

# Identification of novel loci for the generation of reporter mice

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## ABSTRACT

**Deciphering the etiology of complex pathologies at molecular level requires longitudinal studies encompassing multiple biochemical pathways (apoptosis, proliferation, inflammation, oxidative stress). *In vivo* imaging of current reporter animals enabled the spatio-temporal analysis of specific molecular events, however, the lack of a multiplicity of loci for the generalized and regulated expression of the integrated transgenes hampers the creation of systems for the simultaneous analysis of more than a biochemical pathways at the time. We here developed and tested an *in vivo*-based methodology for the identification of multiple insertional loci suitable for the generation of reliable reporter mice. The validity of the methodology was tested with the generation of novel mice useful to report on inflammation and oxidative stress.**

## INTRODUCTION

The next frontier of biological research resides in the creation of reliable tools to investigate the dynamics of interaction among multiple biochemical pathways in living organisms. This is now possible by the combined application of genetic engineering with the use of appropriate reporters (such as radiolabeled, bioluminescent or fluorescent molecules) facilitating the *in vivo* imaging of molecular events.

Prototypes of ‘reporter animals’ (i.e. organisms engineered to carry an easily measurable, surrogate molecule able to portray specific biological events) have demonstrated their significant investigational powerfulness (1), yet more work is required to lead to the generation of more performant models where the ubiquitous expression of multiple reporters may give the visual representation in real time

of the changes and interactions of several biochemical pathways in response to physiological, pathological or environmental stimuli at systemic level. The knock-in integration of a reporter gene downstream of the selected promoter (2–5) has been the methodology of choice to measure *spatio-temporally* the expression of the genes of interest; however, with this method the disruption of the gene in study often perturbs cell metabolism with phenotypic consequences, particularly when the animal is homozygous. More importantly, the knowledge of the dynamics of expression of a single gene seldom gives the necessary insights into biological processes relevant for patho-physiological studies (i.e. inflammation, oxidative stress, cell death). This latter experimental need has been successfully addressed with the creation of ‘path activity reporters’ characterized by the use of a reporter transcriptionally regulated by the transducers of relevant biological processes such as nuclear receptors, NFY, Nrf2 and others (6–12). Ideally, we could generate ‘path activity reporters’ for the investigation of the activity of multiple molecular events at the same time facilitating tremendously physio-pathological studies. For instance, cancer and cancer drugs could be studied in animals where proliferation and apoptosis could be measured at the same time and in different tissues or the understanding of molecular basis of neurodegenerative disorders would advance significantly by analyzing oxidative stress and apoptosis (6). Unfortunately, the key element to obtain this ‘path activity reporters’ requires the availability of genomic loci where the expression of the integrated reporter is ubiquitous and inducible in all the cells of the organism of interest.

This aspect has significantly hampered the expansion of this field because the number of mammalian loci enabling the constitutive and regulated expression of exogenous transgenes is still very limited. At present time the loci most extensively exploited for the generalized expression of a transgene are the *hrpt* and *rosa26* (13,14); both shown to suffer of shortcomings. The *hrpt* gene, located on the X

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chromosome is subject to random X-inactivation, thus, the expression of the integrated gene is guaranteed only in homozygous females; in addition, in mouse tissues like kidney and liver the activity of promoters in this *locus* is low or undetectable (14,15). So far, the *rosa26* (Gt(ROSA)26Sor) is considered as the best available site for the ubiquitous expression of transgenes, but the generation of 'path activity reporters' demands the availability of a multiplicity of reliable sites for the ubiquitous and regulated expression of the transgenes.

To overcome current shortage of *bona fide* permissive loci, we applied a systematic approach aimed at finding and testing novel integration sites for the ubiquitous and regulated expression of exogenous, homologous and heterologous genes. Taking advantage of *in vivo* imaging, we here describe a novel work process that facilitates the identification of suitable *loci*. In addition, we demonstrate the efficacy of such methodology with the full characterization of two of the numerous integration sites identified and the generation of two novel reporter mice.

## MATERIALS AND METHODS

### Reagents

Primers were synthesized by Primm srl (Milan, Italy).

### Cells culture and transient transfections

All cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in media containing streptomycin–penicillin (50 000 U plus 50 mg/l) in a humidified 5% CO<sub>2</sub>-95% air atmosphere at 37°C. RAW264.7 cells were grown in red-phenol free Dulbecco's minimal essential medium (DMEM)-10% DCC serum (DCC) (EuroClone, Milan, Italy) supplemented with 2 g of sodium carbonate per liter, 0.11 g of sodium pyruvate per liter; MCF-7 cells were grown in ATCC-formulated Eagle's Minimum Essential Medium with 0.01 mg/ml bovine insulin (Euroclone, Milan, Italy), fetal bovine serum to a final concentration of 10% (EuroClone, Milan, Italy); NIH-3T3 cells were grown in ATCC-formulated DMEM plus bovine calf serum to a final concentration of 10% (EuroClone, Milan, Italy).

For the transfection, RAW264.7 and MCF-7 cells were seeded in a 24-well plates (100.000 cells/well) and cultured overnight prior transfection. For NIH-3T3 cells, we seeded 25.000 cells/well. All transfections were carried out using FUGENE HD (Roche, Milan, Italy), following the manufacturer's instructions using a DNA: FUGENE HD *ratio* of 2:6.

### Animal treatments

All animal experimentation was carried out in accordance with the ARRIVE and European Guidelines for Animal Care. All animal experiments were approved by the Italian Ministry of Research and University and controlled by a Departmental panel of experts.

The animals were fed ad libitum and housed in individually ventilated plastic cages within a temperature range of

22–25°C, relative humidity of 50% ± 10% and under an automatic cycle of 12 h light/dark (lights on at 07:00 am).

For the lipopolysaccharide (LPS) study, NFκB-*luc2* mice were injected i.p. at indicated time and doses with LPS (LPS from *Escherichia Coli* L4130 Sigma Aldrich, St. Louis, MO 63103, USA) or vehicle (PBS). For the NaArsenite (ASN) study the dose administered i.p. to the ARE-*luc2* mice, was 12.5 mg/kg of ASN (Sodium (meta) arsenite S7400 Sigma Aldrich, St. Louis, MO 63103, USA) or vehicle (PBS).

### Design and identification of a promoter for ubiquitous expression of luciferase

The promoter sequence of *UBC-luc2*, *mUBC-luc2*, Enhanced-Cytomegalovirus-*UBC-luc2* (ECMV-*UBC-luc2*) plasmids was cloned, using standard cloning procedures, in pGL4.10 vector (Promega Corp., Madison, WI, USA):

- i) A total of 1219-bp DNA fragment encoding *UBC* was amplified with Phusion Enzyme (Phusion®) High-Fidelity DNA Polymerase, Euroclone, Milan, Italy) from genomic DNA extracted from MCF-7 cell line, using this primers:  
-1225-f-EcoRV 5'- gatatctatccaccgctcccgggtgcagc-3'  
-6-r-HindIII 5'- aagctttggtggcaacaaaaagccaaaaac-3'  
and cloned into the EcoRV/HindIII restriction sites.
- ii) Minimal *UBC* was amplified from *UBC-luc2* plasmid with Phusion Enzyme using this primers:  
-844-f-EcoRV 5'- gatatctttggtgatcgtgtgatcgtcac-3'  
-6-r-HindIII 5' - aagctttggtggcaacaaaaagccaaaaac-3'  
and cloned into the EcoRV/HindIII restriction sites.
- iii) ECMV was amplified from pGL4.50 plasmid (Promega Corporation Madison, WI, USA), with Phusion Enzyme using this primers:  
225-f-XhoI 5'- ctcgagcgcgttataacttacgg-3'  
632-r-EcoRV 5'- gatatcccaaaacaaactcccattgac-3'  
and cloned in *UBC-luc2* in XhoI/EcoRV restriction sites.

The plasmid for the initial blastocyst injections (random integration), *mUBC-luc2* plasmid was cloned in XhoI/SalI restriction sites in pgk-HPRT plasmid (kindly provided by PolyGene AG, CH).

