Toxoplasma gondii manipulates host cell signaling pathways via its secreted effector molecules

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Abstract

The obligate intracellular parasite *Toxoplasma gondii* secretes a vast variety of effector molecules from organelles known as rhoptries (ROPs) and dense granules (GRAs). ROP proteins are released into the cytosol of the host cell where they are directed to the cell nucleus or to the parasitophorous vacuole (PV) membrane. ROPs secrete proteins that enable host cell penetration and vacuole formation by the parasites, as well as hijacking host-immune responses. After invading host cells, *T. gondii* multiplies within a PV that is maintained by the parasite proteins secreted from GRAs. Most GRA proteins remain within the PV, but some are known to access the host cytosol across the PV membrane, and a few are able to traffic into the host-cell nucleus. These effectors bind to host cell proteins and affect host cell signaling pathways to favor the parasite. Studies on host–pathogen interactions have identified many infection-altered host signal transductions. Notably, the relationship between individual parasite effector molecules and the specific targeting of host-signaling pathways is being elucidated through the advent of forward and reverse genetic strategies. Understanding the complex nature of the host–pathogen interactions underlying how the host-signaling pathway is manipulated by parasite effectors may lead to new molecular biological knowledge and novel therapeutic methods for toxoplasmosis. In this review, we discuss how *T. gondii* modulates cell signaling pathways in the host to favor its survival.

Keywords: *Toxoplasma gondii*, dense granule, rhoptry, immune evasion, immune activation, innate immunity

1. Introduction

Toxoplasma gondii, an obligate intracellular parasite, can infect almost all warm-blooded animals including humans [1]. Toxoplasmosis is generally asymptomatic in immunocompetent people but may become severe and occasionally fatal in the immunocompromised, such as those with HIV/AIDS or pregnant women [2,3]. *T. gondii* infections occur when tissue cysts in uncooked or undercooked meats are ingested by intermediate hosts or when the oocysts shed by the definite hosts (infected felines) are ingested [4]. After acute infection with *T. gondii*, the parasite forms cysts that can persist throughout the life time of the host [5]. It has been reported that *T. gondii* infects approximately one third of humans worldwide [6]. A key to *T. gondii* success as a widespread parasite in intermediate hosts hinges on its ability to persist for the life of the host [7]. *T. gondii* contains three morphologically distinct secretory organelles: micronemes, rhoptries (ROPs) and dense granules (GRAs). The contents of both the micronemes and the ROP neck are required for parasite invasion of the host cell and for forming the parasitophorous vacuole (PV) [8]. When *T. gondii* releases the contents of its ROP bulbs they are injected into the host's cytoplasm at the time of invasion, as are the GRA proteins involved in parasite survival in host cells [8]. GRA proteins are involved in remodeling the PV and cyst wall maturation [9,10]. Early studies of genetic diversity in *T. gondii* strains using multilocus restriction fragment-length polymorphism genetic markers reported that only a few genotypes dominated most of Europe and North America; namely types I, II, and III, some additional lineages, and atypical and recombinant strains [11]. *T. gondii* strains differ in virulence in laboratory mice and likely cause different clinical signs and symptoms in humans [12]. The *T. gondii* factors that determine virulence are mostly related to genetic polymorphisms in ROPs and GRAs [7]. Strain-specific activities of *Toxoplasma* effectors are shown in the table. Moreover, a schematic overview of the differences between type I and II *Toxoplasma* strains is provided in the Figure. As this figure implies, complete understanding the genetic factors influencing *T. gondii* virulence is a major research goal.

Over the last twenty years, the strain-dependent susceptibility of *Toxoplasma* to interleukin (IL)-12-interferon-gamma (IFN-γ) axis-mediated mechanisms has been well described [7,13]. For example, the ROP5 *Toxoplasma* pseudokinase and the ROP18 active kinase determine strain-specific virulence differences in mice by cooperatively inactivating host-specific interferon-regulated GTPases (IRGs) [14,15]. ROP18 is highly expressed in type I strains but not in type III strains [16]. These proteins suppress host immune responses so as to facilitate parasite persistence. Surprisingly, however, GRA15 enhances the host immune response rather than repressing it, making the parasite less virulent and helping host survival [17]. Although it seems that having GRA15 is disadvantageous, these biological approaches enable the parasite to adapt better to long-term persistence in the host. Type II GRA15 strongly activates the nuclear factor-kappa B (NFκB) pathway, whereas type I and type III GRA15s display less or no activity [18]. Importantly, these molecules confer distinct advantages on parasite strains that impact their transmission, host range and

pathogenicity. In this review article we provide an up-to-date overview of the immune evasion and immune activation aspects related to *Toxoplasma* effectors.

2. IFN-γ-dependent resistance to intracellular *T. gondii*

The potent cell-mediated response to invading parasites in the host is characterized by the production of the IL-12 inflammatory cytokine, a key cytokine involved in the development of the type 1 T helper (Th1) response produced by dendritic cells and macrophages [13,19]. IL-12 production promotes early natural killer-cell production of IFN-γ and helps activate $CD8^+$ T cells [20]. Mice lacking IFN-γ signaling components display increased susceptibility to *T. gondii* infection [21–25]. IFN-γ activates the signal transducer and activator of transcription (STAT) 1 and induces many interferon-γ-regulated genes [26]. The latter activates effector mechanisms, including IRGs, inducible nitric oxide synthase (iNOS/Nos2), indoleamine 2,3-dioxygenase (IDO), p65 guanylate binding proteins (GBPs), tryptophan degradation in human cells, and autophagy control of parasite elimination [27–32]. *T. gondii*-related acute virulence in mice largely depends on inactivation of the IRG family [33,34]. IRGs, a family of IFN-γ inducible proteins, are indispensable for host resistance to *T. gondii* [35]. Normally, IRGs are held inactive on the host's endomembrane by regulatory IRGs (called the GMS subfamily) such as Irgm1 and Irgm3 [36]. Upon recognition of pathogen-containing PVs, these IRGs accumulate on the PV membrane (PVM), which leads to its disruption and is followed by rapid killing of the released tachyzoites [27,37]. In immunologically activated mouse cells, the microtubule-associated protein 1 light chain 3 (LC3) and its γ-aminobutyric acid receptor-associated protein (GABARAP) homologs are involved in the IFN-γ dependent recruitment of effector IRGs (called the GKS subfamily), such as Irga6 and Irgb6, to the PV [36,38]. PV-binding effector IRGs promote the translocation of ubiquitin-binding protein p62 and E3 ubiquitin ligases (e.g., tripartite motif-containing 21 and TNF receptor-associated factor 6 (TRAF6)) to the PVM, which destabilizes the PV. p62 also mediates GBP recruitment to the ubiquitinated PV and this leads to PV lysis [39–42].

