Muotri et al., 2005), supporting the hypothetical role of L1-RTP in the plasticity of nerve cells. Moreover, L1-RTP was shown to be a critical event at an early stage of cell division in the fertilized egg, although its exact role remains elusive (Vitullo et al., 2012). While these lines of evidence strongly suggest that L1-RTP is pivotal for early embryogenesis, recent observations indicate that L1-RTP also occurs in somatic cells. Interestingly, some studies suggest that the L1 copy number is increased in the human brain (Muotri et al., 2005; Baillie et al., 2011), and greater L1 activity was detected in patients with defective ataxia telangiectasia mutated (*ATM*) genes (Coufal et al., 2011). Moreover, new somatic inserts were identified in the tumors, suggesting increased activity therein (Iskow et al., 2010; Ting et al., 2011). These observations support the idea that somatic cells possess the machinery that is involved in the induction of L1-RTP, but little is known about the cellular factors required for L1-RTP. Recently, we found that L1-RTP was induced by environmental compounds that included carcinogens (Okudaira et al., 2010, 2011; Ishizaka et al., 2012). L1-RTP induction by these compounds was dependent on members of the basic helix–loop–helix/per–arnt–sim (bHLH/PAS) protein family, which has been proposed to be associated with the environmental adaptation of living organisms (Beischlag et al., 2008; McIntosh et al., 2010). Here, we provide an overview of our current understanding of the mechanism of L1-RTP coupled with bHLH/PAS proteins, and discuss the role of L1-RTP in relation to environmental adaptation and revolution.

L1 AND THE ROLES OF L1-ENCODED PROTEINS

L1 is ~6 kb in length and is composed of a 5′-untranslated region (UTR), two non-overlapping open reading frames (ORFs), and a 3′ UTR with a poly(A) tail (Goodier and Kazazian, 2008). L1 has evolved along a single, unbroken lineage for the past 40 million years (myrs) in primates, and five subfamilies of L1 (L1PA1-5) have developed within the past 25 myrs in hominoid primates (Lee et al., 2007). Interestingly, species-specific L1 subfamilies emerged in *Homo sapiens* and *Pan troglodytes* after their divergence 6 myrs ago. In humans, active L1 (Ta-1) arose from the Ta-0 subfamily

~4 myrs ago, and expanded as a dominant subfamily thereafter. Notably, it has also been shown that ~69% of L1-inserted loci are polymorphic for the presence or absence of the Ta-1 insert, and that ~90% of such loci possess Ta-1d inserts, the youngest subset of Ta-1, which arose ~1.4 myrs ago (Boissinot et al., 2000). Of note, Ta-1d accounts for approximately two-third of the Ta-1 subfamily, indicating that it has been selectively expanded during the evolution of *H. sapiens.* Intriguingly, humans have higher numbers of RTP-competent L1 sequences (80~100 copies) than do chimpanzees (~5 copies), although there are similar numbers of species-specific inserts (1200–2000 copies) in humans and chimpanzees (Lee et al., 2007).

L1 encodes two proteins: ORF1 and ORF2 (Goodier and Kazazian, 2008). ORF1 is a 40-kDa protein that acts on L1-mRNA *in cis* (Wei et al., 2001) and functions as its chaperone (Martin et al., 2005). ORF2 is a protein of about 150 kDa with both reverse transcription (RT; Mathias et al., 1991) and endonuclease (Feng et al., 1996) activities. ORF2 recognizes 5′-TTAAAA hexanucleotides in the genome and induces a nick between 3′-AA and TTTT in the complementary strand (Jurka, 1997; Gilbert et al., 2005). It has been proposed that first-strand DNA is synthesized by target site-primed RT, in which the poly(A) tail of L1 mRNA anneals to the poly-T stretch of nicked genomic DNA (Kazazian, 2004; Babushok and Kazazian, 2007; Goodier and Kazazian, 2008). ORF1 and ORF2 complete the entire process of L1-RTP and also transpose other elements, such as *Alu* and *SVA* (SINE–VNTR–Alus: short interspersed element, SINE; variable number of tandem repeats, VNTR; and Alu; Dewannieux et al., 2003; Wallace et al., 2008; Hancks and Kazazian, 2012; Raiz et al., 2012), indicating that L1 functions as a driving force of genome shuffling.