### NFκB-loxP-STOP1x-loxP-*luc2*-ires-TdTomato design and generation

In the NFκB-loxP-STOP1x-loxP-*luc2*-ires-TdTomato vector each functional cassette was flanked with unique restriction sites to facilitate further manipulations. Each element was sequentially cloned in the pGL4.10 vector (Promega Corp., Madison, WI, USA) using standard cloning procedures.

- i) the NFκB responsive promoter was obtain as previously described (16) first by cloning into AseI/SalI restriction sites of pBluescript II KS+ (Stratagene, Agilent Technologies, Santa Clara, USA) four NFκB responsive elements identified by bioinformatic analysis and named 2A, 2B, 1A and 1B. Each element was synthesized by Eurofins Genomics, Ebersberg Germany as

a dimer. Then the 220-bp DNA fragment of NF $\kappa$ B responsive elements and TATA minimal promoter was excised and cloned into the EcoRV/SnaBI restriction sites in the pGI2 basic vector (Promega Corp., Madison, WI, USA).

- ii) the 3185-bp loxP-STOP1x-loxP fragment was excised from LSL-TOPO plasmid (Addgene, USA cat number 11584) and cloned in the SalI restriction site. To obtain STOP1X we performed a partial digestion with the MfeI restriction enzyme.
- iii) the 641-bp DNA fragment encoding internal ribosome entry site (IRES) element was excised from pIRES vector (Clontech Laboratories, Inc., 1290 Terra Bella Ave. Mountain View, CA 94043, USA) and cloned into the XbaI/FseII sites.
- iv) the 1430-bp TdTomato sequence was excised from pRSET-B tdTomato plasmid (kindly provided by Prof. R. Tsien's lab), and cloned into the XhoI/EcoRV site.

Once obtained, the NF $\kappa$ B-loxP-STOP1x-loxP-*luc2*-ires-TdTomato plasmid was cloned in BstBI/XhoI site in the pTargeting-19 plasmid.

In the pTargeting-19 each element was sequentially cloned, using standard cloning procedures, in the pFlrt vector (kindly provided by CFCM- San Raffaele – DIBIT- Milan, Italy):

- i) the 3' homology region (line 19) was amplified from genomic DNA isolated from embryonic stem (ES) cells v6.4 using these primers:  
3HR\_2231\_xma.f 5'-cagcccggtctctctcttcttcttcttcttccagag-3'  
3HR\_4230\_not-sal.r 5'-aatgtcgacgcccgcagccacagagagtatgtgggaaga-3'  
and cloned in SalI/XmaI restriction sites;
- ii) a single copy of MAR (3-kb DNA fragment) from chicken lysozyme gene was excised with digestion of the pBSKMAR, kindly provided by L.Hennighausen (17) and cloned in the NheI/Claf in place of neomycin cassette in pFlrt vector;
- iii) a second copy of MAR was cloned in the KpnI/XhoI site;
- iv) the 5' homology region (line 19) was amplified from genomic DNA isolated from ES cells v6.4 using these primers:  
5HR-f\_AatII\_Not 5'-atgacgtcggccgccagggttaagccagtaagatggagcca-3'  
5HR-r\_Mlu 5'-atacgcgtgaaggaaggaaggggaaaggcctacagctcag-3'  
and cloned in AatII/MluI restriction sites.

#### ARE-loxP-STOP1x-loxP-*luc2*-ires-TdTomato design and generation

The ARE-loxP-STOP1x-loxP-*luc2*-ires-TdTomato vector was cloned simply by substituting the promoter sequence of the NF $\kappa$ B-loxP-STOP1x-loxP-*luc2*-ires-TdTomato vector with antioxidant responsive element (ARE) promoter sequence.

ARE was obtained by cloning four selected Nrf-2 responsive elements repeated twice into the XhoI/XmaI site of

pBluescript II KS+ (Stratagene, Agilent Technologies, Santa Clara, USA). These responsive elements had been identified by bioinformatic analysis and synthesized by Eurofins Genomics, Ebersberg, Germany. Then the 325-bp DNA fragment of ARE responsive element and the TK minimal promoter were excised and cloned into the XhoI/XmaI of the NF $\kappa$ B-loxP-STOP1x-loxP-*luc2*-ires-TdTomato vector.

#### ES manipulation: random integration of the *mUBC-luc2-pgk-HPRT*

The plasmid *mUBC-luc2-pgk-HPRT* was linearized with NotI, the transgene was separated from plasmid sequences and electroporated into embryonic stem cells 129Ola. The ES cells were grown on murine embryonic fibroblasts in DMEM-N supplemented with 15% fetal bovine serum, 0.1 mM 2-mercaptoethanol and 2 mM L-glutamine. Transfected cells were selected in the hypoxanthine-aminopterin-thymidine (HAT) medium (0.016 mg of hypoxanthine per ml/0.0.1 mM aminopterin/0.0048 mg of thymidine per ml) for around 10 days, at which time individual colonies were picked for expansion. A total of 86 clones were obtained and pooled (3 pools of 10 clones each and 7 pools of 8 clones each). Each pool was injected in C57Bl/6 blastocysts and then implanted in the oviducts of *in vitro* culture-held foster mice. The resulting chimeric mice were crossed to C57Bl/6 to generate the F1.

#### ES manipulation: homologous recombination of NF $\kappa$ B and ARE plasmids

The targeting vector ARE/NF $\kappa$ B-targeting-19 was linearized with NotI and transferred into sv6.4 embryonic stem cells by electroporation: 35  $\mu$ g/DNA each using 15 million cells (Core Facility for Conditional Mutagenesis, DIBIT San Raffaele, Milan, Italy). Positive clones were selected with puromycin (1  $\mu$ g/ $\mu$ l). More than four hundred resistant clones for each transgene were screened for homologous recombination by PCR. One out of six positive clones in the case of NF $\kappa$ B and one out of eight positive clones in the case of ARE, were injected into C57Bl/6NCRl blastocysts which are transferred to pseudo-pregnant CD-1 females. We obtained two chimeric male mice (with 80–90% of chimerism) that were mated to wild-type (WT) C57Bl/6NCRl female mice to produce F1 transgenic mice.

#### *In vivo* and *ex vivo* imaging

*In vivo* imaging: for the semi-quantitative analysis of photon emission, animals were injected i.p. with 80 mg/kg of luciferin (Beetle Luciferin Potassium Salt; Promega, Madison, WI, USA) 15 min prior the imaging session. For the imaging, mice were anaesthetized using Isoflurane (Isoflurane-Vet; Merial, Lyon, France) and kept under anesthesia during the 5 min of the session carried out with a CCD-camera (IVIS Lumina II Quantitative Fluorescent and Bioluminescent Imaging; PerkinElmer, Waltham, MA, USA). Photon emission in selected body areas was measured, respectively, using the Living Image Software (PerkinElmer).

*Ex vivo* imaging: the selected organs were dissected from mice treated with luciferine 15 min prior euthanasia and

subjected to *ex vivo* imaging immediately after death. Imaging analysis was done by 5 min exposures of the tissue explants. Photon emission was quantified with the Living Image Software (PerkinElmer).

### Luciferase enzymatic assay

Organs were homogenized in lysis buffer and lysates were subjected to three cycles of freezing and thawing. Proteins were separated from DNA and lysosomes by centrifugation ( $13000 \times g$  for 30min), after having measured the protein concentration of the extract by the Bradford assays (18), the biochemical assay of Luciferase activity was carried out with a luciferase assay buffer (470  $\mu$ M luciferine, 20 mM Tricine, 0.1 mM EDTA, 1.07 mM  $(\text{MgCO}_3)_4 \cdot \text{Mg}(\text{OH})_2 \times 5\text{H}_2\text{O}$ ; 2.67 mM  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  in  $\text{H}_2\text{O}$ , pH 7.8, with 33.3 mM DTT and 530  $\mu$ M ATP) by measuring luminescence emission with a luminometer. The relative luminescence units (RLU) determined during a measurement of 10 s time was expressed as RLU per microgram protein. RLU were measured in all organs of WT mice; the average RLU measured was 35. The RLU measured in WT mice were routinely subtracted from the RLU measured in the TG mice.