Thus, although IFN-γ-dependent responses play critical roles in host immunity against *T. gondii* infection, *T. gondii* targets these responses to modulate host immunity and this dictates virulence differences between strains [13]. During the early infection stages, type I parasite infections do not activate pro-inflammatory reactions. Moreover, type I strains express genes associated with strong virulence and have the ability to reduce pro-inflammatory cytokine production, resulting in rapid parasite growth. The two parasite proteins secreted by infected cells, profilin and cyclophilin, which are recognized by dendritic cells via toll-like receptor 11 and C-C chemokine receptor 5, respectively, induce NFκB activation and IL-12 production, which activates NK cells, T cells and IFN-γ secretion [43,44]. In contrast, type II strains effectively activate the early immune response, resulting in the production of large amounts of pro-inflammatory cytokines and directing T cells to become the Th1 type. Like type I, type III strains do not activate the initial immune response and limit the production of pro-inflammatory cytokines. However, because these parasites express inactive but highly virulent

factors, intracellular parasites are unavoidably eliminated. In type II and type III strains, the parasite load is controlled by a sufficient Th1 type response, leading to chronic infection. Interestingly, the IRG genes in laboratory mice are all substantially identical, but the IRG genes in wild mice are very diverse . Interestingly, the polymorphisms of IRG in wild mice result in resistance to virulent parasite kinases [45]. The highly polymorphic IRG protein Irgb2-b1 from the South Indian CIM strain bounds directly to ROP5, resulting in efficient IRG accumulation around PV [46]. This may be the result of co-adaptation between host cell resistance and *T. gondii* virulence effectors [46]. Furthermore, IFN-γ priming also leads to PV ubiquitination in human cells but the ubiquitinated substrates remain unknown. For example, while mice have more than 20 IRG family members, humans only possess two IRG genes (IRGC and IRGM), and they are not IFN-γ inducible [47]. By contrast, several studies have shown IFN-γ-dependent nutrient deprivation and cell death to be an anti-*T. gondii* responses in human cells [30,48]. Induction of IDO expression leads to degradation of tryptophan, an essential amino acid for *T. gondii* growth in human fibroblasts such as HFF, Huh7, and HAP1 cells [49,50]. However, other studies have found that IFN-γ-dependent L-tryptophan breakdown results in a minimal inhibition defect of parasite growth in HFF and primary fibroblast cells [48,51].

3. Roles played by ROP proteins in IFN-γ-dependent immune responses

Most *Toxoplasma* strains isolated in Europe and North America fall into just three clonal lines/ strains: type I, II, and III [11]. These lines/ strains differ in their virulence in mice. Type I strains are associated with a 100% lethal dose (LD_{100}) for a single parasite challenge infection in laboratory mice, whereas the LD_{50} doses for type II and III strains are ~10^3 or 10^5 parasites, respectively [12]. Virulence differences between the clonal lines are largely caused by polymorphisms in ROP18 [16,52] and ROP5 [53,54] effectors, together with ROP17 and GRA7 [14,15,55]. Their activities are required to subvert the recruitment of IRGs [56–61] and GBPs [39,40] to the PV. Previous genetic mapping of crosses between type I×II, I×III, and II×III strains revealed that the high virulence of type I parasites was associated with two parasite proteins, ROP18 and ROP5, but intermediately virulent type II strains and avirulent type III strains carry less virulent allele combinations [16,52].

The expression levels of strains carrying the type III ROP18 allele are lower than those carrying type I and II alleles in the order of 10,000-fold, as caused by a 2.1 kb insertion 85-bp upstream of the start codon [52]. Subsequent functional studies have shown that ROP18 from type I strains can phosphorylate host Irga6 at T102 and T108 in the switch I loop, thereby inactivating the GTPase and inhibiting its normal accumulation on the parasite-containing vacuole [56,57]. Thus, IRGs cannot accumulate on the PVM in cells that express sufficient levels of ROP18, and the parasite survives. Contrastingly, very low ROP18 expression levels in cells cause recruitment of IRG to the PVM, resulting in the parasites being efficiently eliminated [7].

Polymorphism in ROP5 is also critical for strain-related virulence differences. The ROP5 locus is encoded by highly divergent, tandemly repeated genes [54,62]. ROP5, which consists of ROP5A, ROP5B, and ROP5C, differs in its copy numbers between strains. Type I has ~ 6 copies, type II has ~ 10, and type III has ~ 4 copies of ROP5 [54]. These paralogues contain all the residues in the canonical kinase except for the catalytic aspartate residue in the HRD (His-Arg-Asp) domain [62,63]. ROP5 isoforms are almost identical between type I and type III parasites, but type II isoforms are distinct from the isoforms of the other two strains [54]. The type II allele of the ROP5B isoform contains a frameshift, which leads to a non-functional protein [54]. The major type I allele of ROP5 acts to cooperatively enhance the ROP18-mediated phosphorylation of Irga6 and Itgb6 [53,60]. Certain type II strains with intermediate virulence have an almost functional ROP18 [52,61] but IRG accumulation in the PVM still progresses [27]. These strains carry ROP5 alleles that ineffectively support ROP18 in the phosphorylation of IRG proteins [59]. Hence, the ROP5 and ROP18 combination can explain *T. gondii* virulence in mice; that is, the high virulence type I phenotype, the intermediate type II phenotype, and the low virulence type III phenotype [7].

Type I ROP17 also interacts with ROP5 and plays a role in preventing IFN-γ-dependent parasite clearance [15]. The other ROP kinases, type I ROP2 and type I ROP8, along with type I dense granule protein GRA7, also exist in a protein complex with ROP18 on the PVM [14]. Type I GRA7 binds to Irga6 and acts synergistically with ROP18 to block IRG proteins [14,64]. Unlike the lack of ROP18, loss of either GRA7 or ROP2/8 alone does not decrease parasite virulence [14]. However, a double knockout mutant of GRA7 and ROP18 had a completely avirulent phenotype in infected mice [14]. This double knockout mutant exhibited an increased recruitment of IRGs and parasite clearance in IFN-γ priming macrophages [14]. The function of these auxiliary factors has been investigated mostly in the type I lineage because the immunosuppressive mechanism used by ROP5 and ROP18 does not work in type II and type III lineages. For example, sequence alignment analysis of ROP17 revealed that type I ROP17 differs at 24 amino acids from the type II and type III ROP17 [15]. It is unclear whether these differences are involved in strain-specific differences in parasite virulence.

The ROP16 ROP kinase influences the Janus kinase/STAT pathway and determines transcriptional response differences between strains [65]. Cells infected with type I or type III strains but not with type II strains phosphorylate STAT3 and STAT6, resulting in induction of a group of genes that polarize the response to type 2 T helper cytokines while downregulating IL-12 expression [65]. Interestingly, comparison of ROP16 from type I RH and type II ME49 showed that a single amino acid substitution (position L503S) determined the difference in STAT3 activation between strains (supplementary material).[66]. STAT3 and STAT6 mediate the effects of IL-4 and IL-13, and induce an alternative activation program in macrophages whereby proinflammatory responses are inhibited [67]. However, the role of ROP16 in virulence remains unclear.

4. GRA proteins mediate parasite susceptibility to host immune responses

Aside from the above-mentioned ROP proteins, several GRA proteins can modulate host immune responses [7]. Importantly, *T. gondii* can differentially modulate the NFκB pathway depending on its genotype. GRA15 from type II parasites was shown to positively activate the NFκB pathway in both mouse and human cells [17]. NFκB consists of homodimers or heterodimers containing p65, p50, 52, c-Rel and RelB subunits, and plays a pivotal role in regulating the expression levels of various inflammatory genes [68,69]. RelB- and c-Rel-deficient mice are lethally susceptible to *T. gondii* infection unlike their wild-type counterparts, indicating the importance of the NFκB response to *T. gondii* infection [70,71]. The number of IL-12p40-producing cells among the peritoneal exudate cells collected on day 2 post-infection was found to decrease in response to *T. gondii* infection in c-Rel-deficient mice, although within 2–3 days this defect is known to no longer be present [71]. Moreover, the increased susceptibility of c-Rel-deficient mice to *T. gondii* infection is rescuable by exogenous administration of IL-12 until 2 days post-infection, suggesting that immediate signaling from IL-12 and subsequent production of IFN-γ are required for host resistance to *T. gondii* [71]. Therefore, NFκB plays a critical role in controlling parasite growth, especially during the initial infection stage.