L1-RTP IN SOMATIC CELLS IS INDUCED BY ENVIRONMENTAL FACTORS

REPORTED INDUCERS

The environmental triggers, gamma irradiation (Farkash et al., 2006), and heavy metals (El-Sawy et al., 2005; Kale et al., 2005), such as mercury, cadmium, and nickel, have been shown to induce L1-RTP. Although it was proposed that L1-RTP induced by nickel occurs at the post-transcriptional level (El-Sawy et al., 2005), the precise mode of induction of L1-RTP remains to be clarified. Stribinskis and Ramos (2006) also reported that benzo[*a*]pyrene (B[*a*]P) induced L1-RTP. They found that B[*a*]P-induced L1-RTP depended on the aryl hydrocarbon receptor (AhR). Interestingly, however, it was shown that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), a well-known ligand of AhR (Beischlag et al., 2008; McIntosh et al., 2010), did not induce L1-RTP. In a proposed mechanism, ligand-bound AhR activates expression of metabolic enzymes encoded by cytochrome P450 (*CYP*) genes, which in turn convert B[*a*]P into a genotoxic compound that induces L1-RTP (Stribinskis and Ramos, 2006). By contrast, TCDD is not a genotoxic compound, and does not induce L1-RTP.

NEWLY IDENTIFIED ENVIRONMENTAL FACTORS AND NOVEL MODES OF L1-RTP

We recently discovered that 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan photoproduct and a putative physiological AhR ligand (Wincent et al., 2009), induces L1-RTP (Okudaira et al., 2010). Experiments using short-interfering RNA (siRNA) along with an AhR inhibitor revealed that the L1-RTP induced by FICZ was not dependent on AhR, but required AhR nuclear translocator-1 (ARNT1; Hoffman et al., 1991). Chromatin recruitment of ligand-bound AhR is dependent on the ARNT1 nuclear localization signal (Eguchi et al., 1997). Moreover, it was also shown that addition of FICZ initiated the molecular interaction between ARNT1 and ORF1, and promoted the recruitment of ORF1 to the chromatin-rich fraction (Okudaira et al., 2010). Given that there have been no reports indicating that ARNT1 by itself functions as a receptor for environmental compounds, it is likely that a novel cellular factor that functions as an FICZ receptor cooperates with ARNT1 in the induction of L1-RTP.

Further studies demonstrated that dimethylbenzoanthracene (DMBA), B[*a*]P, and 3-methylchoranthrene (3-MC) induced L1-RTP (Okudaira et al., 2011; Ishizaka et al., 2012). Interestingly, the results of siRNA-based experiments revealed that the induction of L1-RTP by these carcinogens depended on AhR. Moreover, the induction of L1-RTP by DMBA required ARNT1, whereas L1-RTP induced by B[*a*]P and 3-MC did not. Notably, *ARNT1* siRNA blocked mRNA expression of the CYP gene *CYP1A1*. Additionally, it has been ascertained that the expression of *CYP1A1* mRNA depends on a heterodimer of AhR and ARNT1 (AHRC, AhR complex). These observations indicate that *ARNT1* siRNA efficiently attenuated the biological function of the AHRC, confirming that the induction of L1-RTP by B[*a*]P and 3-MC was independent of ARNT1. Moreover, the expression of *CYP1A1* mRNA in response to environmental factors requires the chromatin recruitment of ligand-bound AhR, which is dependent on the ARNT1 nuclear localization signal (Eguchi et al., 1997), lending support to the notion that a cellular factor, the function of which was similar to that of ARNT1, could contribute to chromatin recruitment of ORF1. Since AhR forms a complex with estrogen receptor α (ERα; Ohtake et al., 2003), we examined whether ERα was involved in induction of L1-RTP. Notably, experiments using *ERα* siRNA indicated that L1-RTP induced by both B[*a*]P and 3-MC depended on ERα (Ishizaka et al., 2012).

It has been shown that B[*a*]P increased the expression of L1 mRNA (Stribinskis and Ramos, 2006), whereas nickel chloride did not (El-Sawy et al., 2005). We also observed no apparent increase in L1 transcripts with FICZ (Okudaira et al., 2010). These observations suggest that the modes of L1-RTP triggered by environmental compounds are regulated differently at the transcriptional and post-transcriptional levels. It is conceivable that the cellular machinery that functions at the post-transcriptional level is regulated differently depending on the compound (**Table 1**). Although further study is required, the data support the idea that genotoxic carcinogens induce L1-RTP in an AhR-dependent manner. Moreover, the modes of L1-RTP induced by environmental compounds were different, suggesting that the integration sites of L1 differ depending on the trigger. We will discuss this possibility further later (**Figure 1**).