### Site finder PCR

Mouse genomic DNA was extracted from the mice tails (19 and 21 mouse lines) in accordance with the manufacture instruction (EUROGOLD Tissue-DNA Mini Kit form Euroclone, Milan, Italy). The steps of site finder PCR were slightly modified from Tan *et al.* 2005 (19) as follows:

- i) SiteFinder annealing: after low temperature priming by a Site Finder, one strand of the target gene was replaced by Phusion Enzyme (Phusion<sup>®</sup> High-Fidelity DNA Polymerase, Euroclone, Milan, Italy) that generated double-stranded target molecules of different lengths;
- ii) exponential amplification and nested PCR: the target DNA was exponentially amplified by nested PCR with gene specific primers 1, 2 and 3 (GSP) and SiteFinder primers 1 and 2 (SFP);
- iii) cloning target sequence: the PCR products (generated by SFP2-GSP3 and SFP2-GSP2) were loaded on agarose gel, the bands with expected molecular weight were excised, purified and cloned in the pCRII-TA vector (Thermo Fisher Scientific, Waltham, MA 02451, USA);
- iv) screening and sequencing: the clones were screened by colony-PCR with GSP3 and SPF2 and the positive clones were sequenced (Primm srl, Milan, Italy).

Site finder 5'- cagcagacgctactcaacacaccactcgcacagcgtcctcaagcggccgcNNNNNNgcct-3'

SPF1 5'- cagcagacgctactcaacac-3'

SPF2 5'- actcaacacaccactcgcacagc-3'

GSP1 5'- cgagcagacatgataagatacatt-3'

GSP2 5'- ggacaacacacaactagaatgagtg-3'

GSP3 5'- tcattttatgtttcaggttcagggg-3'

### Immunohistochemical analysis

ARE-*luc2* mice were sacrificed and perfused transcardially with cold saline solution followed by 4% paraformaldehyde in PBS. Brains were immediately removed, post-fixed in the 4% paraformaldehyde fixative for 24 h and then transferred in solutions of sucrose at increasing concentrations (up to 30%) during the following 72 h. Samples were then frozen and stored at  $-80^\circ\text{C}$  for successive analyses. Serial coronal sections of 40  $\mu$ m were cut throughout the brain using a freezing sliding microtome (Leica SM 2000R) and stored at  $-20^\circ\text{C}$  in a solution containing 30% ethylene glycol, 20% glycerol and 0.05 M sodium phosphate buffer until use. The slide-mounted sections were rinsed in PBS and incubated in PBS containing 10% NGS and 0.3% TX-100 at room temperature. Sections were then incubated at  $4^\circ\text{C}$  in PBS/1% NGS/0.3% TX-100 containing a rabbit polyclonal anti-RFP antibody (diluted 1:400; Abcam, Cambridge, UK). After a 24 h incubation sections were rinsed in PBS and incubated 1 h at RT in PBS/1% NGS containing the Alexa Fluor 594 conjugated goat anti-rabbit IgG antibody (1:300; Molecular Probes, Carlsbad, CA, USA). Finally, sections were rinsed in PBS and covered with Prolong with DAPI (Molecular Probes, Carlsbad, CA, USA).

### Real-time PCR

Total RNAs were extracted after tissue homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA) as suggested by the manufacturer's instructions. For the preparation of cDNA, 1  $\mu$ g of RNA was denatured at  $75^\circ\text{C}$  for 5 min in the presence of 1.5  $\mu$ g of random primers (Promega) in a 15  $\mu$ l final volume. Deoxynucleotide triphosphate (GE Healthcare) and Moloney murine leukemia virus reverse transcriptase (RT; Promega) were added at 0.5 mM and 8 U/ $\mu$ l final concentration, respectively, in a final volume of 25  $\mu$ l. The RT reaction was carried out at  $37^\circ\text{C}$  for 1 h; the enzyme was inactivated at  $75^\circ\text{C}$  for 5 min. For each sample control reactions were done routinely omitting the addition of the reverse transcriptase. A 1:4 cDNA dilution was amplified using SYBR green chemistry. The qPCR was carried out in triplicate on a 96-well plate using GoTaq<sup>®</sup>qPCR Master Mix technology (Promega) according to the manufacturer's protocol with a 7900HT fast real time PCR system (Applied Biosystems, Life Technologies) with the following thermal profile: 2 min at  $95^\circ\text{C}$ ; 40 cycles, 15 s at  $95^\circ\text{C}$ , 1 min at  $60^\circ\text{C}$ . The following primers were used:

36B4: forward 5'-ggcgacctggaagtccaact-3', reverse 5'-ccatcagcaccacagccttc-3';

Enah: forward 5'-gctaagccccatcaacaag-3', reverse 5'-aggtgtgattgggtctgg-3';

Srp9: forward 5'-acgatgcctcagttccagac-3', reverse 5'-gtcttgccgttggtctgttc-3';

Seph2: forward 5'-gacagcccggatcattgaca-3', reverse 5'-cgagttccaacaaccgcta-3';

Itga1: forward 5'-cgtgtcatctcttccctcg-3', reverse 5'-atagccagctctcgtttccg-3';

Il6: forward 5'-ctggatataatcaggaaatttgctt-3', reverse 5'-tgaggtaggaaggactattttatgt-3';

Il1 $\beta$ : forward 5'-tgccacctttgacagtgatg-3', reverse 5'-gctgcgagattgaaagctgg-3';

Cxcl2: forward 5'-tgaacaaaggcaaggctaactgacc-3', reverse 5'-acgatccaggcttcccgggtg-3';

Nrf2: forward 5'-cccagcaggacatggattg-3', reverse 5'-agctcatagctcttctgtcgc-3';

The data were analyzed using the ABI Prism 7000 SDS Software and the  $2^{-\Delta\Delta C_t}$  method or  $\Delta C_t$  using 36b4 as the housekeeping gene.

### Statistical analysis

Was done by ANOVA followed by Bonferroni's test for multiple comparisons (Graph Pad 5 software).

## RESULTS

The strategy we pursued was based a series of sequential steps aimed at identifying *loci* permissible for expression in all tissues; the second had the goal to systemically test the suitability of the *loci* identified for the regulated and generalized expression of a reporter driven by a minimal, inducible promoter. To pursue such an experimental design, we identified three steps: (i) electroporation of ES cells with a construct expressing constitutively a non-mammalian reporter protein of easy detection under the control of a strong, ubiquitous promoter; (ii) bioluminescence (BLI)-based imaging for the insertional screening in cells and then in animal lines to select the mice where the integrated gene was expressed *spatio-temporally* at relatively high level and with the least possible variability; (iii) identification of the new *loci* and testing of their efficacy by the integration of specific reporter genes flanked by insulators sequences to best shield the exogenous transgene from position effects and to ensure that the reporter can be fully regulated by the given transcription factor.

### Electroporation of ES cells with a construct expressing constitutively a non-mammalian reporter

To secure the highest probability to identify the positive ES clones and animal lines we needed a reporter suitable for *in vivo* imaging driven by a promoter ensuring the constitutive, unrestricted, high expression of the reporter. For the reporter, we selected the *luc2* (20), a firefly luciferase engineered to generate a luminescence 4- to 5-fold greater than the native enzyme. This reporter was considered most appropriate for an efficient identification of the positive ES clones after electroporation, but, more importantly, for the rapid quantitative and qualitative analysis of the reporter expression in living animals. To design the promoter first we took into consideration the cytomegalovirus (CMV) promoter generally considered of choice and we generated a construct containing the CMV (Human cytomegalovirus immediate early promoter derived from pGL4.50 plasmid from Promega Corporation cod E1310) (Figure 1A, 1); however, based on our initial experience corroborated by data in the literature we did not feel that this promoter would have guaranteed the ubiquitous expression of the reporter (21–24), therefore, we contemplated the use of a mammalian ubiquitous promoter and, based on an extensive literature search, we selected the human ubiquitin C gene (NG\_027722.2, Gene ID: 7316). We generated three

constructs, the first containing the sequence -1225 to -6 of the human *UBC* promoter as indicated by Schaefer *et al.* (25) for the generation of mice with widespread expression of the reporter together with the CMV enhancer (from 225 to 632 bp of CMV derived from pGL4.50 plasmid from Promega Corporation cod E1310) (Figure 1A, 2); the second the *UBC* promoter alone (Figure 1A, 3) and the third (Figure 1A, 4), a *UBC* promoter shorter version (minimal *UBC*, *mUBC*) where most binding sites for regulatory transcription factors were maintained as indicated by an unbiased sequential informatics search (Transfect®6.0; Al-iBaba 2.1 and Patch 1.0) (Figure 1B). These constructs were transfected in parallel in three different cell lines: NIH-3T3; RAW264.7 and MCF-7 and their efficiency in the production of luciferase was compared with the *ERE-Luc* reporter shown to generate a bioluminescence signal sufficient for *in vivo* imaging by prior studies (1). Figure 1C shows that the extent of *Luc2* synthesis was of the same order of magnitude for all constructs tested; this led us to select the smallest of them *mUBC-luc2* because the efficiency of the electroporation process is known to be inversely proportional to the dimension of the transgene.