GRA15-mediated inflammation can stimulate IL-1β and IL-12 induction in infected macrophages, which leads to IFN-γ production [17]. Mice infected with type II GRA15-deficient parasites reportedly have a higher parasite burden early after infection [17]. Moreover, our recent data have shown that GRA15 deficiency is sufficient to increase acute virulence in mice [72]. GRA15, which contains amino acid polymorphisms, accounts for differences in NFκB activation among different strains [17]. For example, the type I GT1 strain has a functional GRA15, whereas the type I RH strain does not because a frameshift in its GRA15 causes an early stop codon [17,18]. Type I GT1 and type III strains translate all 635 amino acids in GRA15, whereas type II GRA15 produces a truncated GRA15 protein of 550 amino acids [18]. Ectopic expression of either type II or type III GRA15 can strongly activate NFκB, whereas GRA15 from the type I RH strain lacks activity. Additionally, infections with type I (RH or GT1) and type III strains alike show an absence of high-level NFκB-p65 translocation to the nucleus. Recent studies have revealed that GRA15 is not only responsible for NFκB activation differences among strains, but is also involved in strain-dependent susceptibility to IFN-γ-mediated *Toxoplasma*cidal mechanisms [17,18,51,73,74]. It has been reported that type II GRA15 elevates the recruitment of GBP1-5 to the parasite-containing vacuole in murine cells lines [75,76]. Sangaré et al. recently reported that type II GRA15 binds to TRAF ubiquitin ligases, which are key intermediates in the NFκB pathway [18]. TRAF6 ubiquitination is required for p62 recruitment and ubiquitination in the vacuole, which causes disruption of the PV by IRGs and GBPs [77]. Elsewhere, Mukhopadhyay et al. showed that TRAF6 recruitment by type II GRA15 mediates GBP and IRG recruitment [51]. In fact, the type II Pru strain, which expresses the type II copy of GRA15, showed a significantly larger fraction of its vacuole coated with TRAF6, IRGB6, ubiquitin (K63 and K48), GBPs, LC3, and GABARAPs, as compared with the type I RH strain in IFN-γ

stimulated MEF cells [51]. They further demonstrated that a lack of type II GRA15 results in significantly lower growth inhibition in IFN-γ stimulated MEF cells than parental Pru strain [51]. These data show that type II GRA15 increases mouse susceptibility to IRG- and GBP-dependent parasite clearance by recruiting TRAF6 in IFN-γ priming cells [51]. Furthermore, Wang et al. recently reported that type II GRA15 enhances the type I interferon response mediated by the stimulator of interferon genes (STING) protein in murine cells [74]. GRA15 promotes the polyubiquitination of STING at lysine 337 via TRAF molecules [74]. Overall, type II GRA15 activates the host immune system, protecting mice from uncontrollable parasite loads and supporting persistent infection.

Toxoplasma GRA7 induces a strong antibody response; therefore, GRA7 is a potential vaccine candidate and a promising sero-diagnostic marker for toxoplasmosis [78–81]. Previous studies have demonstrated that GRA7 plays important roles in regulating immune evasion and activation. Type I GRA7, which associates with the ROP5/ROP18 protein complex, is known to target IFN-γ-activated IRG [14,64,82]. Type I GRA7 initiates mitogen-activated protein kinase (MAPK) and NFκB signaling through the production of NADPH oxide-dependent reactive oxygen species and Myd88-dependent TRAF6 activation [83,84]. Recombinant type I GRA7 also interacts with the NLR family pyrin domain, which contains the 3 inflammasome complex, to induce IL-1β and IL-18 processing via caspase-1 activation [84]. We recently investigated the role of GRA7 in the disease etiology caused by type II strains. Similar to GRA15, GRA7 deficiency induced much lower cytokine secretion from infected macrophages than seen in cells infected with the parental strain [72]. Our study also showed that a lack of GRA7 results in a comparable increase in mortality via GRA15 [72].

GRA14, which is secreted into the vacuole, can migrate to both the PVM and the intra-vehicular network [85]. Ectopic expression of both type I and type II GRA14 was found to stimulate the NFκB promoter equivalently to GRA7, but GRA15 produced much higher levels of NFκB-dependent luciferase activity than GRA7 and GRA14 [72]. Unlike the other two molecules, type II GRA14 deficiency made a slight difference to mortality [72]. In summary, type II GRA7 and type II GRA15 contribute significantly to virulence in mice, whereas type II GRA14 has a relatively small effect on virulence via NFκB-p65 activity [72].

Interestingly, unlike type II strains, the lack of GRA14 in the type I RH strain resulted in mouse mortality via intra-footpad injection with *T. gondii* [72]. Unlike intraperitoneal inoculation, which causes a rapid and acute systemic infection, intra-foot inoculation allows observation of the gradual spread of *T. gondii in vivo* [86]. GRA14 has only three amino acid differences between type I and II strains (P43S, D323G, and S356V) [72]. Moreover, ectopic expression of type I GRA14 was found to induce comparable reporter activity from the NFκB reporter plasmid unlike that of type II GRA14 [72]. Therefore, deleting GRA14 delays the early immune response and causes excessive parasite growth, possibly leading to mouse death.

A recent study showed that a novel dense granule resident effector in the type II strain called TEEGR (*Toxoplasma* E2F4-related EZH2-inducible gene regulator) selectively suppressed

transcription of IL-1β, IL-6, IL-23A, IL-15, IL-8, and C-C Motif chemokine ligand 20 by negatively regulating the NFκB pathway without affecting the expression of IL-12 and IL-18 [87]. TEEGR has almost the same sequence in type I and type III parasites, whereas type II parasites have an insertion in its middle. The contribution of type I TEEGR to virulence is unknown because type I infections are lethal, but it suppresses the expression of some genes (iNOS, Alox12, and Kiss1R) in a strain-independent manner. Overall, immune activation via these effector molecules limits parasite tissue invasion and ensures host survival, but paradoxically this process helps the parasite transform into the muscle and brain tissue sustainable form: the bradyzoite [88].

Conversely, other studies have shown that type I strains interfere with the host's NFκB pathway to promote their survival. NFκB-p65 is a known target protein of ROP18-mediated NFκB suppression. Type I ROP18 phosphorylates p65 and promotes ubiquitin-dependent degradation [89], and the ROP16 type I polymorphic kinase can suppress the IL-12 response in lipopolysaccharide-stimulated infected macrophages, thereby inhibiting NFκB transcriptional activity [17,65]. However, it is unclear how these effectors in the type I strain act on virulence control via the NFκB pathway. After secretion of GRAs into the PV lumen, some GRAs associate with the PVM or the intravacuolar network. They are also exported beyond the PVM into the host cell cytoplasm, and reach to the host cell nucleus. However, little is known about the transport mechanism of GRAs after exportation into the PV lumen. Several molecules involved in GRA trafficking after secretion to PV lumen have been identified [90]. For example, MYR1/2/3/4 are important molecules for the transport of GRAs across PVM. GRA16, GRA18, GRA24, TgIST, and TGEEGR do not translocate into the host nucleus when MYR1 is deficient [87,91]. ROP17 is also involved in the translocation of these GRAs across PVM [92]. On the other hand, MYR1 is not involved in the function of GRA15 localized in PVM [91]. GRA7 and GRA15 are secreted onto and beyond the PVM into the host cell cytoplasm. However, the subsequent transport mechanism is not understood. How post-secreted GRA molecules interact with host transcription factors should be investigated in the future.