MITOGEN-ACTIVATED PROTEIN KINASES ARE REQUIRED FOR RTP INDUCED BY ENVIRONMENTAL COMPOUNDS

In mammalian cells, six groups of MAPKs – extracellular signal-regulated protein kinase1/2 (ERK1/2), ERK5, JNK, p38, ERK3/4,

Table 1 | Summary of cellular factors required for L1-RTP by environmental compounds.

Cellular factors	Inducers			
	FICZ	B[*a*]P	3-MC	DMBA
AhR	–	○	○	○
ARNT1	○	–	–	○
ERα	N.T.	○	○	–
MAPKs				
SB202190	○	○	○	–
SP600125	○	○	–	–

○, dependent; –, independent; N.T., not tested.
The induction of L1-RTP was examined by a PCR-based assay.

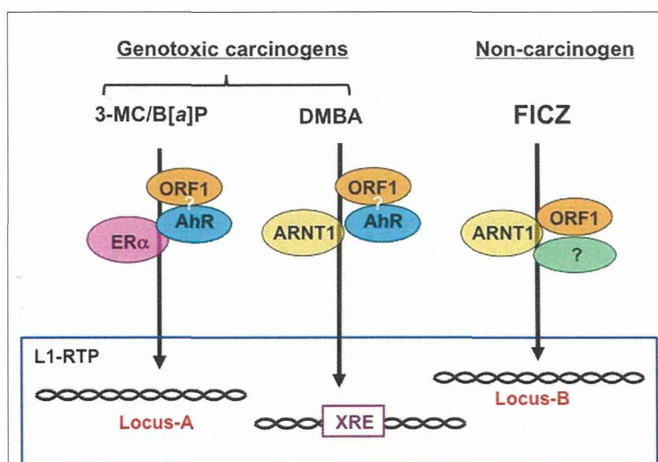

FIGURE 1 | Schematic modes of L1-RTP triggered by environmental compounds. Given that ORF1, which associates with retroelements, is recruited to the chromatin, regions of L1-RTP are likely determined by the binding partners of ORF1. As shown in **Table 1**, the induction of L1-RTP by environmental compounds depended on different sets of bHLH/PAS proteins. The induction of L1-RTP by DMBA depended on AhR and ARNT1, whereas that by 3-MC and B[*a*]P required AhR. Interestingly, L1-RTP by 3-MC and B[*a*]P did not require ARNT1, but depended on ERα. By contrast, FICZ-induced L1-RTP depending on ARNT1, whereas it did not require AhR. It is plausible that the regions where these compounds insert L1 are different: DMBA induces L1-RTP in the vicinity of XRE, whereas 3-MC and B[*a*]P induce L1-RTP in the region determined by AhR and ERα. The locus of L1-RTP by FICZ would differ from those determined by these genotoxic carcinogens. To prove this, it is necessary to identify the association of ORF1 and AhR and genome regions where L1 is inserted in response to each environmental compound.

and ERK7/8 – have been identified and shown to be activated by intracellular and extracellular stimuli (Cargnello and Roux, 2011). Among these MAPKs, the cellular signal cascades activated by ERK1/2, p38, and JNK have been well characterized because of the availability of inhibitors. PD98059, SB202190, and SP600125 are inhibitors of ERK1/2, p38, and JNK, respectively. To examine the involvement of MAPKs in the induction of L1-RTP, the effects of these inhibitors on the induction of L1-RTP have been analyzed. SB202190 blocked FICZ-induced L1-RTP, and siRNA specific for cyclic-AMP responsive element binding protein (*CREB*) mRNA efficiently attenuated FICZ-induced L1-RTP (Okudaira et al., 2010). Additionally, FICZ-induced phosphorylation of CREB in an ARNT1-dependent manner, and SB202190 blocked the FICZ-induced phosphorylation of CREB and chromatin recruitment of ORF1. These observations verified that p38 is required for the induction of L1-RTP by FICZ at the step of chromatin recruitment of ORF1. Further analysis on the mode of L1-RTP induced by 3-MC and B[*a*]P revealed that the L1-RTP induced by 3-MC was coupled to CREB phosphorylation, and that both *CREB* siRNA and SB202190 abrogated L1-RTP induction by the compound. The effects of MAPK inhibitors are summarized in **Table 1**. The next challenge is to determine how MAPKs and the phosphorylation of MAPK substrates are involved in the induction of L1-RTP. One plausible role of these molecules is driving the chromatin recruitment of ORF1 or retroelements in response to environmental compounds (Chiu and Greene, 2008). Several studies suggest that retroelements, such as Alu, are present in a cytoplasmic high-molecular weight complex due to the function of APOBEC3 proteins, an innate restriction molecule (Chiu and Greene, 2008).