### BLI-based imaging for the insertional screening in animal lines

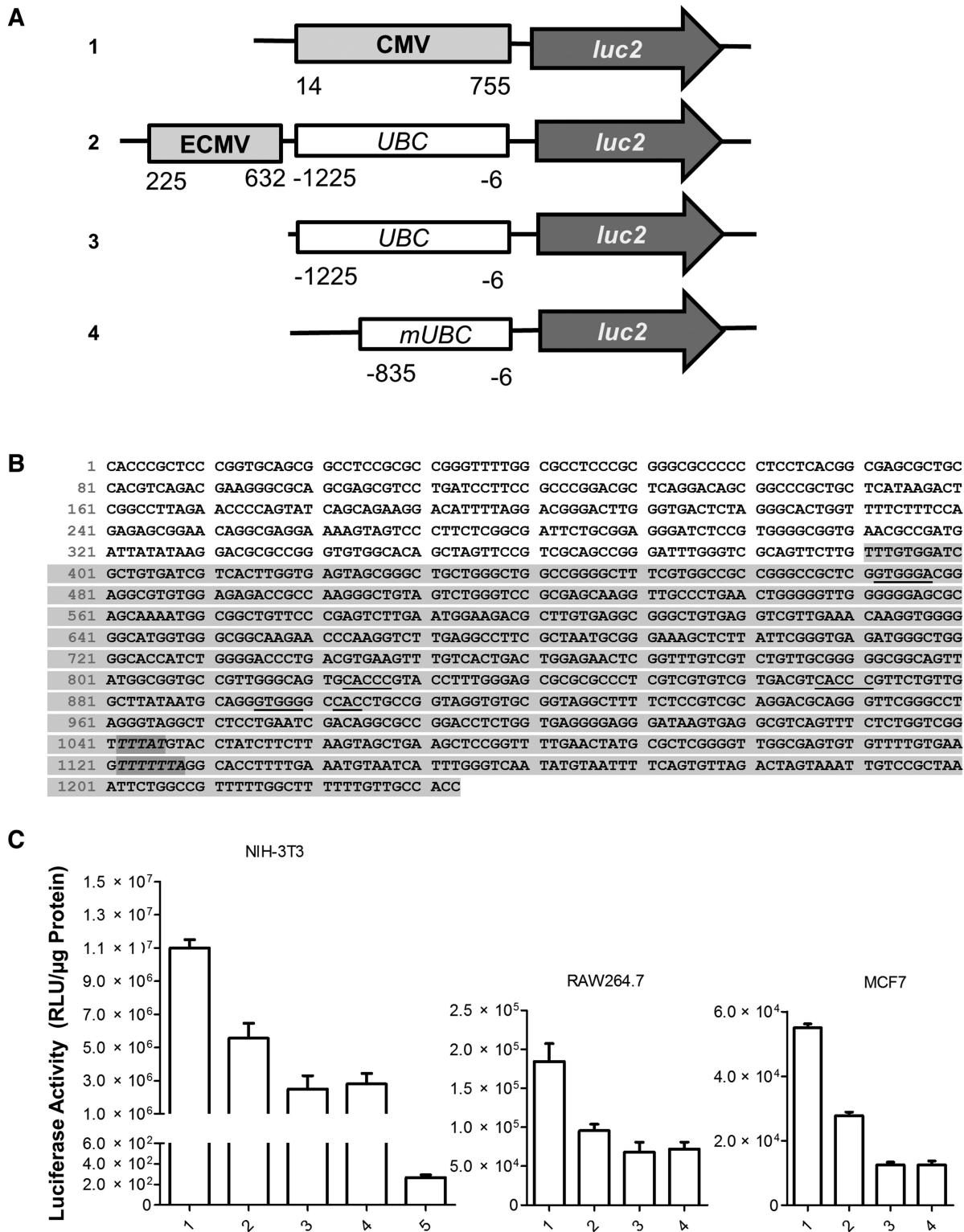
After electroporation, the 129Ola ES cells were selected in the HAT medium; we obtained a total of 86 positive clones. To reduce the number of injections into blastocysts we pooled the positive clones (3 pools with 10 clones each and 7 with 8 clones). Each pool was injected in a C57Bl/6 blastocyst to produce a founder that was bred into C57Bl/6. Each of the F1 mice had the potential to originate multiple transgenic mouse lines; therefore, we needed a robust methodology to identify the mouse lines in which the transgene was expressed ubiquitously, trans-generationally, at all stages of life. This process would not have been possible without the aid of *in vivo* imaging that allowed us to measure the expression of the reporter in space and time.

Based on the above assumption the criteria for the 'insertional screening' we set up as follows: (i) widespread presence of BLI, (ii) intensity and stability of BLI during the entire life span; (iii) reproducibility of BLI expression in the progeny; (iv) sex-independence of BLI expression.

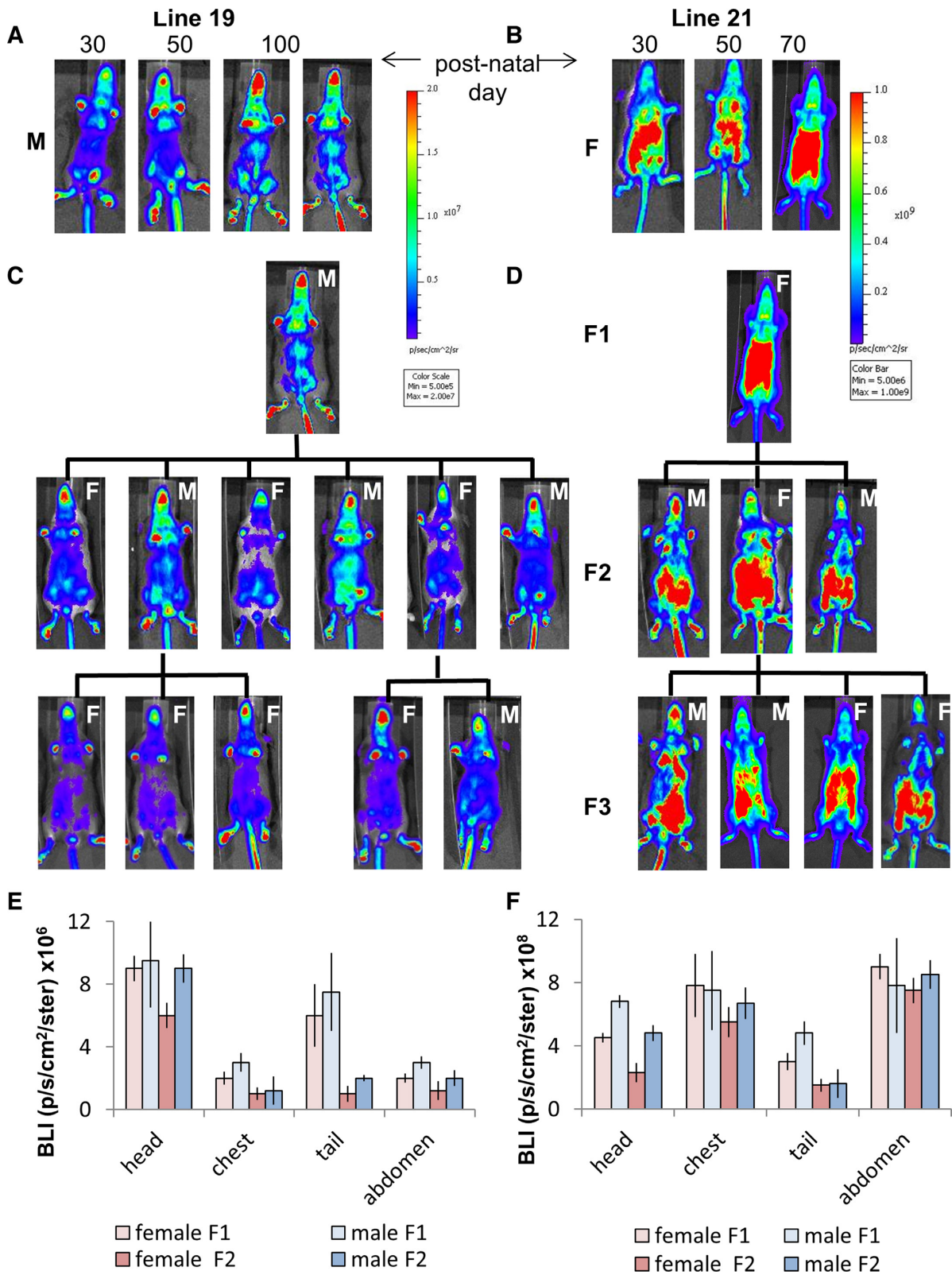
The number of animal lines that met the criteria for the insertional screening stated above were 22 (Supplementary Figure S1) and all these animal lines showed single integration of the transgene, as demonstrated by quantitative analysis of *luc2* DNA content by real time PCR (data not shown) and as expected after ES cells electroporation (26). In each of these lines the pattern of BLI expression was different: this was the predictable outcome of a random integration of the transgene.

