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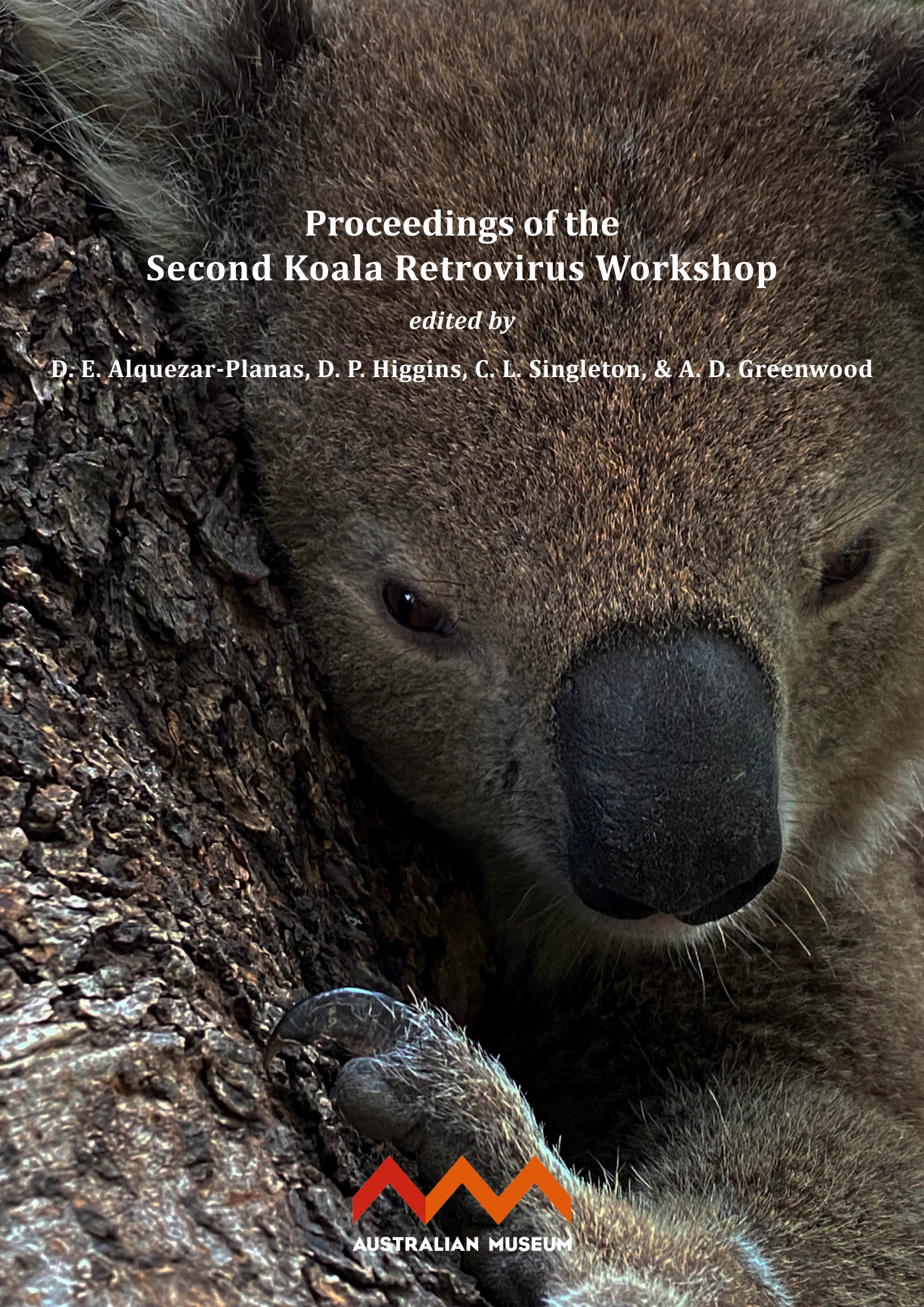

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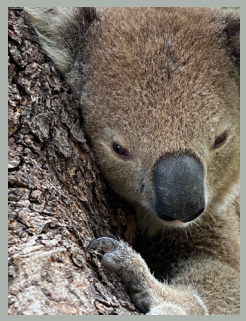

Cover photo
by Damien P. Higgins

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The Role of Koala Retrovirus Integrations in Promoting Neoplasia in Koalas (*Phascolarctos cinereus*)

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Abstract. Koalas suffer unusually high rates of neoplasia. There has been a long-standing correlation between koalas with the koala retrovirus (KoRV) and development of neoplasia which has lacked a mechanistic explanation. We describe recent results that demonstrate that many KoRV integrations lead to neoplasia by (*I*) inserting into somatic cells preferentially near oncogenes, (*II*) inserting into germ cells near oncogenes predisposing koalas to cancer, and (*III*) transduction—replacing KoRV genes with oncogenes, resulting in drastic upregulation of the transduced gene. The high mortality associated with integration-driven promotion of neoplasia may explain the increased prevalence of dysfunctional recombinant KoRVs (recKoRVs), which, over time, could replace KoRV, thereby slowing the production of novel detrimental integration sites.