5. Other *Toxoplasma* proteins that influence virulence

Various other *T. gondii* proteins that are secreted across the PVM can induce host cell signaling changes. For example, type II GRA24 drives MAPK-p38 activation and induces IL-12 and chemokine production in *T. gondii*-infected bone marrow-derived macrophages [93]. Type I GRA6 stimulates the nuclear factor of activated T cells 4 (NFAT4), which is mediated through the calcineurin activator calcium regulatory ligand in host cells [86]. GRA6 activation of NFAT4 induced chemokine (C-C motif) ligand 2 and chemokine (C-X-C motif) ligand 2, which are required for inflammatory monocyte and neutrophil recruitment [86]. GRA16, which is well conserved among type I, II and III strains, can bind to host enzymes such as herpesvirus-associated ubiquitin-specific proteases and PP2A-B55 phosphate to alter tumor suppressor p53 levels, and it positively modulates the expression of genes involved in cell cycle progression and the p53 pathway [94]. Thus, GRA16 promotes host cell survival

under stress conditions [94]. Interestingly, comparative genomic expression profiling of ROP kinases identified differential expression of them between strains [95]. The expression difference for ROP38 was 8 times higher in the type II ME49 strain and 64 times higher in the type III VEG strain than in the type I RH strain [95]. ROP38 is a putative functional kinase with a predicted signal peptide. Type I ROP38 modulates apoptosis and cell proliferation by downregulating host genes associated with the MAPK pathway [95]. The TgIST *Toxoplasma* effector has recently been shown to be secreted from GRAs and eventually localize in the host cell nucleus. By secreting type II TgIST, *T. gondii* potently inhibits the expression of STAT1-dependent genes including IRGs, GBPs, iNOS, and chemokines, leading to enhanced virulence in mice [96,97]. Furthermore, the mechanism of IFN-γ-induced cell autonomous immunity response varies widely between species [98–100]. In human cells, ROP5 and ROP18 have no effect on parasite's survival due to lack of IFN− γ -inducible IRGs [61]. Additionally, IFN-γ induces IDO1-mediated tryptophan breakdown in human cells, whereas this pathway did not play any role in parasite growth restriction in murine cells [101]. TgIST is able to inhibit IDO1 gene expression to promote parasite growth in IFN− γ -activated human cells [49].

6. Conclusions

We have summarized both immune evasion and immune activation in terms of *Toxoplasma* effectors. The question of how secreted effector molecules affects *Toxoplasma* virulence is a highly complex one. Regulation of the host's innate immune response by *T. gondii* is important for establishing both acute and persistent latent infections. Therefore, the outcome of infection determined by the parasite strain depends on a combination of often polymorphic effectors and the genetic backgrounds of its hosts. This resulting diversity may contribute to the high prevalence and wide distribution of this parasite. Interestingly, accumulating numbers of studies have revealed that *T. gondii* needs to balance its host's immune response in order to increase the likelihood of a successful infection. Further insight into the exact roles performed by these molecules may help to delineate the pathogenesis of toxoplasmosis.

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Figure caption

Fig. Overview of the differences between type I and II *Toxoplasma* strains and how their effectors modulate the NFκB-IFN-γ axis-related host immune response.

Toxoplasma effectors secreted from rhoptries and dense granules modulate host immune pathways. During the early infection phase type I parasites do not activate the NFκB pathway. Type I ROP16 induces sustained activation of STAT3 and STAT6, dampening the production of IL-12 and IL-1β. *Toxoplasma* EEGR negatively regulates the NFκB signaling pathway, selectively repressing transcription of a subset of NF-kB-regulated genes including IL-1β. ROP18 promotes the degradation of NFκB-p65 by phosphorylating p65. Profilin and cyclophilin parasite proteins are secreted by infected cells and are recognized by DCs via TLR11 and CCR5, respectively, causing NFκB activation and IL-12 production while activating NK and T cells and secreting IFN-γ. IFN-γ binds to its receptor and triggers the STAT pathway, leading to activation of IRGs and GBPs. IRGs and GBPs cause destruction of the PV. The ROP5/ROP18/ROP17 complex, together with GRA7, cooperatively prevents the accumulation of IRGs at the PV membrane. ROP18 has also been shown to degrade ATF6β. TgIST binds to STATs and blocks induction of the IFN-stimulated genes normally up-regulated by IFN-β.

In contrast, type II strains express a functional form of GRA15, which activates NFκB. GRA7 and GRA14 contribute to the efficiency with which the NFκB pathway is activated. GRA7 activates the inflammasome and promotes caspase-1 activity, thereby converting pro-IL-1β into the active cytokine. NFκB activation triggers the massive production pro-inflammatory genes including IL-12 and IL-1β, both of which potentiate IFN-γ production in NK cells and T cells. Unlike type I strains, type II and type III strains encode combinations of avirulent ROP5 and ROP18 alleles, respectively. Type II and III parasites are unable to block the recruitment of IRGs and GBPs to the PV. GRA15 mediates IFN-γ-dependent growth inhibition through TRAF ubiquitin ligases.

NFκB, nuclear factor-kappa B; IL, interleukin; TLR, toll-like receptor; CCR5, C chemokine receptor 5; NK, natural killer; DC, dendritic cell; IFN-γ, interferon-gamma; IFN-β, interferon-beta; ATF6β, activating transcription factor 6 beta; STAT, signal transducer and activator transcription; ROP, rhoptry; GRA, dense granule; PV, parasitophorous vacuole; TEEGR, *Toxoplasma* E2F4-associated EZH2-inducing gene regulator.

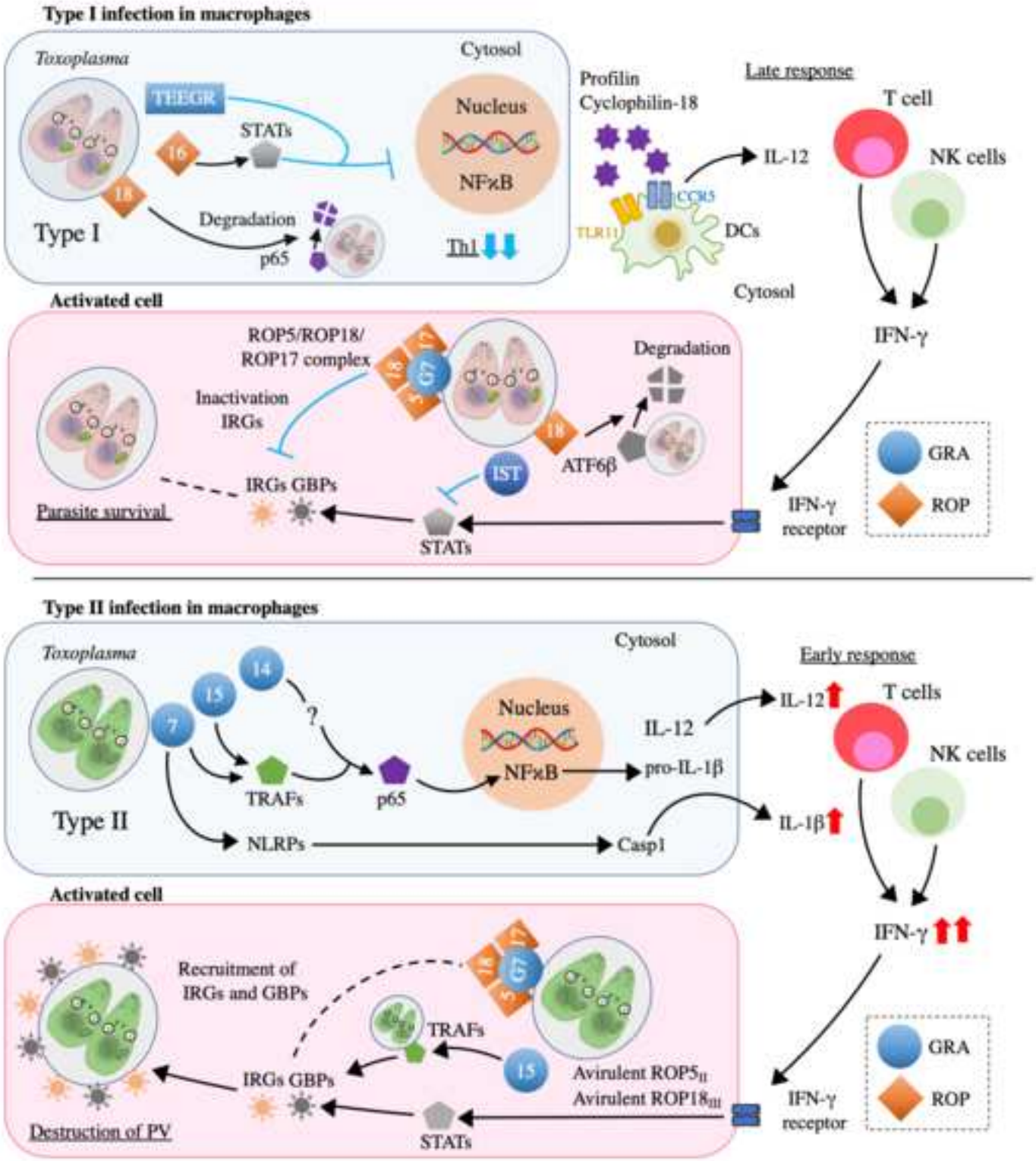
733 **Table. Strain-specific activities of *Toxoplasma* effectors on the murine immune response.**