### Identification of the new, permissive integration loci

To ensure that the screening done had led to the identification of permissive *loci* we continued the study on 2 of the 22 lines available: lines 19 and 21 where the luciferase was found to have the same pattern of expression from birth to maturity in male and females for at least three generations



**Figure 1.** Identification of a eukaryotic promoter for the ubiquitous expression of the luciferase reporter. (A) Schematic representation of plasmid vector tested: (1) CMV-*luc2* (pGL4.50 plasmid from Promega Corporation cod E1310); (2) ECMV-*UBC-luc2* in which ECMV derived from cytomegalovirus (CMV) (position 225–632) plus the promoter of human Ubiquitin C (position –1225 to –6 with respect to the start site), (3) promoter of human Ubiquitin C (position –1225 to –6 with respect to the start site), (4) *mUBC* minimal promoter of the human Ubiquitin C (position –835 to –6 with respect to the start site). (B) DNA sequence of human Ubiquitin C promoter region, the minimal region of Ubiquitin C used to generate *mUBC-luc2* vector is highlighted and the binding sites for general transcription factors were indicated with following code: light grey CAC binding site; grey TATA binding site as defined by Transfect 6.0; AliBaba 2.1 and Patch1.0. (C) Transient transfection of the constructs shown in (A) in three cell lines: NIH-3T3, RAW264.7 and MCF7, plus the plasmid ERE-*luc* (lane 5) The transfections were standardized by co-transfecting the plasmid CMV- $\beta$ -gal. Data are expressed as luciferase activity (RLU/ $\mu$ g of total proteins), bars show means  $\pm$ SEM (n = 6). The experiment was repeated twice with superimposable results.



**Figure 2.** *In vivo* analysis by bioluminescence (BLI) of luciferase expression in males and females of lines 19 and 21: F1–F3 generations. Pseudocolor images of each individual mouse were obtained 15 min after the injection of 80 mg/kg luciferin with 5 min exposition time for line 19 and 3 mg/kg luciferin with 1 s exposition time for line 21. (A and B) *In vivo* imaging of a single, representative mouse indicates the pattern of luciferase expression of F1 mice at different post-natal days. The mice analysed in total were 6; (C and D) BLI of adult male (M) and female (F) F1, F2 and F3; (E and F) Quantification of photon emission from indicated body areas or regions of interest (ROIs). Data are expressed as BLI bioluminescence (photon/cm<sup>2</sup>/s/ster), mean ± SEM (n = 6).

(Figure 2). Next, lines 19 and 21 were bred to homozygosity to ascertain that the mice carrying the transgene in both alleles were viable, fertile, had normal size and not obvious phenotypic alterations.

In the homozygous mice, the generalized expression of the reporter was confirmed *ex vivo* in 24 organs isolated from anesthetized mice injected i.p. with luciferin 15 min prior of euthanasia. Figure 3A–E shows that in both lines, 19 and 21, all the organs tested expressed the integrated reporter. However, the extent of LUC2 expression varied significantly. In line 19, the expression measured in thymus and testis was about 15-fold higher than in liver, muscle, spleen and lung. In line 21, the reporter expression was generally higher than in line 19 and the organs with the highest expression (liver and muscle) showed a content of LUC2 about 100-fold higher than heart and brain where the expression was lowest (Figure 3C). What observed has to be ascribed to position effects as the transgene was not flanked by insulators (8). Indeed, in the absence of insulators even the expression of reporters integrated in the *locus Rosa26* is quite variable (27). In fact the integration in this *locus* of Enhanced Green Fluorescent Protein (EGFP) reporter under the control the chicken  $\beta$ -actin promoter had an inter-organ variability of about 35-fold in the 9 organs tested and the cytomegalovirus promoter did not show ubiquitous EGFP expression (27).

Next, we proceeded with the identification of the site of integration in the two transgenic line using a methodology previously described (28) and illustrated in Supplementary Figure S2.

In both mouse lines the transgene integration had occurred in noncoding, intergenic DNA regions with unknown function. More in detail, line 19 integration mapped in chromosome 1, 10 208 bp 5' of the *Enah* gene (enable homolog isoform 1) and 94 173 bp 3' of the *Srp9* (signal recognition particle 9kDa protein) (Figure 3D). The integration site of line 21 was in chromosome 7, 5445 bp 5' of *Sephs2* gene (seleno phosphate synthetase 2) and 16 886 bp 3' of *Itgal* (integrin alpha 11) (Figure 3E). To ensure that in the two lines the transgene integration did not interfere with the activity of the proximal genes we measured their expression in WT and transgenic lines. Supplementary Figure S3 shows that the integration did not significantly affected the expression of the 4 genes 5' and 3' of the integration site: *Enah*, *Srp9*, *Sephs2* and *Itgal*.

### Generation of mice with a generalized and regulated expression using the newly identified locus

To finally provide the necessary proof of concept that the methodology we had developed had indeed delivered novel integration sites for the ubiquitous and regulated expression of transgenes we tested locus 19 by generating two novel reporter mice in which the reporter was driven by different promoters to ensure that the expression of the reporter dependent on the promoter used and not simply associated with the locus utilized. Thus, we conceived two mice model: one for the study the activity of the inflammatory path (NF $\kappa$ B-*luc2*) and the second for the oxidative stress (ARE-*luc2*).

As previously stated (6), the ideal 'path reporter' should provide a reliable measure of the process in study in all organs and cells of the organism independently of the sex and stage of development or age. As a consequence, the expression of the reporter should be strictly dependent from the desired stimulus and occurring in all responsive organs at all stages of development of the animal.