Introduction

Koalas develop neoplasia at rates at least an order of magnitude higher than humans and many other mammals (Gonzalez-Astudillo *et al.*, 2019). Approximately 3% of wild koalas from southeast Queensland brought into veterinary clinics had lymphoma and 7% had lymphoid neoplasms (Gonzalez-Astudillo *et al.*, 2019; Fabijan *et al.*, 2020). This is likely an underrepresentation as it is unlikely that wild koalas manifesting late stage leukaemia or lymphoma will be found when they die. In contrast, in zoos where observation of koalas is continuous, 25% developed lymphoma (Gillett, 2014). This tremendous cancer burden has been associated correlatively with koala retrovirus (KoRV) infection, either as a quantitative increase of KoRV expression in koalas suffering from neoplasms or as an increase in the number of variants of KoRV expressed (Tarlinton *et al.*, 2005; Quigley *et al.*, 2019). A causative relationship and mechanistic explanation for the elevated cancer risks faced by koalas with KoRV has not been provided until now.

In general, there are four major mechanisms by which KoRV as a gammaretrovirus could cause cancer in koalas: I. insertional mutagenesis in somatic cells, II. non-lethal insertional mutagenesis in the germline in or near genes that promote the development of cancer later in life, III. transduction of oncogenes whereby the retrovirus incorporates an oncogene sequence in its genome and expresses it above the normal host rate, and IV. immune suppression by expression of the immunosuppressive domain in the envelope protein preventing host immune cell recognition and clearing of cancerous cells. We have recently provided evidence supporting a role for I-III (McEwen *et al.*, 2021). Ten paired tumour and neoplastic tissues were obtained from koalas from Queensland, Australia. Seven of the animals died or were euthanized because of advanced lymphoma. Three animals, all from one population, died of osteosarcoma. The integration site (IS) profile for each tissue sample and assorted positive and negative controls was determined using a novel long inverse PCR approach followed by large fragment high throughput sequencing

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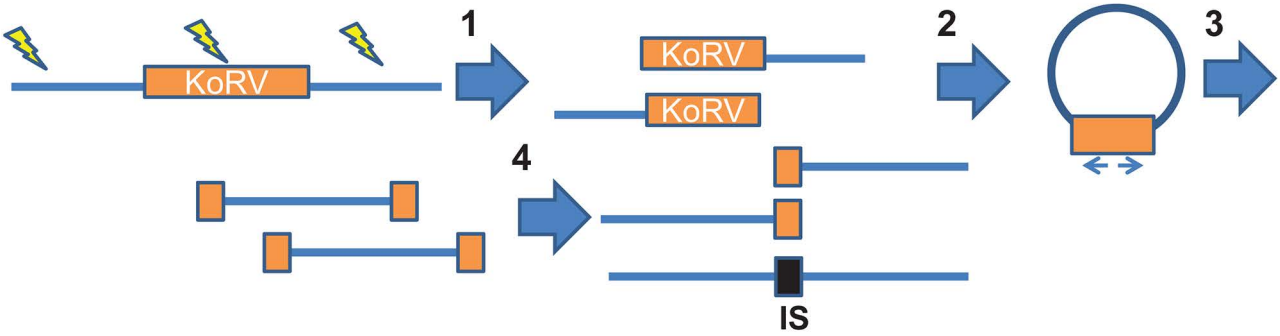

Figure 1. Summary strategy for comprehensive determination of integration sites by sonication inverse PCR (SIP). 1. DNA is randomly fragmented to an average size of 3 kb using high frequency sound waves. 2. The DNA molecules are turned from linear DNA into circular DNA. 3. Inverse PCR with primers based on the viral long terminal repeats (LTRs) are used to amplify fragments that extend partially into the virus and partially into the flanking sequence. 4. The resulting PCR products are sequenced on a long fragment high throughput sequencing platform and then mapped to the koala reference genome to identify the integration sites (Alquezar-Planas *et al.*, 2021).

(PacBio) called sonication inverse PCR (SIP) (Alquezar-Planas *et al.*, 2021). The end result of this sequencing is a comprehensive profiling of the endogenized KoRV IS in an average fragment size range of 2–3 Kb, yielding partial retroviral genome information at the 5' and 3' ends of the virus and several hundred to thousand base pairs of flanking sequence information. At higher sequencing depth, low coverage somatic cell integrations (which, unlike germline integrations, occur in only some cells of the body) can be detected (Fig. 1).