FUNCTIONAL INTERACTION OF bHLH/PAS PROTEINS AND L1-RTP

BIOLOGY OF bHLH/PAS PROTEINS

The bHLH/PAS family is composed of numerous transcriptional factors with PAS domains consisting of approximately 275 amino acids. Family members have sequence homology to the clock gene *period* (*per*) from *Drosophila melanogaster*, which is involved in the control of circadian rhythms; the *arnt* gene in mammals, which is required for signaling pathways activated in response to dioxin or polyaromatic hydrocarbons; and the *single-minded* (*sim*) gene, a neurodevelopmental regulator in flies (Beischlag et al., 2008; McIntosh et al., 2010). It is interesting to note that the bHLH/PAS family is involved in these three apparently different biological responses. The PAS domain has two sub-domains, PAS-A and PAS-B, each of which comprises approximately 70 amino acids. The PAS-A domain functions as a binding site for other PAS-A domain-containing proteins (McIntosh et al., 2010). In contrast, the PAS-B domain is involved in interactions with other classes of proteins, such as heat shock protein 90 and small molecules. One of the best-characterized functions of bHLH/PAS proteins is in the response to environmental pollutants, such as polyaromatic hydrocarbons (PAH; Beischlag et al., 2008). When PAH binds to the PAS-B domain of AhR, a heterodimeric complex of ligand-bound AhR and ARNT1 (AHRC) is formed. AHRC is then recruited to chromatin, where it recognizes a xenobiotic responsive element (XRE; Probst et al., 1993) and activates the expression of genes encoding xenobiotic metabolic enzymes (Beischlag et al., 2008). Interestingly, the AHRC also activates expression of AhR repressor (AhRR), which has both bHLH and PAS-A domains, but not a PAS-B domain. It has been proposed that the expression of AhRR down-regulates the activity of the AHRC by a feedback mechanism (Mimura et al., 1997).

CHROMATIN RECRUITMENT OF ORF1 IS DEPENDENT ON bHLH/PAS PROTEINS

Experiments involving the forced expression of a plasmid DNA encoding *ORF1* cDNA revealed that ORF1 is present in cytoplasmic stress granules in ribonucleoprotein complexes (Hohjoh and Singer, 1996; Goodier et al., 2007). Given that ORF1 acts *in cis* on L1-mRNA, it is conceivable that ORF1 is recruited from the cytoplasm to chromatin. Consistently, we observed that FICZ-induced enrichment of ORF1 in the chromatin-rich fraction and also triggered the physical association of ORF1 and ARNT1 (Okudaira et al., 2010). Additional experiments revealed that carcinogen-induced L1-RTP was dependent on AhR (Stribinskis and Ramos, 2006; Okudaira et al., 2011). These observations led us to hypothesize that ORF1 is functionally associated with bHLH/PAS proteins, and that both bHLH/PAS proteins and MAPK are involved in the chromatin recruitment of ORF1.

The importance of bHLH/PAS proteins in L1-RTP suggests that L1-RTP in the genome induced by environmental compounds may be directed. As summarized in **Table 1**, environmental carcinogens induce L1-RTP in an AhR-dependent manner. By contrast, FICZ-induced L1-RTP was not dependent on AhR. Instead, it requires ARNT1 and an additional cellular factor. Interestingly, the dependence of carcinogen-induced L1-RTP on ARNT1 differed among compounds: L1-RTP induced by DMBA required ARNT1, whereas that induced by 3-MC and B[*a*]P depended on AhR and ERα. These observations suggest that DMBA induces L1-RTP in the vicinity of XRE, which the AHRC targets for induction of mRNA expression of genes, such as *CYP* genes. In contrast, 3-MC and B[*a*]P would induce L1-RTP in the genome region determined by AhR and ERα. Moreover, the region in which FICZ-induced L1-RTP occurs differs from that in which carcinogen-induced L1-RTP occurs (**Figure 1**). It is important to analyze the regions of the newly integrated L1 to obtain novel information on the roles of L1-RTP in carcinogen-induced genetic alternations.