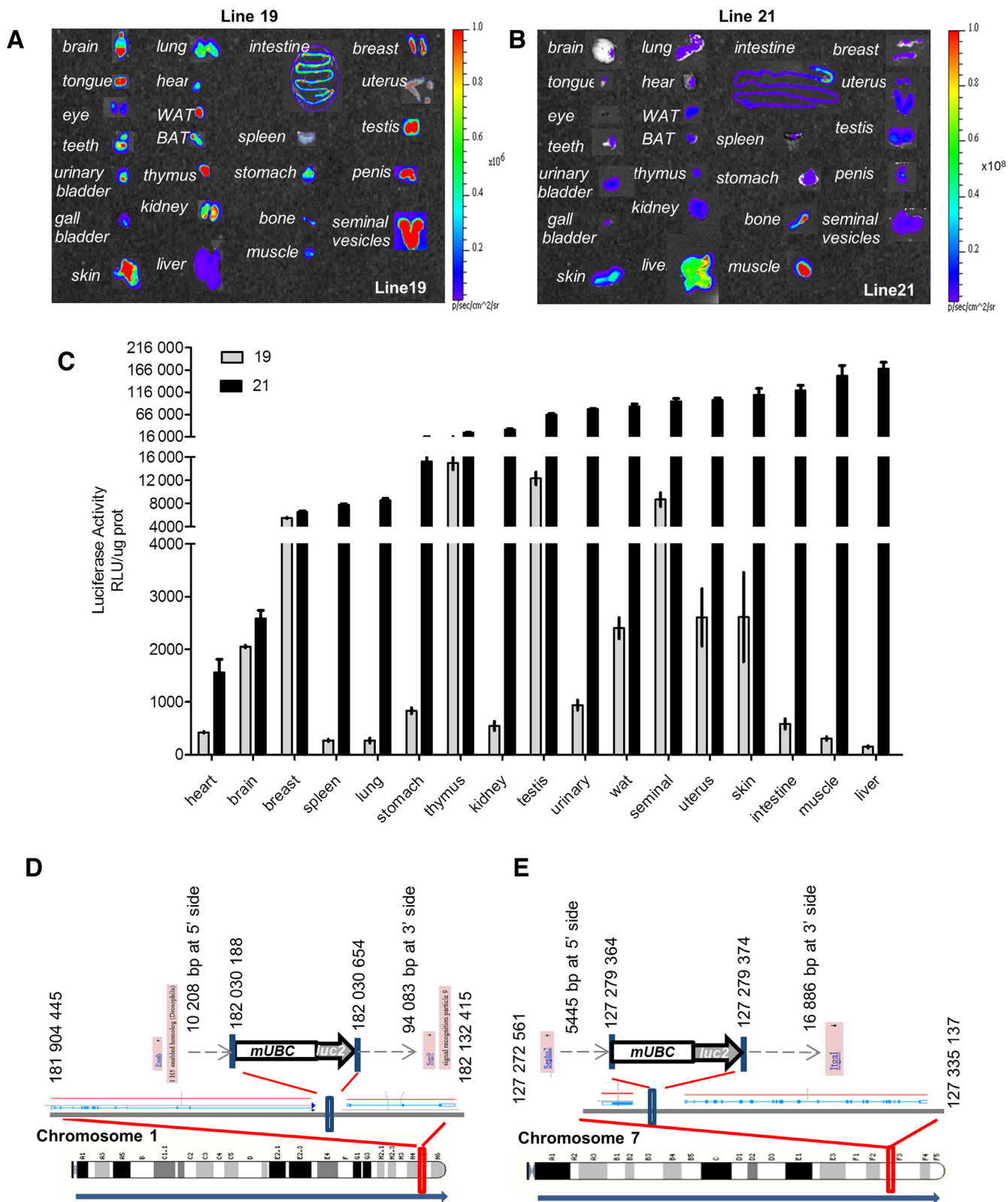
To test the two *loci* we therefore generated a construct with: (i) multiple reporter systems to enable *in vivo* studies as well as *ex vivo* analysis of the expression of the reporter at the cellular level; (ii) cre-Lox sequences for the selection of the tissues where the transgene is expressed and, in addition (iii) to further decrease the probabilities of position effects we also decided to flank the reporter constructs with insulator sequences (refer European patent No. EP 1298988B1; US patent No. 7 943 81 (Matrix attachment Regions, MAR)).

As reporters we selected a bicistronic system conceived to contain a gene for *in vivo* BLI (*luc2* gene) and a gene encoding a protein ideal for *ex vivo* studies at single cell level (td-Tomato, a monomeric red fluorescent protein derived from the *Discosoma* sp. red fluorescent protein) separated by an IRES.

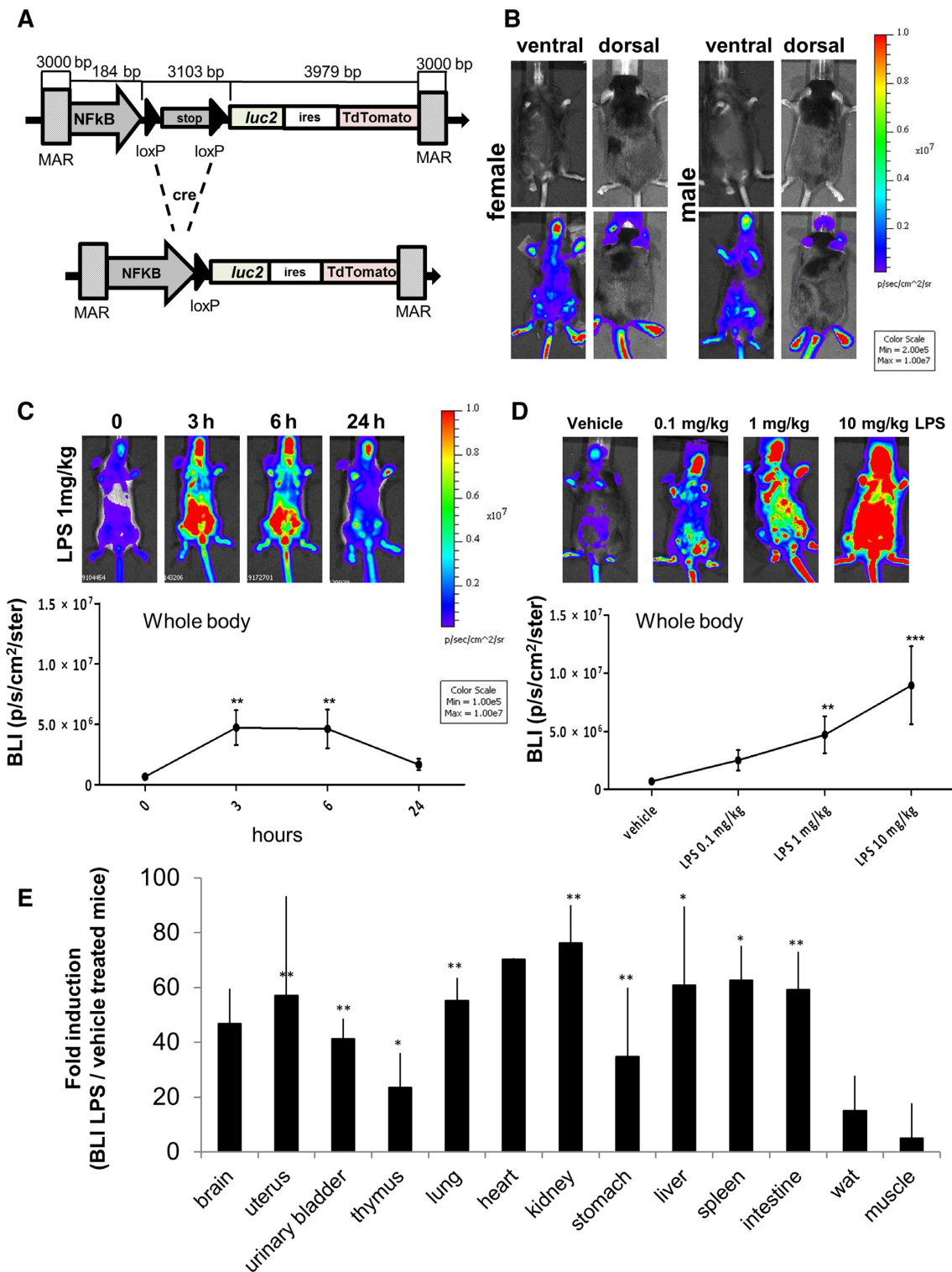
For the tissue specific expression of the reporter, we used the cre-loxP system integrating a floxed STOP sequence between promoter and the bicistronic reporter (29) (Figure 4A). To limit the dimension of the cassette, we tested the efficiency of different multimers of the original STOP sequence (RNA PolIII termination signal) proposed by Lasko *et al.* (30) and demonstrated by transient transfection, that a monomer of the STOP sequence was sufficient to completely block the activity of PolIII (Supplementary Figure S4).