Mechanisms by which IS result in neoplasia

We detected 1002 unique IS among the 10 koalas. Of these 793 were endogenous retroviruses (ERVs), identified in both paired tissue types. There was an average of 100 KoRV ERVs per koala. The remaining IS were tumour specific (172) or healthy specific (37) representing somatic cell integrations. The vast majority of IS represented KoRV-A integrations and a smaller group of recKoRV integrations. KoRV-B and other variants were exceptionally rare among the individuals tested. In the neoplasms studied (also summarized in Fig. 2), KoRV IS were associated with the following mechanistic pathways:

I **Insertional mutagenesis**: The 172 tumour specific integrations identified were both associated with and enriched for genes known to be involved in the development of cancer (reaching statistical significance).

II **ERVs promoting cancer**: Of the ERVs identified, sharing of IS was directly correlated with spatial proximity to where the koala was sampled. Three koalas from one wild managed population (Lone Pine Sanctuary) all shared integrations in oncogenes associated with osteosarcomas, the type of cancer from which all three individuals died. Additional shared IS among the koalas in the data set were associated with oncogenes which may greatly increase the lifetime risk of developing cancer in a heritable manner. In both cases of somatic IS and ERV IS, multiple "hotspot" genes were identified where the same gene in several koalas had unique IS as either an ERV or a somatic integration. These were statistically significantly more likely to contain IS than other genes and the majority of these genes were known oncogenes such as c-myc and c-myb. In most cases, we observed that the IS near the various identified oncogenes promoted increased expression of these oncogenes, sometimes strongly increased above the level observed in koalas lacking such IS in the same tissues.

III **Transduction**: In one koala the KoRV LTRs and interrupted KoRV protein coding sequences flanked the *BCL2.1-xl* gene, a known promoter of invasion by various neoplasms (Trisciuoglio *et al.*, 2017). The oncogene interrupted most of the *env* gene of KoRV with the *gagpol* region experiencing deletions and interruptions by a small portion of the ZBTB18 gene. The result of this transduction was a greater than 500 fold increase in expression of *BCL2.1-xl*.

Somatic IS, ERVs and transduced oncogenes all resulted in increased expression of oncogenes in tumour tissues of affected koalas providing a molecular link between KoRV and cancer in koalas. This mutational excess likely puts enormous pressure on the koala host to purge deleterious KoRV IS from the population and on KoRV to attenuate. There is some evidence that this process is occurring and from a population genetic evolutionary perspective, quickly (Löber *et al.,* 2018).

KoRV recombinants (recKoRVs) and long term defence against novel IS

Most large-bodied species take several years to reach sexual maturity. In such species, neoplasms occurring before or during the optimal breeding years will likely be purged as individuals dying of cancer prior to having offspring, or having fewer offspring as a consequence, will be poorly or not represented in the subsequent generations. Thus, large-bodied long-lived species typically have ERVs which are highly degraded either by mutation, deletion or recombination preventing the expression of full-length viral transcripts or proteins (Katzourakis *et al.*, 2014). A class of recombinant KoRVs (recKoRVs) which replace much of the KoRV protein coding sequences with the highly disrupted genome of an ancient marsupial ERV (PhER) have been observed (Hobbs *et al.*, 2017; Löber *et al.*, 2018) (Fig. 3).

Some recombinant KoRV's, such as recKoRV1, have up to 10 copies, comprising approximately 10% of the total KoRVs in an individual. Seventeen distinct recKoRVs