L1-RTP IS LINKED TO THE DEVELOPMENT OF VARIOUS DISEASES

GENETIC ERRORS

L1-RTP accidentally disrupts structures of functional genes and gives rise to inborn errors (Goodier and Kazazian, 2008). Since the discovery of aberrant insertions of L1 in the gene encoding factor VIII in 2 of 240 sporadic hemophilia A patients (Kazazian et al., 1988), at least 20 genetic disorders have been identified as resulting from L1 insertional mutagenesis (Hancks and Kazazian, 2012). Including genetic alterations by *Alu* and *SVA*, 96 genetic disorders have been so far identified (Hancks and Kazazian, 2012). In addition, abnormal L1 insertion was detected in a patient with branchio-oto-renal syndrome, in which L1 disrupted the locus responsible for the disease (Morisada et al., 2010). Of note, a recent study of 18 unrelated patients with neurofibromatosis type 1 (*NF1*) identified insertions of 14 *Alu*, three L1, and one poly(T) stretch within the *NF1* gene, indicating the retrotransposon insertions account for ~0.4% of all *NF1* mutations (Wimmer et al., 2011). Importantly, inserted L1, which was responsible for *NF1* disruption, was Ta-1, which is the youngest subfamily of human L1 (Boissinot et al., 2000; Lee et al., 2007; Wimmer et al., 2011). Moreover, six different insertions were identified within the 1.5-kb region between exons 21 and 23. As the *NF1* gene is 280 kb in length, it was proposed that the insertions of endogenous retroelements into the *NF1* gene occurred non-randomly (Wimmer et al., 2011).

TUMORIGENESIS

During early studies of the alterations of cancer-related genes, aberrant L1 insertions in the c-*myc* and *APC* genes were found in breast and colon carcinoma, respectively (Morse et al., 1988; Miki et al., 1992). It was recently proposed that L1 functions as a natural mutagen for genetic alterations, and L1-RTP has been detected in lung carcinomas and pancreatic adenocarcinomas (Iskow et al., 2010; Ting et al., 2011). Interestingly, L1-RTP is associated with the expression of major satellite repeats and malignant tumor phenotypes (Ting et al., 2011). L1-RTP induces a variety of genetic alternations, including gene deletions, inversions, and inter/intra-chromosome translocations (Gilbert et al., 2002, 2005; Symer et al., 2002). Moreover, ORF2 induces DNA damage (Gasior et al., 2006). In addition to these direct effects of L1, the human genome is susceptible to genetic alterations by *Alu–Alu* non-allelic homologous recombination (NAHR; Belancio et al., 2010; Konkel and Batzer, 2010). For example, it was shown that *Alu–Alu* NAHR induced structural alterations in the tumor suppressor gene *BRCA1*, and the *MLL-1* and *Myb* genes, in cancers cells (Strout et al., 1998; Mazoyer, 2005; O'Neil et al., 2007). Notably, the frequency of deletion via *Alu–Alu* NAHR under conditions of p53 deficiency was 40–300 times higher than that observed in the presence of wild-type p53 (Gebow et al., 2000), suggesting the importance of *Alu–Alu* NAHR during multistep carcinogenesis (Hanel and Moll, 2012). Environmental stimuli possibly function as risk factors for carcinogenesis by modulating genetic alternations via L1-RTP.

Although there is no model system to evaluate the association of L1-RTP with tumorigenesis, we recently showed that L1-RTP was common in skin tumors induced by DMBA and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Balmain et al., 1984; Okudaira et al., 2011). Of 15 skin tumors, 13 were positive for L1-RTP. It has been proposed that DMBA induces *H-ras* activation (Nelson et al., 1992), whereas TPA promotes the growth of transformed cells in a manner depending on TPA susceptibility locus (*Psl*) that is located on chromosome 9 (Angel et al., 1997). Recently, glutathione *S*-transferase α4 was identified as a candidate *Psl* gene (Abel et al., 2010). Interestingly, it was recently reported that activation of the *H-ras* gene in normal cells activates an ATM-dependent growth-arrest signal (Bartkova et al., 2006; Di Micco et al., 2006). Since ATM-dependent signaling causes senescence, an additional genetic alteration is required for tumor development; otherwise, tumor growth would be prevented. It remained elusive to clarify how TPA-induced L1-RTP is linked with the functional modification of the *Psl* gene product and attenuation of the ATM-dependent growth-arrest signal. Although it remains unclear whether L1-RTP is really involved in tumorigenesis, DMBA/TPA-induced skin carcinogenesis would be a good animal model for clarification of the role of L1-RTP in tumor development.