To generate the mouse to study the activation of the inflammatory process, we used a promoter cassette developed in prior experiments that had led us to identify a sequence regulated by the NF $\kappa$ B TF in cells of the innate immunity selectively; such promoter contained a synthetic stretch of DNA in which the 4 NF $\kappa$ B-binding motifs was repeated twice (16). Such a promoter was associated with the construct above described (Figure 4A) and used for the generation of the reporter mouse by homologous recombination into the line 19 locus. The mice carrying the transgene did not expressed luciferase (Figure 4A) before they were crossed with the B6.C-Tg(CMV-cre)1Cgn/J mouse expressing the Cre recombinase in germ cells (31). Further demonstration of the tightness of the floxed STOP sequence was obtained with the quantitative analysis of photon emission of selected organs dissected from mice prior (NF $\kappa$ B-STOP-*luc2*) and after the breeding with B6.C-Tg(CMV-cre)1Cgn/J mice (NF $\kappa$ B-*luc2*). Prior removal of the STOP sequence the expression of the reporter was in the order of thousands p/s/cm<sup>2</sup>/ster, after the removal was at least one order of magnitude higher (Supplementary Figure S5A). In both male and female NF $\kappa$ B-*luc2* mice, the basal expression of the reporter appeared to be generalized (Figure 4B). A time-course study with a low dose of pro-inflammatory molecule LPS showed that the reporter reached the maximal activity at 3–6 h and was back to the basal level at 24 h after induction (Figure 4C). Most relevant, however, was the response of the reporter to increasing concentrations of





**Figure 3.** Identification of the integration *loci* of lines 19 and 21. (A and B) Representative images that indicate the intensity of photon emission in organs explanted from mice of line 19 and 21. The total mice analyzed were 4. Mice were injected with of 80 mg/kg and 3 mg/kg luciferin, respectively, for line 19 and line 21 and tissues were explanted 15 min after the luciferine injection and the exposition time at the CCD was 5 min. (C) Luciferase activity measured in tissue extracts and expressed as relative luciferase units (RLU) in mice of the lines 19 and 21. The relative luminescence units (RLU) determined during a measurement of 10 s is expressed as RLU–RLU measured in corresponding *w.t.* tissues per microgram protein. Data are expressed as mean  $\pm$  SEM (n = 4). (D and E) Graphical representation of line 19 (chromosome 1) and line 21 (chromosome 7) *loci*.



**Figure 4.** Regulation of luciferase reporter activity in the NFκB-*luc2* mouse. (A) Schematic representation of the plasmid vector utilized to generate NFκB-*luc2* mouse prior and after excision of STOP sequence. (B) Ventral and dorsal luciferase emission of NFκB-*luc2* mice (female and male) before (upper panel) and after (lower panel) breeding with the B6.C-Tg(CMV-cre)1Cgn/J. (C) *In vivo* BLI: time-course luciferase expression in NFκB-*luc2* mice after injection of 1 mg/kg lipopolysaccharide (LPS) (i.p.). BLI is measured ventrally, whole body and expressed as photon emission (p/s/cm<sup>2</sup>/sr). The image is representative of 4 individual animals. In the graph, each point corresponds to mean ± SEM (n = 4). (\*\*) *P* < 0.01 versus 0 h; (\*\*\*) *P* < 0.001 versus 0 h. (D) *In vivo* BLI: dose-response luciferase expression in NFκB-*luc2* mice after 6 h of i.p. injection of increasing doses of LPS. BLI is expressed as photon emission (p/s/cm<sup>2</sup>/sr), and the image is representative of four individual animals. In the graph, each point corresponds to mean ± SEM (n = 4). (\*\*) *P* < 0.01 versus 0h (\*\*\*) *P* < 0.001 versus 0 h. (E) Photon emission measured in 13 organs dissected from NFκB-*luc2* mice 6 h after i.p. injection of LPS (1 mg/kg). Each bar corresponds to ratio between BLI measured from four treated mice versus BLI measured from four vehicles. mice (n = 4). (\*) *P* < 0.05 versus vehicle; (\*\*) *P* < 0.01 versus vehicle (\*\*\*) *P* < 0.001 versus vehicle.

LPS. The results in Figure 4D showed a strict correlation between the dose of LPS administered and whole body BLI. To better evaluate the ubiquitous expression of the reporter we carried out an *ex vivo* analysis on 13 organs explanted 6 h after 1 mg/kg i.p. injection of LPS. The fact that not all tissues expressed the same amount of luciferase was ascribed to differences in the presence of cells for the innate immunity and in the distribution of i.p. injected LPS (e.g. brain) (Figure 4E). To demonstrate that the activation of the reporter paralleled that of target genes, we also compared the expression of luciferase and with three NF $\kappa$ B endogenous target genes. Supplementary Figure S6A shows that *Il1 $\beta$*  and *Il6* were induced in lung (27-fold and 63-fold, respectively) and uterus (34-fold and 95-fold, respectively) more than in adipose tissue (17-fold and 33-fold, respectively), this was closely reflected by the action of the reporter gene that was induced in lung (53-fold induction) and uterus (26-fold induction) more than in adipose tissue (15-fold induction) (Supplementary Figure S6B). Interestingly, another target of NF $\kappa$ B, *Cxcl2* was maximally induced in adipose tissue (390-fold induction) and less induced in uterus (98-fold induction) and lung (32-fold induction) (Supplementary Figure S6A).

For the generation of the reporter of oxidative stress we designed the promoter using the same strategy prior applied to the identification of appropriate ARE for the Nrf-2 transcription factor. Briefly, we analyzed a panel of 14 genes known to be involved in the oxidative stress pathway. Bioinformatic comparative analyses of the sequences recognized by the Nrf-2 factor (Supplementary Table S1) led us to select a synthetic promoter sequence with four ARE repeated twice (Figure 5A). Likewise NF $\kappa$ B-*luc2*, a floxed STOP codon was placed between promoter and bicistronic reporter; therefore, the reporters were expressed after breeding ARE-STOP-*luc2* with B6.C-Tg(CMV-cre)1Cgn/J mouse (Figure 5B and Supplementary Figure S5B). Injection of a well-known oxidant, ASN (12.5 mg/kg i.p. for 6 h) (32), demonstrated that expression of the reporter was appropriately regulated in the different body areas as indicated by *in vivo* and *ex vivo* studies (Figure 5C–E). The results in Figure 5E were in line with observations on the toxic effects of ASN where major effects were observed in urinary tract, respiratory system, gastro-intestinal apparatus, cardiovascular system, adipose tissue and renal system (33–35). We also measured *Nrf2* mRNA content in different tissues to investigate the extent to which *Nrf2* mRNA abundance reflected the activity of the reporter. In WT mice *Nrf2* resulted more expressed in lung and adipose tissue than in the uterus: this was in line with the extent to which the luciferase reporter was induced (Supplementary Figure S6C and D). ASN treatment affected differently the expression of these two endogenous, target genes: the change in *Homox1* mRNA induced by the treatment was very pronounced in muscle (12-fold), wat (9.5-fold) and lung (8.9-fold); for *Nqo1* gene was in lung (7.5-fold) and uterus (5.9-fold) (Supplementary Figure S7A). When we studied the effect of the treatment with ASN on the reporter we measured the highest induction in wat (15.3-fold), uterus (8.5-fold) and lung (7.6-fold). In these tissue the activation of the transcription factor was reported also with the study of the endogenous genes, how-

ever, we did not see activation of BLI in muscle reproducing what seen with *Hmox1*, but not *Nqo1*.

Finally, immunohistochemical analysis demonstrated that also the second reporter TdTomato was transcribed in the brain regions known to have a high oxidative potential. We selected the brain for this study because we wanted to test the selectivity of the reporter in a complex tissue where the regions where oxidation is very circumscribed, due to the high synthesis induced by the presence of dopamine. In fact Figure 5F shows that the reporter is mainly present in *pars compacta* of *substantia nigra* rich of dopaminergic neurons, while it is very little expressed in the *substantia nigra*, *pars reticulata* poor of dopaminergic neurons. Subsequent staining with anti-Nrf2 antibody showed that this transcription factor is highly expressed in the *pars compacta* but not in the *reticulata* (data not shown).