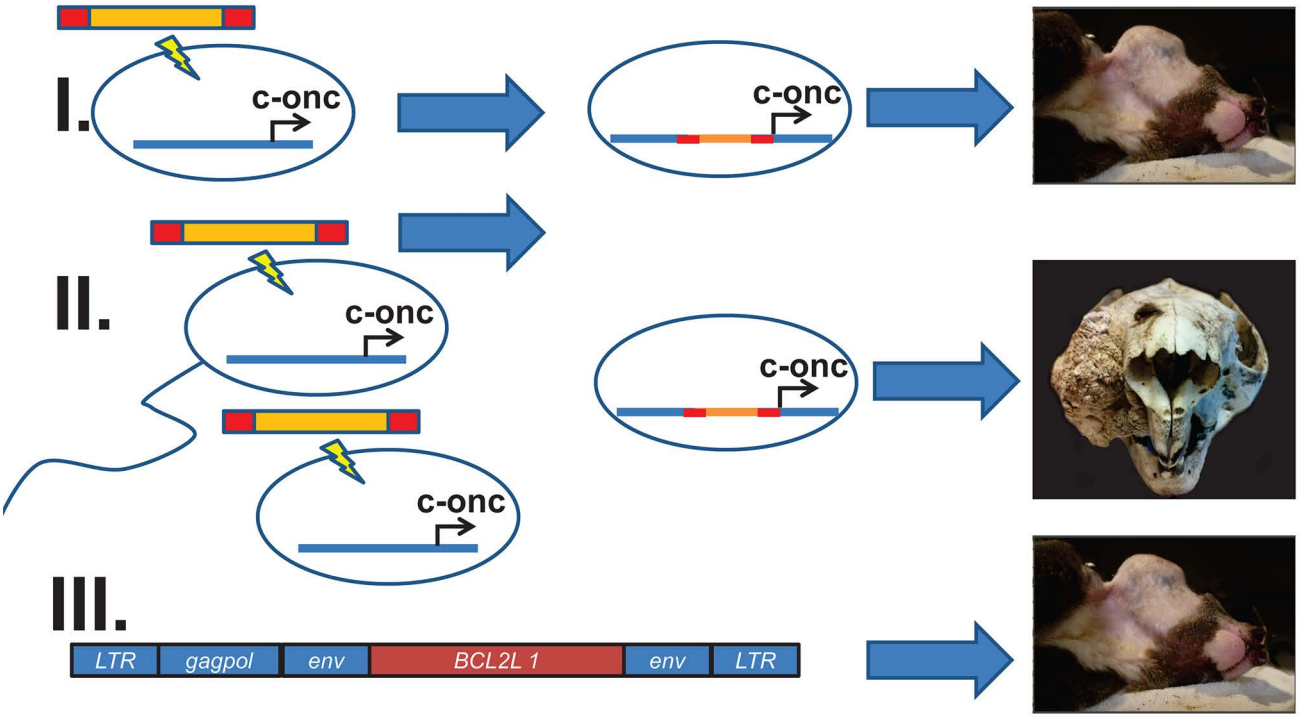

Figure 2. Three mechanisms identified whereby KoRV integrations or transduced KoRV lead to neoplasia (McEwen *et al.*, 2021). I. Somatic integrations lead to lymphoma II. Inherited KoRVs lead to development of osteosarcomas. III. Transduced KoRV results in lymphoma. Photographs kindly provided by Amber Gillett.

that had different spatial distributions, have been observed (Löber *et al.*, 2018). This suggests recKoRVs arise frequently and independently among koala populations. Over the long term, the more recKoRVs established in the koala genome, the fewer functional copies of KoRV will be available. As recKoRVs are likely dependent on KoRV for replication, it will become exceedingly rare for the recKoRV to mobilize autonomously. As recKoRVs are similar to disrupted endogenous retroviruses in other large-bodied species, it is likely that koalas are in a transition phase whereby intact retrovirus remains prevalent in the population, but the disrupted copies begin to increase in frequency, likely under strong selection. However, until competent KoRV becomes uncommon, both novel KoRV and recKoRV integrations will present considerable mutagenic and hence, cancer risks to koalas.

Practical aspects: selective breeding

While somatic mutations in captive populations cannot be controlled or predicted, selective breeding could help prevent or reduce the number of shared IS in oncogenes. This would require IS determination for the entire captive (or at least breeding) population of koalas, an undertaking that could be completed within a year. Selective breeding or introduction of wild koalas lacking specific IS could produce populations that have reduced numbers of ERVs in oncogenes that are heritable which may reduce, but not eliminate the elevated cancer risk. Somatic mutations will remain a problem. Monitoring of expression of specific genes with IS in existing koalas could potentially indicate if they are at risk of developing neoplasia with potential interventions that could be explored.

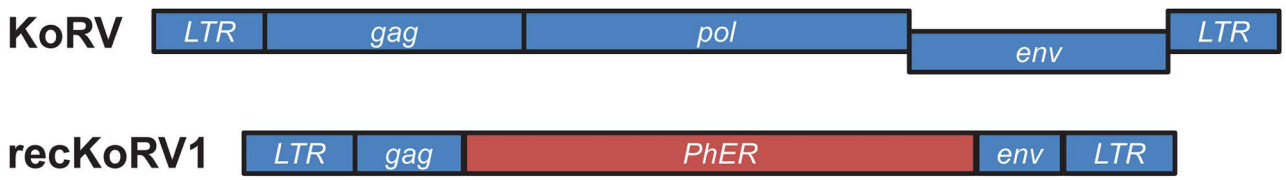

Figure 3. Structure of a common recombinant KoRV (recKoRV1). PhER is an ancient marsupial endogenous retrovirus that interrupts the KoRV genome removing much of the *gag* gene, all of the *pol* gene, and all but approximately 100 bp of the *env* gene. The LTRs and *gag* leader sequence remains intact (Löber *et al.*, 2018).

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