Name	ToxoDB	Virulence factor activities			
		Type I: High virulence	Type II: Intermediate virulence	Type III: low virulence	Reference
ROP5	TGGT1_308090 TGME49_308090 TGVEG_308090	Binds Irga6, enhance ROP18 activity	Does not enhance ROP18 activity	Binds Irga6, enhance ROP18 activity	15,53,54,62
ROP16	TGGT1_262730 TGME49_262730 TGVEG_262730	Activate STAT3/6, induces alternative macrophage activation response	Does not induce long-tem activation of STAT3/6	Activate STAT3/6, induces alternative macrophage activation response	65,66
ROP17	TGGT1_258580 TGME49_258580 TGVEG_258580	Binds Irga6, enhance ROP18 activity	Has not been analyzed directly	Has not been analyzed directly	15
ROP18	TGGT1_205250 TGME49_205250 TGVEG_205250	High expression, phosphorylation of IRGs	High Expression	Very low expression	15,16,52
GRA7	TGGT1_203310 TGME49_203310 TGVEG_203310	Binds Irga6, accelerating turn-over	Activates NFκB, induces classical macrophage activation	Has not been analyzed directly	14,64,72,83.84
GRA14	TGGT1_239740 TGME49_239740 TGVEG_239740	Has not been analyzed directly	Activates NFκB, induces classical macrophage activation	Has not been analyzed directly	72,86

GRA15	TGGT1_275470 TGME49_275450 TGVEG_275470	Does not activate NFκB	Activates NFκB, induces classical macrophage activation	Low expression	17,18

734

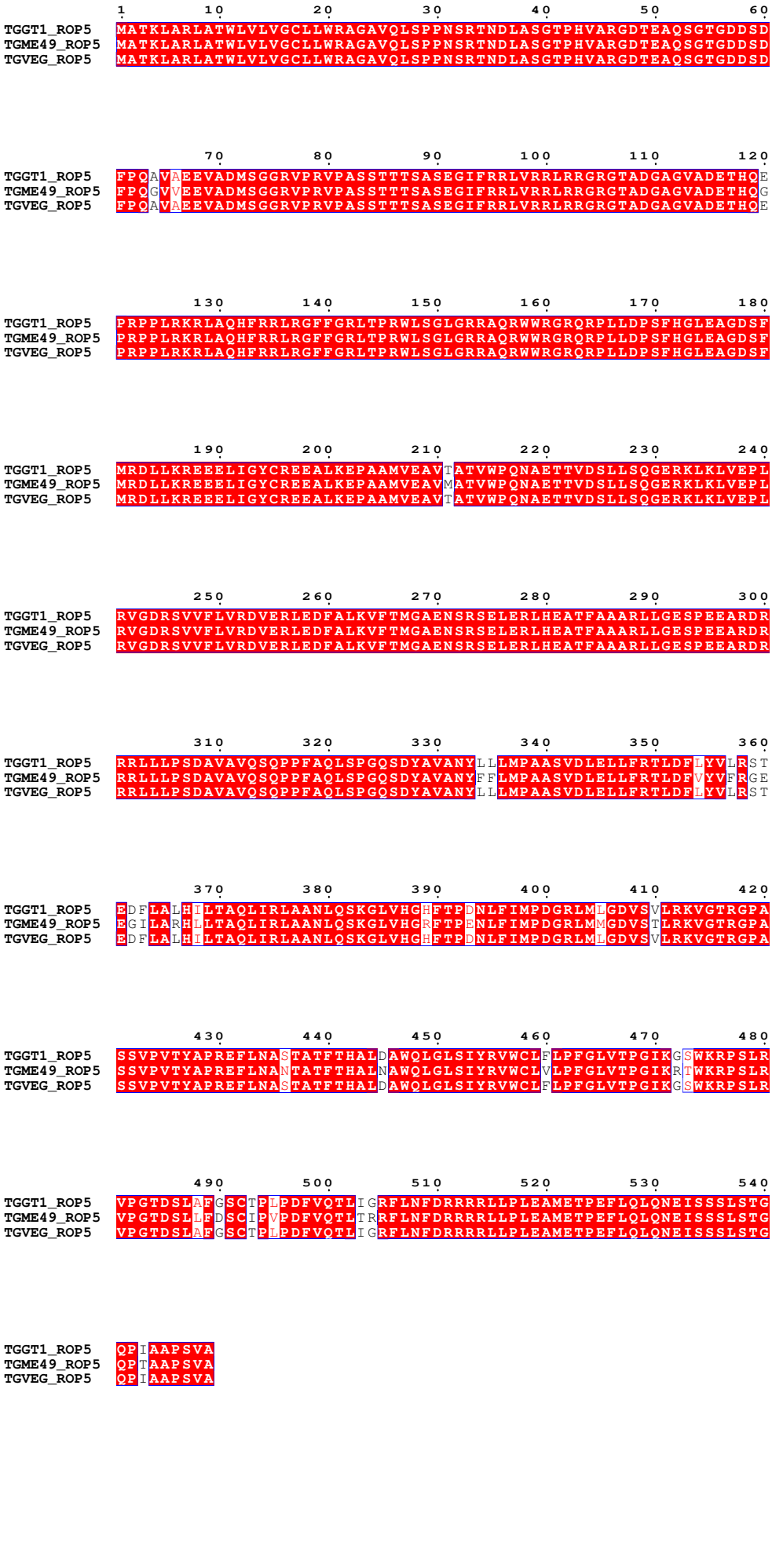
Figure


Type I infection in macrophages
Toxoplasma
Cytosol
THEGR
STATs
16
18
Type I
Degradation
p65
Nucleus
NFκB
Th1
Profilin
Cyclophilin-18
CCR5
TLR11
DCs
Late response
IL-12
T cell
NK cells
Cytosol
IFN-γ
Activated cell
ROP5/ROP18/
ROP17 complex
Inactivation
IRGs
Degradation
ATF6β
IST
IRGs GBPs
Parasite survival
STATs
IFN-γ
receptor
GRA
ROP
Type II infection in macrophages
Toxoplasma
Cytosol
14
15
7
?
TRAFs
p65
Nucleus
NFκB
IL-12
pro-IL-1β
Type II
NLRPs
Casp1
Early response
IL-12
T cells
NK cells
IL-1β
IFN-γ
Activated cell
Recruitment of
IRGs and GBPs
TRAFs
IRGs GBPs
Avirulent ROP5II
Avirulent ROP18III
Destruction of PV
STATs
IFN-γ
receptor
GRA
ROP