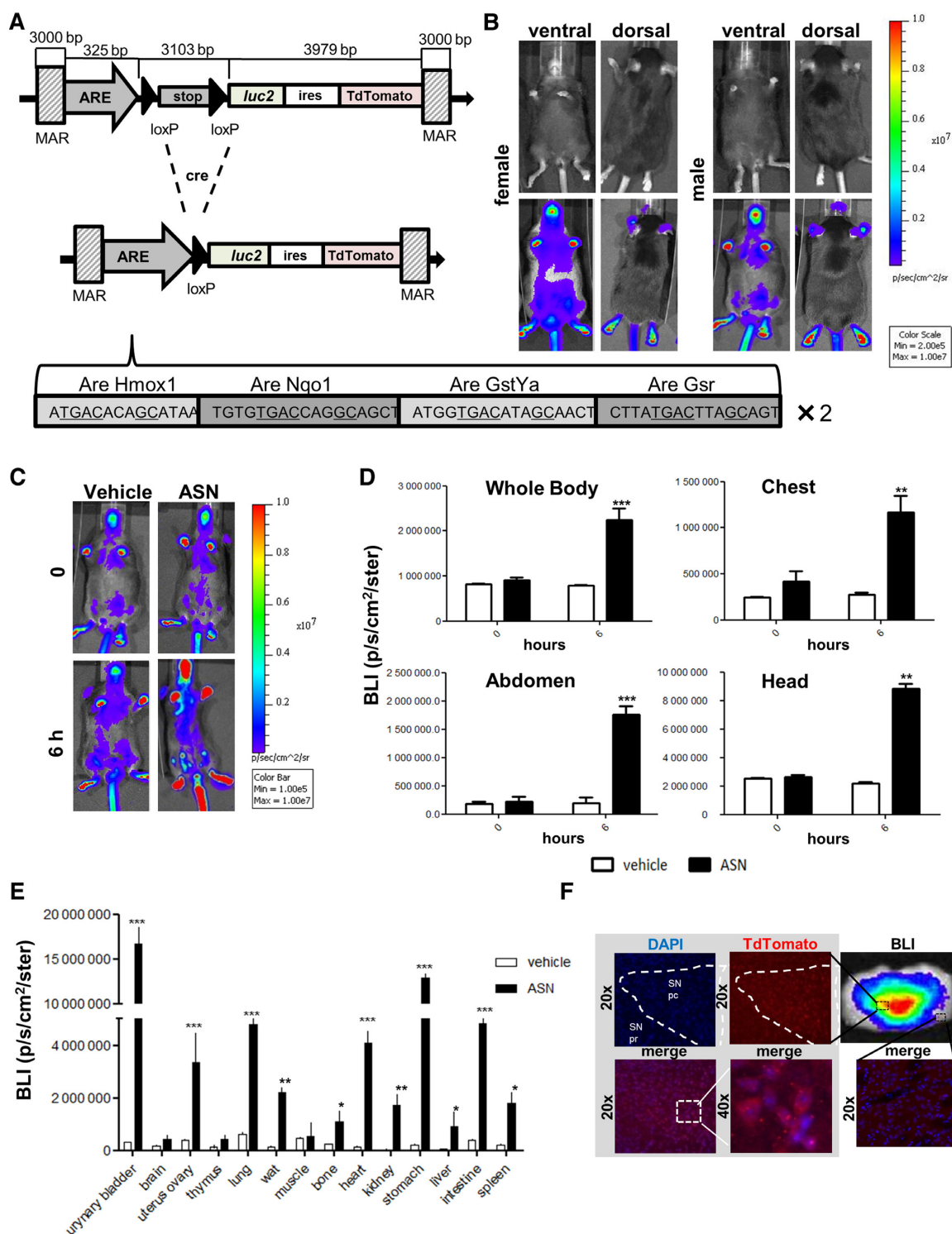
## DISCUSSION

We here developed and applied a systematic approach for the identification of permissive *loci* for the generation of ‘path reporter mice’ and tested its validity with the validation of one of the *loci* identified.

The key feature of such methodology is the use of *in vivo* imaging for the identification of the *loci* enabling the generalized and regulated expression of the integrated transgenes in mice at different developmental stages, age, sex and trans-generationally. The present study demonstrated the validity of the methodology set up with the generation of two novel reporter systems very valuable for the *spatio-temporal* study of the activity of two transcription factors master regulators of the inflammatory (NF $\kappa$ B) and the antioxidant and phase II detoxification responses (Nrf2). The NF $\kappa$ B-*luc2* reporter mouse was validated to show its *spatio-temporal* responsiveness to a strong inflammatory stimulus (LPS). In the validation procedure, we carried out a comparative analysis the extent of expression of luciferase and three endogenous genes after LPS administration. Each endogenous NF $\kappa$ B target showed a tissue-dependent sensitivity to LPS. This is not surprising because the transcription of those genes is under the control of complex promoters controlled by a multiplicity of transcription factors that act differently *spatio-temporally*. Thus, considering the expression of endogenous targets, it is difficult to obtain a conclusive indication of the extent of transcriptional activation of NF $\kappa$ B in each tissue. We believe that the use of a minimal, synthetic promoter best measures what is the state of activation of the TF in study because directly proportional to the TF action independently from other signaling molecules. Thus, this experiment further underlined the usefulness of the use of a surrogate reporter of TF activity as less susceptible than complex promoters to unpredictable influences able to enhance/suppress the activity of the TF on that promoter, but not on other targets.

We also developed appropriate vectors for the tissue specific expression of such transgenes and proved as successful the use of a shorter, monomeric version of the STOP signal proposed by Lasko *et al.* (30).

Thanks to the ever growing selection of fluorescent and bioluminescent reporter genes available, the identification of multiple integration sites is the key element for the rapid



**Figure 5.** Regulation of luciferase and TdTomato reporter activity in the ARE-*luc2* mouse. (A) Schematic representation of the plasmid vector and antioxidant responsive element (ARE) sequence utilized to generate ARE mouse prior and after excision of STOP sequence. (B) Ventral and dorsal luciferase emission of ARE-*luc2* mice (female and male) before (upper panel) and after (lower panel) breeding with the B6.C-Tg(CMV-cre)1Cg/J. (C) Activation of luciferase expression by the administration of ASN. *In vivo* bioluminescence imaging was done 6 h after i.p. injection of 12.5 mg/kg ASN. The image is representative of four individuals analyzed. (D) BLI measurement in selected body areas after ASN injection. Each bar corresponds to mean  $\pm$  SEM (n = 4), expressed as photon emission ( $p/\text{s}/\text{cm}^2/\text{sr}$ ). (\*\*\*)  $P < 0.001$  versus vehicle; (\*\*)  $P < 0.01$  versus vehicle. (E) Photon emission measured in 14 organs dissected from ARE-*luc2* mice 6 h after i.p. injection of ASN (12.5 mg/kg). Each bar corresponds to mean  $\pm$  SEM of tissues obtained four separate individual mice and BLI is expressed as  $p/\text{s}/\text{cm}^2/\text{sr}$ . (\*)  $P < 0.05$  versus vehicle; (\*\*)  $P < 0.01$  versus vehicle (\*\*\*);  $P < 0.001$  versus vehicle. (F) BLI and immunohistochemical analysis of the brain of the mouse reporter for the oxidative stress. Immunohistochemical analysis of brain slices at two magnification (20x and 40x): in blue DAPI staining, in red TdTomato protein and merging of the stainings showing cytoplasmic presence of TdTomato. SNpc substantia nigra pars compacta; SNpr substantia nigra pars reticulata. Images are representative of two animals analyzed separately.

creation of reliable pluripotent, multiplex reporter systems facilitating the *spatio*-temporal analysis of disparate biological processes. These novel systems will represent a significant step forward for our understanding of the molecular bases of complex and multifactorial pathologies. For instance, interbreeding three reporter systems (e.g. for inflammation, oxidative stress and neuronal death) in a model of neurodegeneration will clarify the causal relations of neuroinflammation and oxidative stress with neuronal death, or the use of a dual systems expressing reporters for apoptosis and proliferation will facilitate tremendously the screening of novel and more specific anti-cancer drugs.

We believe therefore that the methodology set up and the *loci* identified will significantly improve the development of the field.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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