Supplementary material

Sequence alignment results of *Toxoplasma* effectors

The sequence alignments of these molecules against type I GT1, type II ME49, and type III VEG strains were performed using ClustalW. The output files were configured using ESPrint. The amino acids conserved in all strains are highlighted in red.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TGVEG_ROP5 MATKLARLATWLVLVGCLLWRAGAVQLSPPNSRTNDLASGTPHVARGDTEAQSGTGDDSD
70 80 90 100 110 120
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TGME49_ROP5 FPQGVVEEVADMSGGRVPRVPASSTTTSASEGIFRRLVRRLRRGRGTADGAGVADETHQG
TGVEG_ROP5 FPQAVAEEVADMSGGRVPRVPASSTTTSASEGIFRRLVRRLRRGRGTADGAGVADETHQE
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TGVEG_ROP5 PRPPLRKRLAQHFRRLRGFFGRLTPRWLSGLGRRAQRWWRGRQRPLLDPSFHGLEAGDSF
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TGVEG_ROP5 MRDLLKREEELIGYCREEALKEPAAMVEAVTATVWPQNAETTVDSLLSQGERKLKLVEPL
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TGME49_ROP5 RVGDRSVVFLVRDVERLEDFALKVFTMGAENSRSELERLHEATFAAARLLGESPEEARDR
TGVEG_ROP5 RVGDRSVVFLVRDVERLEDFALKVFTMGAENSRSELERLHEATFAAARLLGESPEEARDR
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TGVEG_ROP5 VPGTDSLAFGSCTPLPDFVQTLIGRFLNFDRRRRLLPLEAMETPEFLQLQNEISSSLSTG
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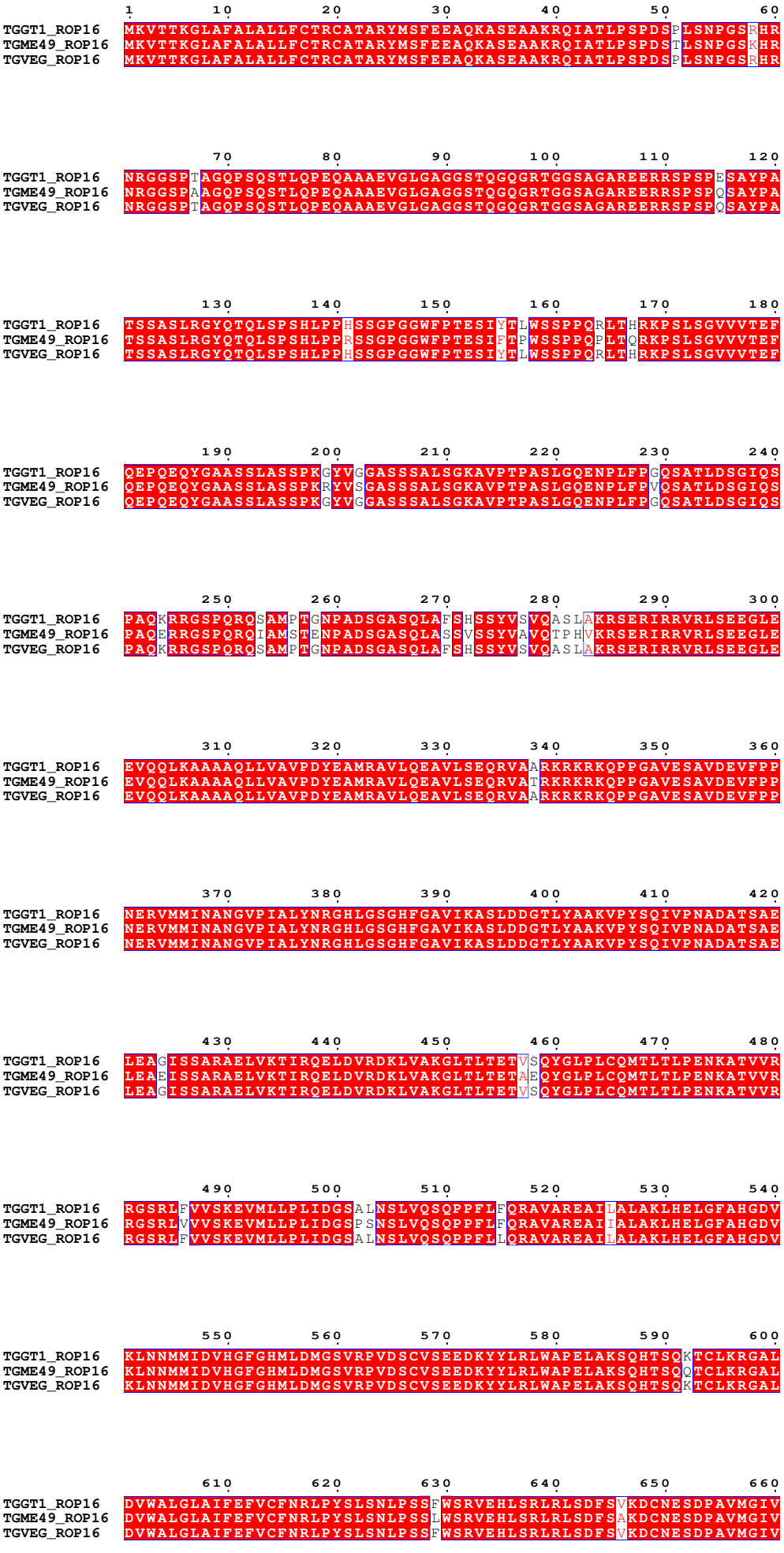
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TGME49_ROP16 NRGGSPAAGQPSQSTLQPEQAAAEVGLGAGGSTQGQGRTGGSAGAREERRSPSPQSAYPA
TGVEG_ROP16 NRGGSPTAGQPSQSTLQPEQAAAEVGLGAGGSTQGQGRTGGSAGAREERRSPSPQSAYPA
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TGVEG_ROP16 TSSASLRGYQTQLSPSHLPPHSSGPGGWFPTESIYTLWSSPPQRLTHRKPSLSGVVVTEF
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TGME49_ROP16 QEPQEQYGAASSLASSPKRYVSGASSSALSGKAVPTPASLGQENPLFPVQSATLDSGIQS
TGVEG_ROP16 QEPQEQYGAASSLASSPKGYVGGASSSALSGKAVPTPASLGQENPLFPGQSATLDSGIQS
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TGVEG_ROP16 PAQKRRGSPQRQSAMPTGNPADSGASQLAFSHSSYVSVQASLAKRSERIRRVRLSEEGLE
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TGVEG_ROP16 EVQQLKAAAAQLLVAVPDYEAMRAVLQEAVLSEQRVAARKRKRKQPPGAVESAVDEVFPP
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TGVEG_ROP16 KLNNMMIDVHGFGHMLDMGSVRPVDSCVSEEDKYYLRLWAPELAKSQHTSQKTCLKRGAL
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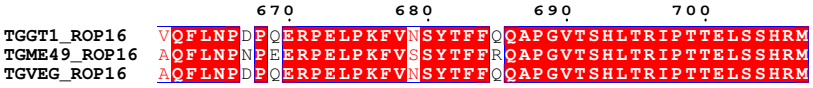
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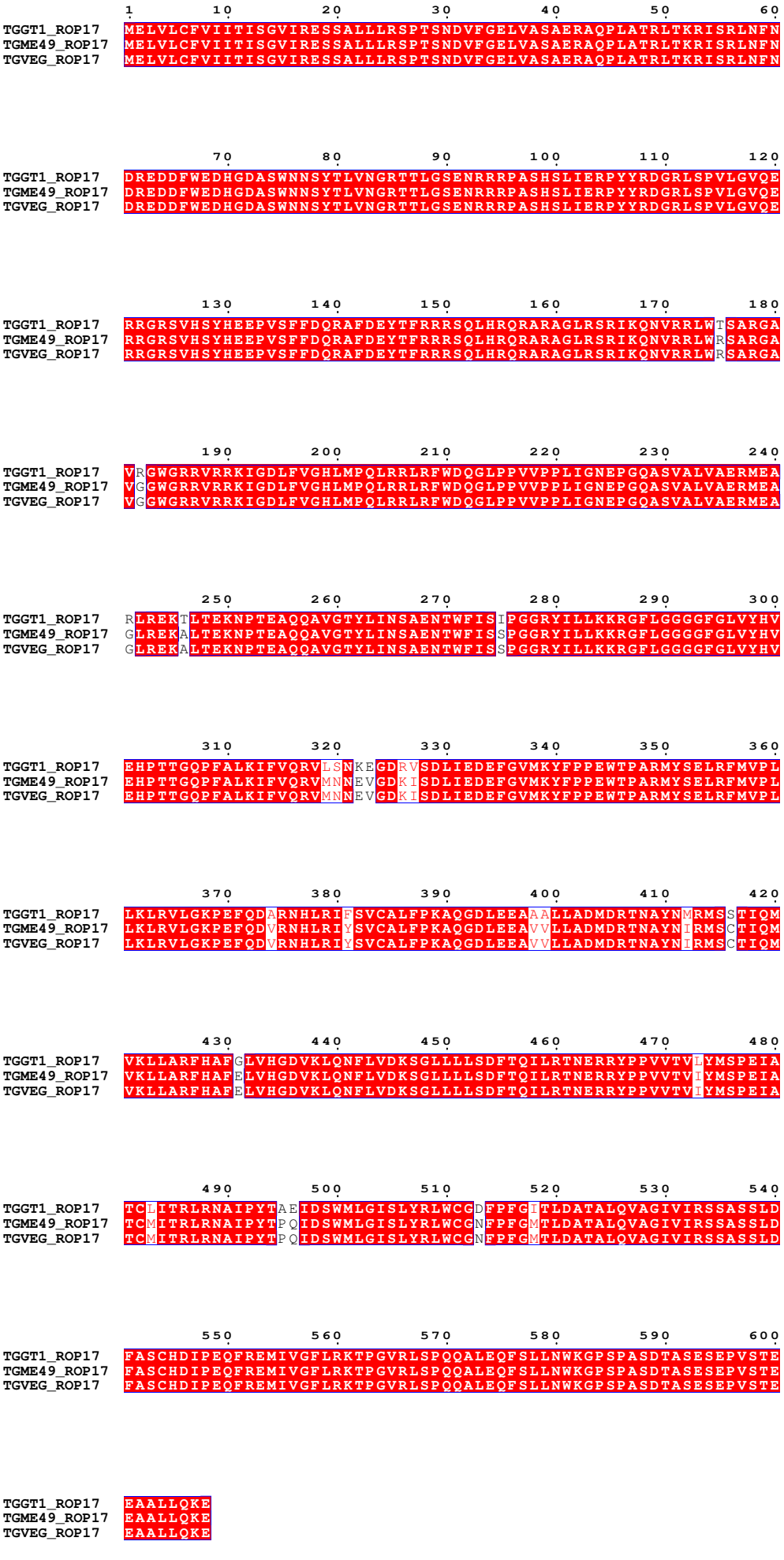
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TGVEG_ROP17 MELVLCFVIITISGVIRESSALLLRSPTSNDVFGELVASAERAQPLATRLTKRISRLNFN
70 80 90 100 110 120
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TGME49_ROP17 DREDDFWEDHGDASWNNSYTLVNGRTTLGSENRRRPASHSLIERPYYRDGRLSPVLGVQE
TGVEG_ROP17 DREDDFWEDHGDASWNNSYTLVNGRTTLGSENRRRPASHSLIERPYYRDGRLSPVLGVQE
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TGVEG_ROP17 RRGRSVHSYHEEPVSFFDQRAFDEYTFRRRSQLHRQRARAGLRSRIKQNVRRLWRSARGA
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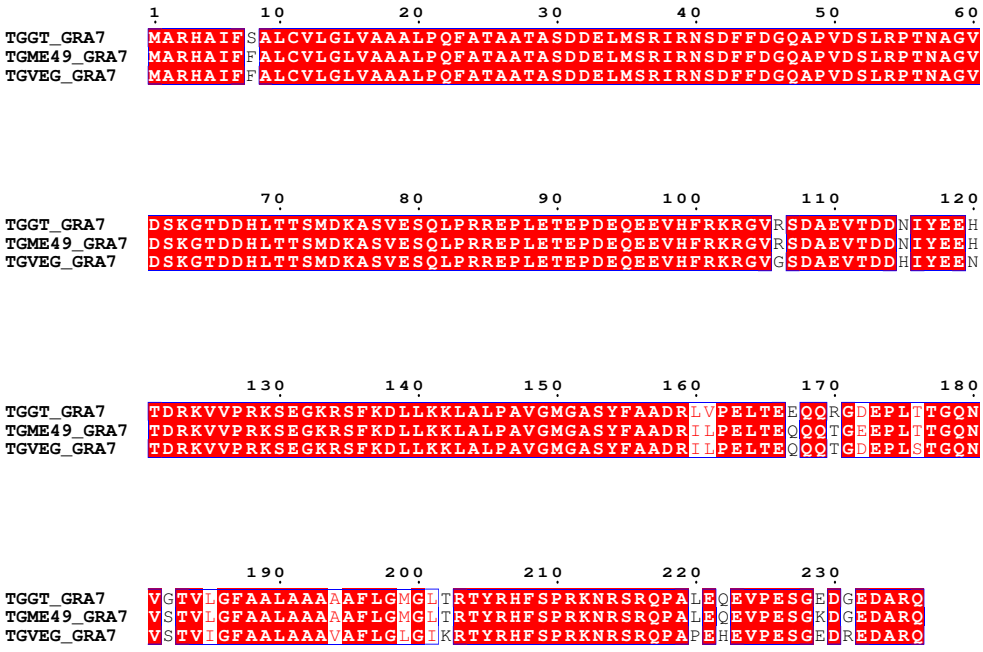
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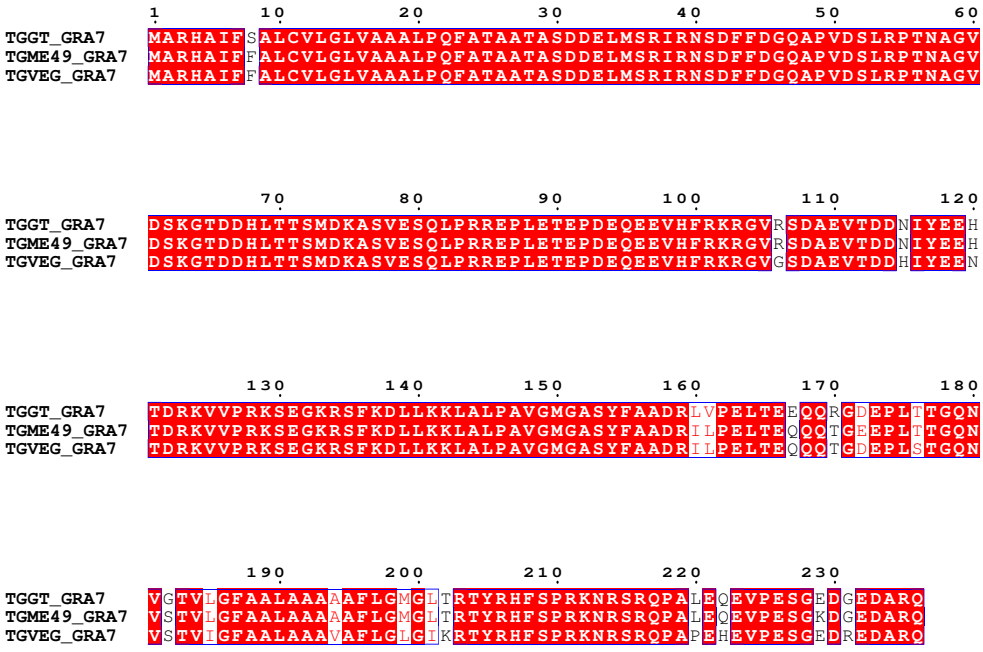
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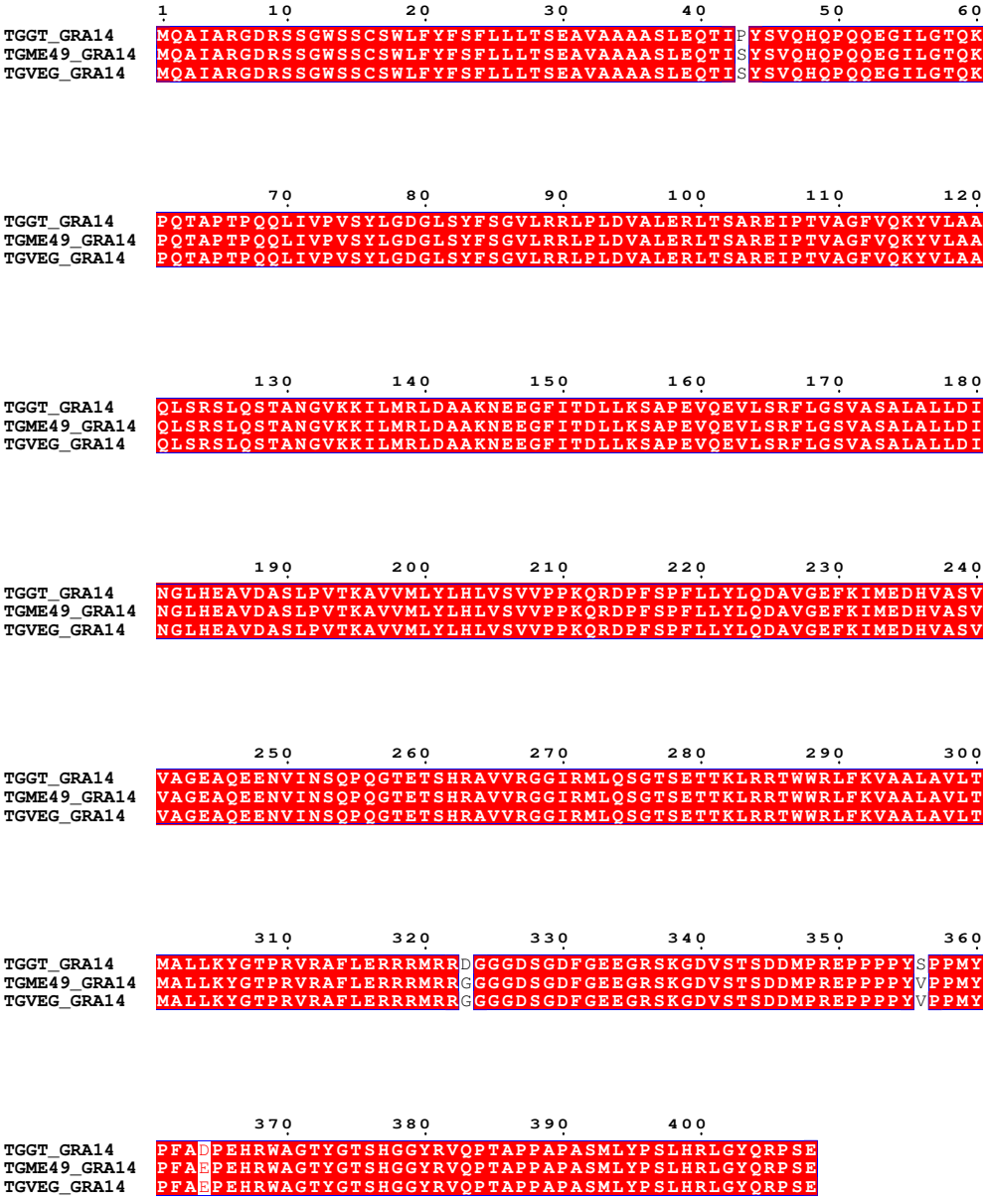
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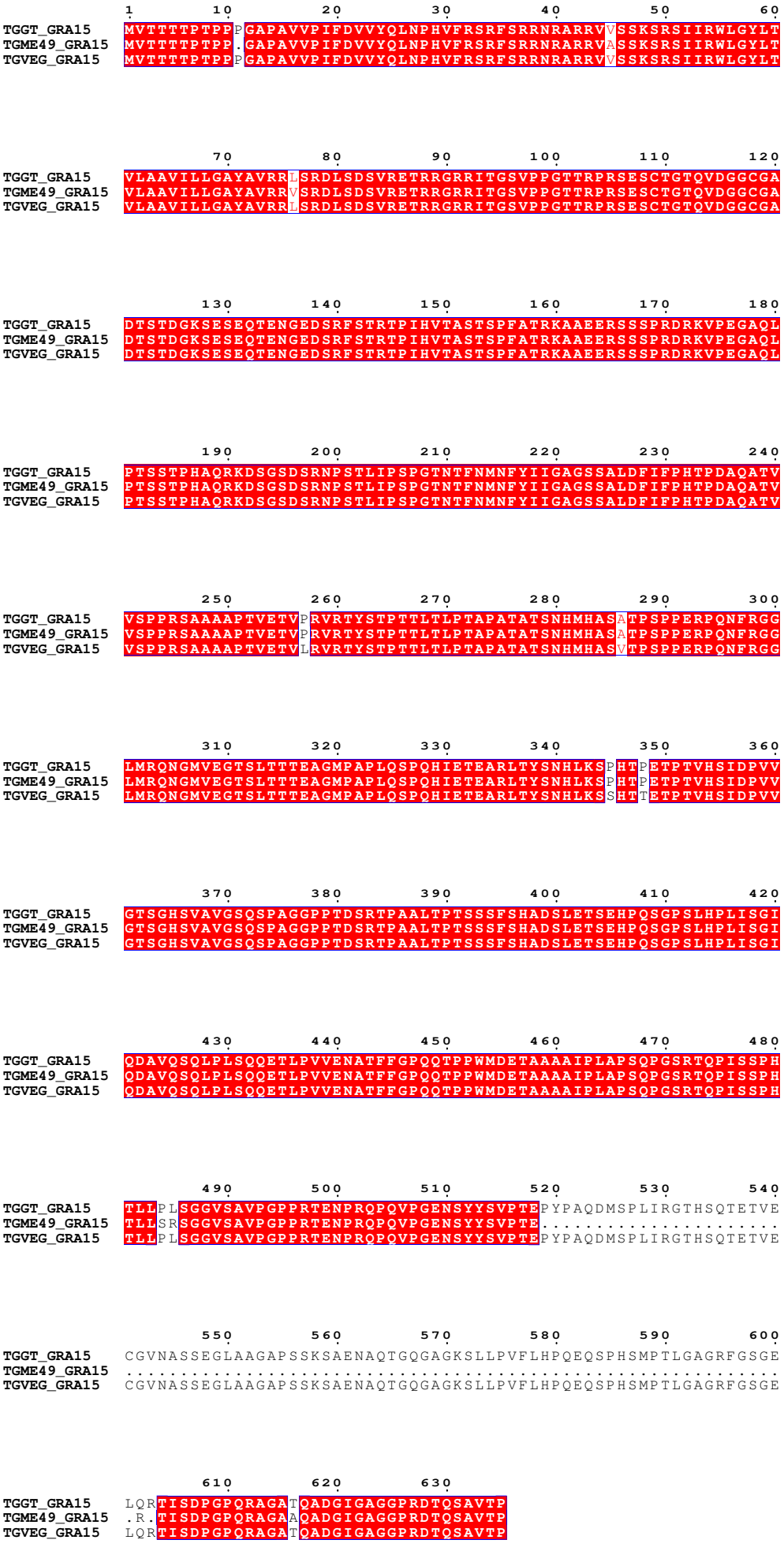
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TGVEG_GRA15 MVTTTTPTPPPGAPAVVPIFDVVYQLNPHVFRSRFSRRNRARRVVSSKSRSIIRWLGYLT
70 80 90 100 110 120
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TGVEG_GRA15 TLLPLSGGVSAVPGPPRTENPRQPQVPGENSYYSVPTEPYPAQDMSPLIRGTHSQTETVE
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